Chapter 1

General introduction

Diabetes mellitus

In the second century AD, a Greek physician called the disease that was characterized by extreme loss of urine 'diabetes', meaning siphon. In 1679, the physician Thomas Willis added the word 'mellitus' to diabetes, referring to the sweet taste of the urine (1). Diabetes mellitus is a disease of metabolic dysregulation, in particular dysregulation of glucose metabolism. In healthy individuals, blood glucose concentrations are tightly balanced by two counteracting hormones, insulin and glucagon, that are secreted by β- and α-cells, respectively, which are endocrine cells located in the pancreas (Fig. 1). Diabetes is characterized by an impaired glucose homeostasis due to an absolute or relative deficiency of insulin. Symptoms include polyuria, polydipsia, polyphagia, and weight loss. The diagnosis of diabetes is based on one of the following criteria: percentages of glycated hemoglobulin (HbA1c) ≥ 6.5%, fasting plasma glucose (FPG) ≥ 7 mmol/l, or a 2-hour plasma glucose ≥ 11.1 mmol/l after a 75-g oral glucose tolerance test (2).

In 2013, 382 million people had diabetes worldwide and by 2035 this is expected to increase to 592 million people (3). In The Netherlands approximately 1 million people are diagnosed with diabetes (4). The two major forms of diabetes are type 1 and type 2, although diabetes can also manifest during pregnancy and under other conditions including drug or chemical toxicity and genetic disorders. In type 1 diabetes (~10% of patients) the majority of β-cells is lost due to autoimmune destruction. A combination of genetic and environmental factors activates an immune response against β-cells. In type 2 diabetes (~85% of patients) β-cell function slowly decreases over time and is associated with a loss of β-cell mass up to 65% (5) probably due to metabolic and/or inflammatory factors.

Patients with type 1 diabetes require life-long insulin replacement therapy by multiple-dose insulin or insulin pump therapy. In patients with type 2 diabetes, initially lifestyle changes as healthy eating, weight control and increased physical activity are stimulated to obtain an HbA1c <7.0%. If this target is not met, pharmacological therapy can be initiated, starting with metformin therapy that improves insulin sensitivity and suppresses glucose production by the liver. Many patients will ultimately require insulin therapy due to the progressive nature of the disease (2).

Despite intensive treatment with diets, antihyperglycemic oral agents or insulin injections, normalization of glycemic control can often not be achieved. Patients are at risk to develop acute and long-term complications. Acute complications include diabetic ketoacidosis from persistent hyperglycemia, and hypoglycemic events. Long-term complications include a wide range of microvascular complications such as retinopathy, nephropathy and neuropathy. Furthermore, patients with diabetes have an increased risk for cardiovascular and cerebrovascular disease (6). Therefore, therapies that restore, maintain or prevent loss of functional β-cells are needed for all types of diabetes. For that reason it is critical to better understand mechanisms that regulate β-cell mass growth and function.

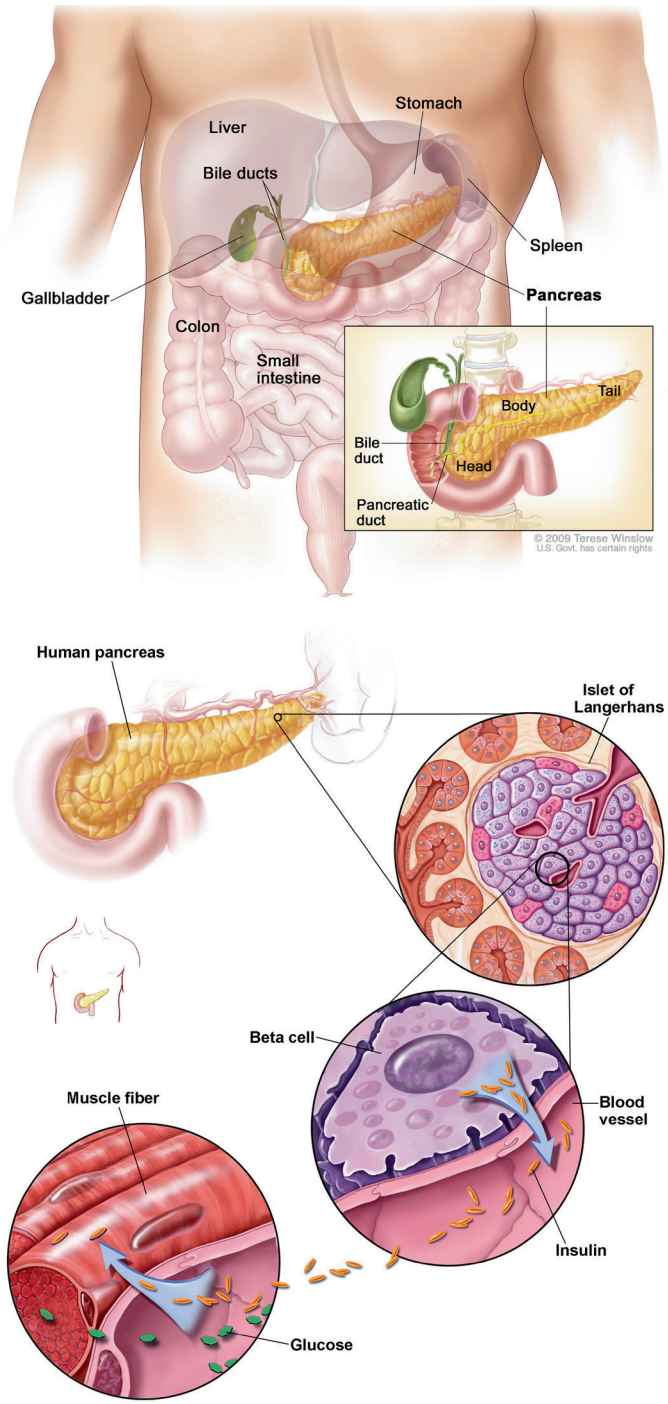

Figure 1. A. Illustration of the pancreas and nearby organs. Inset: Illustration of the head, body and tail of the pancreas. B. Illustration of the insulin-secreting β-cell. *Reprinted with permission from Terese Winslow.*

Glucose metabolism

The blood glucose concentration is determined by the rate of glucose entering the circulation balanced by the removal of glucose out of the circulation. The primary action of insulin is to remove glucose from the circulation whereas its counterpart glucagon stimulates the entering of glucose into the circulation (Fig. 1B). In a fed state β-cells are triggered to secrete insulin, which facilitates the uptake of glucose from blood into cells. Inside the cell glucose will be metabolized via glycolysis, a multistep process of which pyruvate is the end product. Under aerobic conditions, pyruvate enters the citric acid cycle in the mitochondrion resulting in the production of NADH and $FADH_2$ that are subsequently oxidized in the respiratory chain to generate energy (7). Excessive glucose can be stored in the form of the polymer glycogen in the liver and muscle. Also, insulin promotes lipogenesis and protein synthesis and inhibits the oxidation of free fatty acids and protein breakdown (8). During fasting, blood glucose concentrations are increased by the hormone glucagon that stimulates glucogenolysis from glycogen stores and gluconeogenesis from non-carbohydrate sources (9). Furthermore, it counteracts insulin by restraining the synthesis of glycogen, glycolysis and lipid storage. If fasting continues for several days, glucagon can stimulate lipolysis of adipose tissue and proteolysis from muscle tissue of which the substrates can be used to generate glucose by gluconeogenesis in the liver (10). At the same time, glucagon can stimulate ketogenesis, providing ketone bodies that can be used as an alternative fuel for the brain when glucose is sparse (9).

β-Cells

The islets of Langerhans are clusters of endocrine cells that are scattered throughout the exocrine pancreas. Islets represent only 1 to 2% of the pancreas. The islets are composed of insulin-producing β-cells (60-70%), glucagon-producing α-cells (20-30%), somatostatin-producing δ-cells, pancreatic polypeptide (PP) producing PP cells and ε-cells that produce ghrelin (11, 12). The remainder of the pancreas consists of exocrine cells that secrete digestive enzymes that are transported to the duodenum via the pancreatic duct system. Pancreatic islets are highly vascularized to enable efficient secretion of hormones into the circulation and densely innervated allowing control of the glucose homeostasis by the autonomic nervous system (13).
The gene for insulin is located on chromosome 11 in humans and its product is a 110-amino acid precursor peptide called preproinsulin. The signal peptide of the protein brings preproinsulin into the lumen of the rough endoplasmic reticulum (ER) where it is removed generating proinsulin. Within the ER, proinsulin folds into a three-dimensional structure and is brought via vesicular transfer to the Golgi apparatus. In the Golgi, proinsulin enters immature secretory vesicles and is cleaved by prohormone convertases generating insulin and C-peptide. Insulin and C-peptide

are stored in secretory granules together with islet amyloid polypeptide. These insulin granules accumulate in the cytoplasm of the β-cell awaiting a signal to be released from the β-cell (14, 15). The insulin secretory pathway becomes activated when glucose enters the cell through the glucose transporter-2. Oxidative metabolism of glucose leads to an increase in the ATP/ADP ratio. This leads to closure of ATP-dependent potassium channels. The subsequent membrane depolarization results in an influx of Ca^{2+} through the opening of voltage-gated calcium-channels. The increase in intracellular calcium concentration stimulates the fusion of insulin granules to the cell membrane and exocytosis of insulin (16, 17).

Embryonic development and topological heterogeneity of islets in the pancreas

During embryonic development the pancreas originates from two epithelial buds, a ventral and dorsal bud, which protrude from the embryonic gut epithelium and converge to form the definitive pancreas (Fig. 2). The developing pancreatic duct epithelium consists of multipotent pancreatic progenitor cells that will give rise to all mature pancreatic cell types and undergoes extensive branching into a highly organized tubular network. Within the ductal epithelium endocrine progenitor cells arise, characterized by expression of the transcription factors *Pdx1* and *Ngn3*, which delaminate from the duct and migrate into the surrounding mesenchyme and aggregate into cell clusters that ultimately form the islets of Langerhans (18–21). The highest increase in endocrine tissue during development occurs in the second and third trimester of pregnancy in humans. During this period β-cell proliferation is relatively low and data suggest that the majority of new islet cells arise from precursor cells, a process called neogenesis (20, 22). The ventral bud gives rise to the posterior part of the mature pancreatic head and uncinate process, and the dorsal bud forms the anterior part of the head, the body and tail (Fig. 1A). A systematic study of the rat pancreas revealed that in the lower part of the head of the pancreas contains islets with a high percentage of PP-cells, whereas few glucagon-positive cells are observed in this same region (23). In humans, similar PP-rich lobules are identified in the pancreatic head region, most likely the part originating from the ventral pancreatic bud during embryogenesis (24–26). Also, islets derived from the dorsal bud secrete more insulin after a glucose stimulus compared to islets derived from the ventral bud in rats (27). Furthermore, in adult human pancreas the density of islets is higher in the tail-region compared to the head and body-region of the pancreas (28–30). Altogether this shows that islet morphology and function can be different throughout the pancreas.

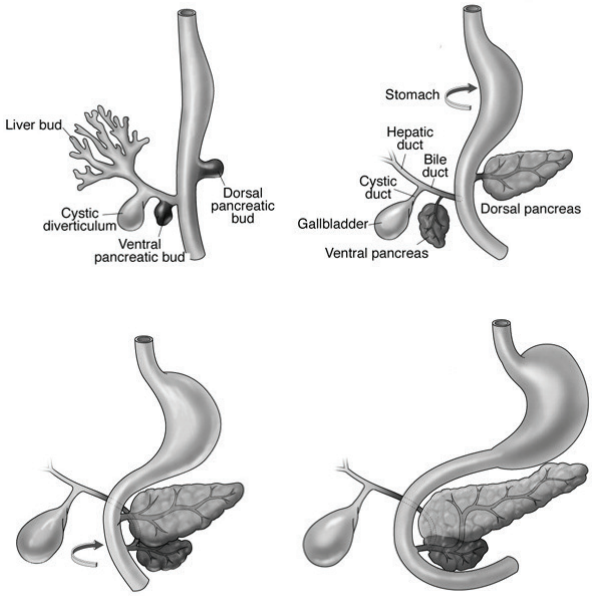

Figure 2. Organogenesis of the pancreas. The pancreas originates from 2 buds, the ventral and dorsal pancreatic bud. The ventral and dorsal pancreas fuse to form the mature pancreas. *Adapted with permission from Cano et al. Gastroenterology 2007* (19).

β-Cell mass adaptation

In adults the β-cell mass is tightly controlled in order to maintain blood glucose concentrations within a narrow range. When the demand for insulin is chronically increased by physiological or pathological changes, such as obesity, pregnancy, glucocorticoid treatment and pancreatic damage, β-cells can adapt by enhancing insulin secretion via increased β-cell function and/or increased β-cell mass (Fig. 3). Inadequate β-cell adaptation leads to the development of hyperglycemia and eventually diabetes mellitus (28, 31).

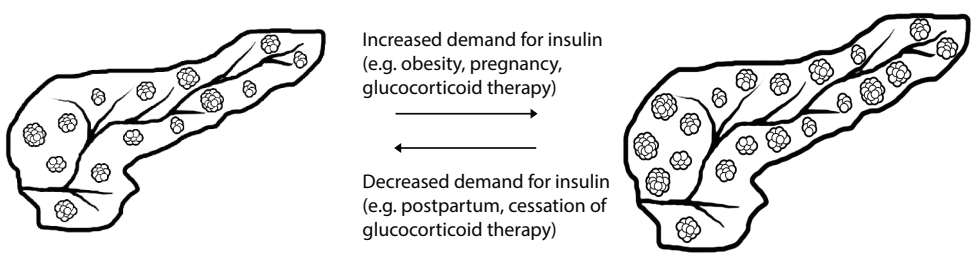

Figure 3. Changes in the demand for insulin are associated with an adaptation of the β-cell mass.

Obesity

Obesity in humans is associated with an increased insulin secretory response following a glucose or meal challenge (32, 33). In 1933 Ogilvie reported that islets in humans with obesity were enlarged (34). Later this observation was confirmed by several studies comparing β-cell mass between non-diabetic obese and lean individuals. These studies have reported increases in β-cell mass ranging from 20 – 100% in obese subjects (28, 31, 35, 36). Recently, Saisho et al. (37) studied the largest population of pancreas donors. A 50% increase in β-cell mass was found when comparing 61 obese vs. 53 lean subjects. Obesity is associated with insulin resistance, which results in an increased demand for insulin from β-cells (16). Mice fed a high-fat diet become obese and develop insulin resistance that is associated with an increased β-cell mass (38, 39). In young (5 - 6 weeks old) mice the β-cell mass increase ranges from 2 – 3.5 fold after a high-fat diet for 8 weeks (39, 40). Also, genetic mutations in rodents that lead to obesity and insulin resistance, such as leptin-deficient *ob/ob* mice and *db/db* mice or Zucker *fa/fa* rats that have a defective leptin-receptor, are associated with a compensatory increase of the β-cell mass (41–44). The development of diabetes in *Macaca mulatta* is associated with obesity and insulin resistance and in normoglycemic, hyperinsulinemic monkeys associated with an increased β-cell area (45).

The importance of insulin resistance generating an environment of increased insulin demand is illustrated by mice that are double heterozygous for null alleles in the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) genes. These mice develop severe insulin resistance that is associated with a massive increase of the β-cell mass, up to 30-fold (46). Specific knockout of the insulin receptor in the liver (LIRKO) also results in dramatic insulin resistance and is associated with a 6-fold increase in β-cell mass (47). Mezza et al. (48) reported an increased islet size in insulin resistant non-diabetic humans, that was inversely correlated with insulin sensitivity. Recently it was shown that transplantation of human islets isolated from non-obese donors into insulin resistant mice results in β-cell mass adaptation of the human islet graft *in vivo* (49).

Together these studies show that in obesity-related, diet- or genetically-induced insulin resistance there is a compensatory growth of the insulin producing β-cell mass. Since about 80% of obese humans remain non-diabetic (50), this adaptation of the β-cell mass is successful in most cases.

Pregnancy

Pregnancy is accompanied with series of metabolic changes including a progressive development of insulin resistance that requires adaptation of the β-cells to maintain normoglycemia (51). Several studies have reported increases of the islet mass during pregnancy in rodents (52). The extent of the increase varies from 1.5- to 2-fold (53–55). Interestingly, postpartum the β-cell mass involuted to prepartum levels (53, 55).

Obviously the possibility to study pancreas material from pregnant women is limited and until today only a few groups have studied the β-cell mass in pregnant women. Van Assche et al. reported an enlargement of the islets of Langerhans and hyperplasia of β-cells resulting in a

2.4-fold increase of β-cell area when comparing 5 pregnant with non-pregnant women (56). More recently, Butler et al. (57) reported a 1.4-fold increase of the β-cell area in the pancreas of 18 pregnant women. Interestingly, this latter group also studied β-cell area in a few pancreas samples from post-partum women and observed a strong tendency for a decrease in β-cell area compared with pregnant women suggesting that, similar to rodents, the β-cell mass returns to baseline levels postpartum. It is thought that failure to compensate β-cells during pregnancy may contribute to the development of gestational diabetes in women, which is a risk indicator for the development of type 2 diabetes later in life (58). Together these studies show that during pregnancy the β-cell mass increases to compensate for the increased insulin demand, a compensation that seems to be reversible postpartum.

Glucocorticoid therapy

Glucocorticoids are essential to the adaptation of the body to fasting, injury, and stress. Their receptors are expressed on most cells, by which they can influence a variety of physiological processes. Therefore, glucocorticoids are used extensively as therapeutic agents, especially for their anti-inflammatory actions. However, patients become glucose intolerant and they have an increased risk to develop diabetes because glucocorticoids antagonize the action of insulin (59). This change in glucose metabolism increases the demand for insulin. In rodents and non-human primates glucocorticoid treatment induces insulin resistance, which is associated with increased insulin secretion and compensatory β-cell mass growth (60–64). Interestingly, discontinuation of the therapy in rats resulted in involution of the β-cell mass to pretreatment levels (64).

Pancreatic damage

When (part of) the endogenous β-cell mass is removed surgically or by physical or chemical damage to the pancreas, an increased demand for insulin on remaining β-cells arises. Multiple animal models have been developed to study the response of the β-cell mass in situations of pancreatic damage. β-Cells can be destroyed using cytotoxic agents, such as streptozotocin (STZ) or alloxan. Following STZ treatment and in the presence of insulin treatment, the β-cell mass in mice regenerated to about 50% after 6 days (65). In STZ-treated newborn rats the β-cell mass was regenerated to 39% of the normal value by day 20 (66). However, no regeneration of the β-cell mass was observed in male vervet monkeys treated with STZ (67). Also removal of 50 – 90% of the pancreas is associated with a (partial) regeneration of the pancreas after a couple of weeks (68–70). So, depending on the treatment regimen, chemically induced destruction or (partial) removal of the β-cells can lead to regeneration of the β-cell mass in rodents (68).
In humans, glucose concentrations begin to rise when the β-cell mass is reduced by approximately 50% (71), which fits the observation that in patients with type 2 diabetes β-cell mass is reduced to 65% compared to non-diabetic subjects (5). No compensatory growth of the β-cell mass was observed in patients that underwent a 50% pancreatectomy because of chronic pancreatitis or

pancreatic cancer (71). Also, hemipancreatectomy in healthy donors resulted in deterioration of insulin secretion and glucose intolerance in 25% of the donors one year after surgery (72). Of 22 children diagnosed with nesidioblastosis who had undergone 90-95% pancreatectomy, 55% showed complete pancreatic regeneration (assessed by ultra-sound, no histology data available) resulting in a pancreas normal in size for the age (73). Since these patients were normoglycemic, this suggests that the regenerated pancreas consisted of an adequate number of endocrine cells. Altogether this shows that, in contrast to rodents, there is no evidence in adult humans that the β-cell mass regenerates in response to pancreatic damage.

Mechanisms of β-cell mass adaptation

The β-cell mass is determined by the balance of β-cell renewal and loss. Mechanisms potentially involved in β-cell mass regulation are proliferation and apoptosis of existing β-cells and the formation of new β-cells from precursor cells (neogenesis). More recently it was reported that mature cells (e.g. α-cells or acinar cells) are able to transdifferentiate into insulin-producing cells (74, 75).

β-Cell proliferation

In young mice (5 - 6 weeks) basal β-cell proliferation detected by Ki67 staining is ~2.5% (76, 77). β-Cell proliferation is restricted with advanced aging and drops to 0.1 – 0.3% in older (> 1 year) mice (38, 77). β-Cell mass adaptation or regeneration in response to diet-induced obesity, pregnancy, glucocorticoid-induced insulin resistance and pancreatic damage have all been associated with increased number of proliferating β-cells in rodents. β-Cell proliferation was found to be increased from ~0.6% to 4.5% Ki67 positive β-cells after a high-fat diet for 8 weeks (40). Recently, Stamateris et al. (78) showed that β-cell proliferation already increases within the first week of high-fat diet feeding. In rodents and non-human primates, glucocorticoid treatment increases β-cell proliferation (60, 63, 79). In pregnant rodents, β-cell proliferation increases until mid gestation and then declines to prepartum levels (53, 55, 80). DNA analogue-based lineage-tracing in mice showed that β-cell mass adaptation following pregnancy was the result of β-cell replication and that no specialized progenitor cells were involved (81). Partial destruction of the β-cell mass using STZ resulted in an increased β-cell proliferation rate from ~0.6% Ki67 positive β-cells in control to 2.5% in STZ-treated mice 7 days after the treatment (40). In rats, β-cell regeneration following 90% pancreatectomy was associated with an increased mitotic index of β-cells that was about 3 - 4-fold higher than sham animals already after 3 days (82). Several lineage-tracing studies have demonstrated that proliferation of pre-existing β-cells are the major source for β-cell regeneration after a partial (50 – 70%) pancreatectomy or destruction of 70-80% of the β-cell mass in mice (81, 83, 84). The regenerative capacity of β-cell proliferation is

restricted with advanced age. Partial pancreatectomy, STZ and diet-induced obesity did not result in increased β-cell proliferation in aged mice (40, 76).

In humans, it is estimated that in normal individuals the β-cell mass is established in the second or third decade of life (85, 86) and that the highest peak of postnatal β-cell proliferation (1 - 2% of β-cells positive for the proliferation marker Ki67) occurs within the first months of life (22, 87, 88). This means that under normal conditions turnover of adult human β-cells is very low and that individual β-cells are long-lived (22, 85, 86). The reported occurrences of β-cell proliferation in adult human β-cells range from 0 - 0.07% (22, 37, 89, 90). Remarkably, the percentage of replicating β-cells in samples obtained directly at surgery was 0.5% and from frozen biopsies was 0.18% as determined by Ki67 staining (71, 91). This raises the question whether the ability to detect Ki67 is lost during certain conditions of tissue preparation and may therefore explain the differences in β-cell proliferation rates observed.

In humans, the increased β-cell mass observed in obese and pregnant subjects did not correlate with an increase of β-cell proliferation, using Ki67 as a marker for cell proliferation (31, 37, 57). Conversely, a study by Hanley et al. (36) reporting an increased β-cell mass in obese subjects found an increased percentage of β-cells positive for the marker proliferating cell nuclear antigen (PCNA). However, PCNA is also involved in DNA repair, which makes it a less specific marker for cell proliferation than Ki67 (92, 93). These studies have led to the current view that the replicative capacity of adult human β-cells is very limited and that other mechanisms may be responsible for the increased β-cell mass observed in obesity and during pregnancy. However, given the static nature of these cross-sectional studies, it cannot be excluded that the window of β-cell proliferation was missed. Furthermore, an increased number of proliferating β-cells in adult humans have been reported in areas adjacent to gastrinoma (94) and in patients with recent onset type 1 diabetes (95, 96) with a reported β-cell proliferation rate of 0.7% (Ki67 staining) in an 89-years-old patient (96). These studies show that adult human β-cells are able to proliferate under certain conditions.

Altogether the results from human and animal studies show that in both species the rate of β-cell proliferation reduces strongly with advanced age during both basal situations and in response to an increased insulin demand (Table 1). In young rodents, physiological or pathophysiological changes that result in a higher demand for insulin are associated with an increased number of proliferating β-cells. Whether this is also true for β-cell mass adaptation in young humans has not been investigated.

		Baseline	**Obesity/HFD**
Humans	*Young (2 months)*	1 – 2%	Not determined
	Adult (>20 years)	0 – 0.07%	0.02%
Mice	*Young (5 – 6 weeks)*	2.5%	4.5%
	Adult (~1 year)	0.1 – 0.3%	0.1 – 0.3%

Table 1. Reported percentages of β-cell proliferation (detected by Ki67 staining) in mice and humans.

β-Cell apoptosis

Another mechanism by which the β-cell mass can be regulated is apoptosis. In rodent models of diabetes that have a reduced β-cell mass, an increased rate of β-cell apoptosis is observed. In obese mice transgenic for human islet amyloid polypeptide (IAPP) that develop islet amyloid deposits similar to human type 2 diabetes, β-cell proliferation and neogenesis were increased comparable to non-transgenic mice (97). However, in transgenic mice a 10-fold increase in β-cell apoptosis prevented adequate β-cell mass expansion. Also, despite a similar β-cell proliferation in normoglycemic obese Zucker *fa/fa* rats and Zucker diabetic fatty (ZDF) rats, the latter develop diabetes most likely due to an increase in β-cell apoptosis (44). In humans, the decrease in β-cell mass observed in humans with type 2 diabetes is associated with an increased percentage of apoptotic β-cells, about 3-fold in obese and 10-fold in lean diabetic subjects compared to non-diabetic controls (31). Also Yoneda et al. (89) recently reported that the percentage of β-cells positive for the apoptosis marker TUNEL was 0.12% in patients with long-standing type 2 diabetes compared to 0% in healthy controls and newly diagnosed patients.

Reduction of the β-cell mass as a physiological response to a decreased insulin demand is associated with an increase of apoptotic β-cells in rodents. Involution of the β-cell mass in rats that had been infused with glucose for 2 days to expand the β-cell mass, was associated with an increased number of apoptotic β-cells (98). Also, transplantation of insulinomas in rats resulted in a reduction of the endogenous β-cell mass and an increase in β-cell apoptosis (99). Involution of β-cell mass postpartum was associated with an increase in β-cell apoptosis in rats (53). In the pancreas of postpartum women, β-cell apoptosis was rarely detected and similar to non-pregnant women (57). Because of the low frequency of β-cell apoptosis and the small number of women in the post-partum group, no conclusion could be drawn for the involvement of apoptosis in the involution of the β-cell mass in human pregnancy. Altogether, β-cell apoptosis is one of the mechanisms involved in decreasing the β-cell mass in type 2 diabetes. Studies in rodents have shown that β-cell apoptosis also plays a role in normal physiology when involution of the β-cell mass is required.

β-Cell neogenesis

β-Cell neogenesis, or the formation of new β-cells from pancreatic progenitor/stem cells, is a process that occurs during embryonic development of the endocrine pancreas and has been suggested to play a role in normal growth and β-cell adaptation (100). In rodents, regeneration of the endocrine pancreas after pancreatic damage coincides with an increased number of proliferating duct cells that seem to recapitulate embryonic development of the pancreas (101–103). This ductal origin of the regenerating β-cell mass has been challenged by several lineage tracing studies in rodents after pancreatic damage (104–106) and by a recent publication that did not notice regeneration of β-cell mass in response to duct ligation (107). Recently a systematic lineage tracing study showed that β-cell neogenesis predominantly occurs during embryogenesis

and is completely absent in adult mice using different models to stimulate β-cell regeneration: pregnancy, partial pancreatectomy, pancreatic duct ligation and chemical β-cell injury by STZ or Alloxan (108).

Measuring the extent of neogenesis in human cross-sectional histological studies is difficult since there is no marker to identity newly formed cells. The most common criteria for identification of neogenesis are insulin-positive cells in the pancreatic duct epithelium or tiny clusters (1 - 3 cells) of scattered insulin-positive cells in the pancreas (100). During human pancreatic development the percentage of insulin positive cells emerging and/or associated with ductal cells and duct cells positive for insulin is the highest in the prenatal period and drops to ~0.5% postnatal (22, 87). Both human pregnancy and obesity are associated with an increased number of insulin-positive duct cells, ~0.75% in obese and ~1% in pregnant subjects (31, 57). The latter percentage was not reverted postpartum. In patients with impaired glucose tolerance or with newly diagnosed type 2 diabetes an increase in β-cell neogenesis was reported (36, 89) suggesting that this represents an attempt for β-cell mass compensation. Also Mezza et al. (48) recently reported an increased number of cells positive for both insulin and the duct marker CK19 in insulin resistant non-diabetic subjects.

Altogether this has led to the current view that β-cell neogenesis from cells in the ductal compartment occurs during embryogenesis of the endocrine pancreas. However, so far, a major role in β-cell mass regeneration has not been shown using adult animal models (109). The contribution of β-cell neogenesis to β-cell mass adaptation in adult humans remains subject for future investigations.

Transdifferentiation

Transdifferentiation, or direct conversion, is a process characterized by the conversion of one mature cell into another mature cell without an intermediate pluripotent or progenitor state. Previously, it was thought that once cells become fully differentiated they could not switch their phenotype. It was shown that β-cells can be generated by forced expression of key transcription factors from pancreatic non-β-cells (74, 75, 110). The potential of transdifferentiation to contribute to β-cell mass regeneration was shown by Thorel et al. (111) in a model of near-total (>99%) β-cell ablation in which lineage tracing revealed that 65% of the regenerated β-cell mass originated from α-cells. Recently, the β-cell mass of alloxan-induced diabetic mice was regenerated by acinar-to-β-cell reprogramming through transient cytokine exposure (112). These studies have led to the suggestion that transdifferentiation of acinar- or α-cells may contribute to alterations in β-cell mass. In obese non-diabetic human donors, Hanley et al. found an increased number of acinar-associated insulin positive cell clusters that was related to an increased β-cell mass (36). Also, several studies have reported increased numbers of cells positive for both insulin and glucagon in patients with diabetes (113), newly diagnosed diabetes patients (89) or insulin resistant subjects (48). It is evident that there are cells present in the human pancreas

that are positive for both insulin and glucagon. Whether these double positive cells are newly formed endocrine cells derived from progenitor cells or α- or β-cells converting into β- or α-cells, respectively, remains an open question and difficult to assess given the limitations of human tissue samples. Future research should elucidate the role of transdifferentiation in β-cell mass adaptation.

Factors involved in β-cell mass adaptation

Numerous factors have been suggested to play a role in adaptation of the β-cell mass to changes in insulin demand. The first stimulus suggested was obviously glucose, however, the discovery of many hormones and other growth factors that can influence β-cell proliferation and function suggested that glucose has many coworkers (Fig. 4).

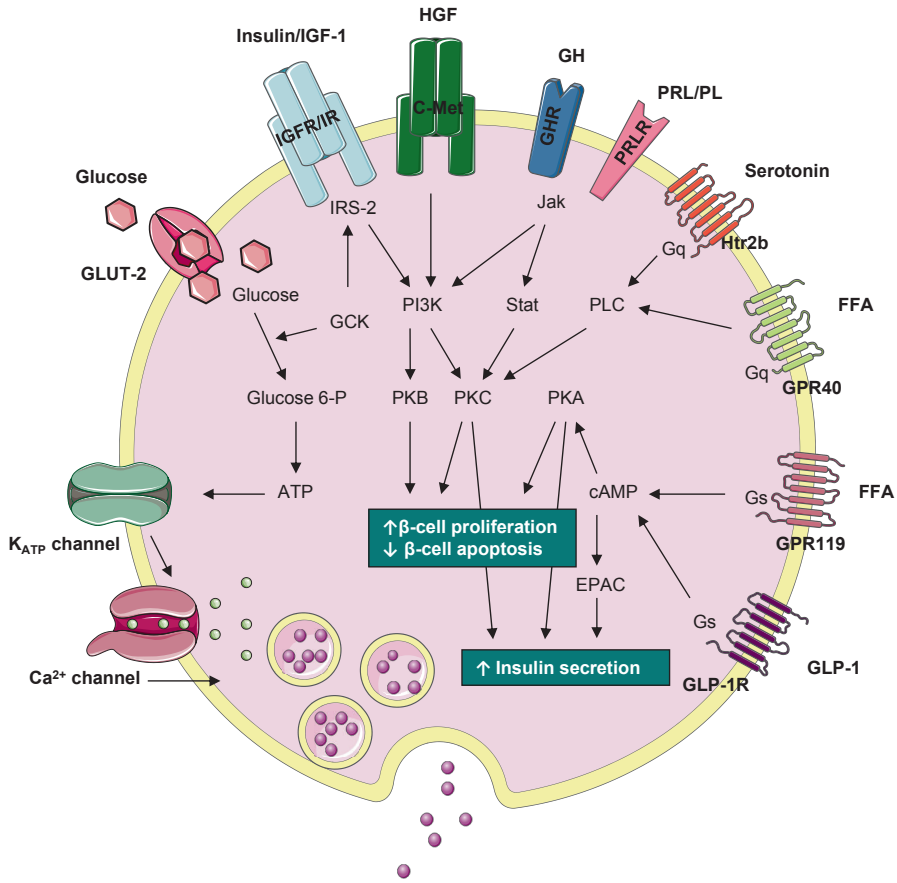

Figure 4. Illustration of several potential factors and some of the multiple signaling pathways that have been reported to be involved in adaptation of β-cells (16, 114, 115). Oxidative metabolism of glucose entering the cell via glucose transporter 2 (Glut-2) leads to an increase in the ATP/ADP ratio. This results in closure of ATP-dependent potassium channels. The subsequent membrane depolarization results in an influx of Ca^{2+} through the opening of voltage-gated calcium-channels. The increase in intracellular calcium concentration stimulates the fusion of insulin granules to the cell membrane and exocytosis of insulin. Increased glucokinase (GCK) activity and activation of the insulin/IGF-1 receptor (IGFR/IR) lead to phosphorylation of insulin receptor substrate 2 (IRS-2) activating a cascade of downstream molecules including phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB) that are associated with increased β-cell proliferation and decreased β-cell apoptosis. Hepatocyte growth factor (HGF) binding to the HGF receptor (HGFR) is also associated with β-cell proliferation via activation of PI3K/PKB signaling. The binding of growth hormone (GH) to the GH receptor (GHR), and prolactin (PRL) or placental lactogen (PL) to the prolactin receptor (PRLR), are associated with activating Jak/Stat signaling pathway leading to activation of protein kinase C (PKC) that is associated with increased β-cell proliferation, decreased β-cell apoptosis, and enhanced insulin secretion. Activation of the G_q-protein coupled receptors Htr2b and GPR40 leads to activation of phospholipase C (PLC), which then activates PKC. Binding of glucagon-like peptide-1 (GLP-1) to the G_s-protein coupled receptor for GLP-1 (GLP-1R) leads to an increase in cAMP levels. cAMP signals are transduced via the exchange protein activated by cAMP (EPAC) or cAMP-dependent protein kinase A (PKA) leading to augmentation of glucose-induced insulin secretion. Activation of PKA also activates signaling pathways involved in β-cell proliferation and survival. Activation of the G_s-protein coupled receptor GPR119 is associated with increased levels of cAMP and improved insulin secretion.

Glucose and insulin

Infusion of glucose results in an increase of the β-cell mass in rats (98, 116). However, there is much debate whether it is glucose itself or the accompanying increase of insulin that is the main trigger for β-cell mass to adapt (117). The critical role for insulin signaling in β-cells became apparent in a model of β-cell specific deletion of the insulin receptor (IR) (βIRKO) in mice (118) that results in glucose intolerance and impairment of high-fat diet-induced β-cell mass adaptation (119). Also, agonism of IR results in signaling through insulin receptor substrate (IRS)-1 and IRS-2. Mice globally deficient in IRS-1 become insulin resistant, but not diabetic because of a compensatory growth in β-cell mass whereas failure of compensation in IRS-2 knock-outs results in diabetes (120). Together this points to an important role for insulin-stimulated IR-IRS-2 signaling in β-cell mass adaptation. However, insulin by itself does not lead to an increase in the β-cell mass. Transplantation of insulinomas in rats results in profound hypoglycemia and a reduction of the endogenous β-cell mass (99, 121). Therefore, it is thought that insulin signaling pathways in β-cells play a more permissive role for β-cell expansion (117). A double knockout of the genes encoding insulin 1 and 2 in mice resulted obviously in fetal growth retardation, diabetes and neonatal lethality, however, these mice exhibited an increased islet mass showing that even in absence of insulin β-cell mass can increase (122).

The importance of glucose metabolism for β-cell mass adaptation was shown in mice haploinsufficient for β-cell glucokinase (GCK+/- mice) (123). In β-cells, GCK catalyzes the rate-limiting step in glucose metabolism and is considered to be the glucose-sensor for regulating glucose-induced insulin secretion (117). GCK+/- mice were unable to increase their β-cell mass when challenged with a high-fat diet, an effect that was mediated by IRS-2 (123). This study illustrates the involvement of glucose metabolism in β-cell mass adaptation that includes cross talk to the insulin signaling pathways. More recently, Porat et al. (124) confirmed the key role for glucose metabolism in regulating β-cell proliferation by showing that β-cell specific knockout of GCK in mice decreases β-cell proliferation and mass and that treatment of mice with a GCK activator resulted in increased β-cell proliferation and mass.

Incretins

The observation that intrajejunal infusion of glucose resulted in a higher insulin secretory response compared to an intravenous glucose injection resulted in the hypothesis that the intestinal wall may be the origin of an insulinogenic mechanism (125). The two most important gut hormones responsible for this effect are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (126). GIP is secreted by intestinal K-cells, whereas GLP-1 is secreted by intestinal L-cells in response to carbohydrate or fat intake. Both hormones are rapidly inactivated by the enzyme dipeptidyl-peptidase-IV (DPP-IV) *in vivo* (127). Mice with a double knock-out for the GLP-1 receptor (GLP-1R) and the GIP receptor showed less β-cell adaptation in response to high-fat diet feeding than control mice, which emphasizes the role for these hormones in the

regulation of β-cell mass (128). GLP-1R agonists, more than agonists of the GIP receptor, appear to be involved in β-cell survival and regeneration following pancreatic damage by STZ (129).
In animal models of diabetes GLP-1 or GLP-1R agonist treatment results in increased β-cell proliferation and β-cell mass (130, 131). Also, studies have reported that GLP-1R activation improves regeneration of the β-cell mass following partial pancreatectomy and STZ-induced pancreatic damage in rodents (132–134). However, activation or inactivation of the GLP-1R does not attenuate the endogenous β-cell mass adaptation in insulin resistant *ob/ob* mice (131, 135). Also, it has been shown that aging negatively affects the ability of the β-cell mass to expand in response to incretins in mice (40, 76). Altogether this shows that GLP-1 receptor agonism contributes to β-cell mass regeneration in animal models of diabetes, especially in younger rodents.
In the past decade, numerous GLP-1-based therapies have become available for patients with type 2 diabetes, leading to an improvement of glycemic control (136, 137). There is some evidence that this improved glycemic control may partly be attributed to an improvement of β-cell function (138, 139). *In vitro* it has been shown that incretin therapy has a beneficial effect on survival of isolated human islets by decreasing islet-cell apoptosis (140). In pancreas tissue from donors with type 2 diabetes a 6-fold increase in β-cell mass was observed in patients receiving incretin-based therapies (113). Moreover, the β-cell mass was 3-fold higher compared to non-diabetic controls. No difference in β-cell proliferation was observed. However, these β-cells were probably not functional since the patients still had diabetes. This paper has been criticized because of methodological deficiencies, which may limit interpretation of the results (141). Whether incretins or incretin-based therapies can increase or stabilize the β-mass in adult patients with diabetes remains an open question.

Adipose tissue-derived factors

Obesity is associated with an increased release of free fatty acids (FFA), adipokines such as leptin and resistin, and proinflammatory cytokines from adipose tissue that may affect insulin sensitivity of the muscle and liver leading to insulin resistance (16). Some of these factors have also been described to affect β-cell mass regulation. Leptin is a key hormone in the control of food intake, energy expenditure, metabolism, body weight, and glucose homeostasis. *Ob/ob* and *db/db* mice, which have defects in leptin or in the leptin receptor, respectively, become severely obese, insulin resistant and have an increased β-cell mass. Since β-cells express leptin receptors, leptin has been implicated as a negative regulator of β-cell mass (142). In a mouse model of pancreas-specific (using the Pdx1 promoter) knock-out of the leptin receptor β-cell mass adaptation was hampered after feeding a high-fat diet may point to a direct effect of leptin on β-cell turnover (143). Furthermore, Park et al. (144) reported that central infusion of resistin increased β-cell mass by β-cell proliferation in pancreatectomized diabetic rats. Also, β-cells abundantly express the nutrient sensing G-protein coupled receptor GPR40, which can bind medium- and long-chain

fatty acids leading to glucose-dependent insulin secretion (145, 146). Similarly, the 'fat sensor' GPR119 is highly expressed on β-cells and activation results in release of incretins leading to enhancement of insulin secretion (147, 148). Whether activation of these FFA receptors can also directly affect β-cell mass and proliferation remains to be investigated. Altogether these studies show that several adipose tissue-derived factors can directly modulate β-cell function and mass.

Liver-derived factors

The liver plays a central role in glucose homeostasis, as it is one of the primary sites for storage of glucose and generation of glucose from noncarbohydrate precursors, processes that are regulated by insulin and glucagon (8). Liver-specific insulin receptor knock-out (LIRKO) mice have severe insulin resistance and marked hyperinsulinemia due to an increased β-cell mass (47). This suggests that the liver plays an important role in regulating the β-cell mass. It was shown that regulation of β-cell mass occurred through neuronal signals from the liver (149). Furthermore, several factors produced in the liver have been reported to increase β-cell mass adaptation. Hepatocyte growth factor (HGF) is increased in mice fed a high-fat diet and pharmacological inhibition of HGF resulted in impaired β-cell mass adaptation to diet-induced obesity (150). It was shown that one of the stimuli for β-cell adaptation in LIRKO mice, is a systemic hepatocyte-derived growth factor(s) that was also able to increase proliferation of human β-cells *in vitro* (151). Finally, chemically induced insulin resistance resulted in the discovery of betatrophin, a protein that is enriched in liver and fat tissues, and potently stimulates β-cell proliferation (152). Also, this study reported that expression of increased betatrophin in the liver of pregnant mice and in diabetic *ob/ob* and *db/db* mice, which emphasizes its involvement in β-cell mass adaptation (152). However, transplantation of human islets into insulin resistant mice with elevated concentrations of betatrophin, did not enhance human β-cell proliferation (153). Recently, a study reported that both patients with type 1 and type 2 diabetes have increased circulating concentrations of betatrophin compared to healthy controls (154, 155). This may suggest that a potential stimulus for β-cell proliferation is present in patients with diabetes; however, this is insufficient to increase the number of β-cells.

Pregnancy-related factors

Placental lactogen and prolactin are both members of the growth hormone/prolactin/placental lactogen family and have been described to be involved in the regulation of β-cell mass adaptation during pregnancy in rodents. Placental lactogen is secreted by the placenta and prolactin and growth hormone by the pituitary gland. Placental lactogen and prolactin can both bind the prolactin receptor (PRLR), which is expressed on β-cells (156). In rats, gene expression of PRLR and the growth hormone (GH) receptor were increased in the pancreas during pregnancy (157). Recently it was reported that deletion of the GH receptor in β-cells was associated with a lack of compensatory β-cell mass adaptation in response to HFD-induced obesity (158). β-Cell

proliferation in pregnant mice followed the same temporal pattern as serum concentrations of placental lactogen, suggesting a causal relationship (80). Overexpression of placental lactogen in normal mice resulted in increased β-cell proliferation and islet mass that was associated with hypoglycemia (159). Also, β-cell proliferation and β-cell mass adaptation was impaired in pregnant mice carrying a heterozygous PRLR null mutation (160). Furthermore, it was demonstrated that lactogenic signaling is associated with an increase in serotonin production by β-cells that activates β-cell proliferation in a paracrine/autocrine way (161). Inhibition of serotonin synthesis blocks β-cell mass expansion in pregnant mice. Interestingly, during pregnancy the expression of the stimulatory G_q-coupled serotonin receptor Htr2b was high but normalized at the end of gestation, while expression of the inhibitory G_i-linked serotonin receptor Htr1d was increased shortly before parturition and associated with cessation of β-cell proliferation and regression of β-cell mass (161). This suggests that the effect of serotonin on β-cell adaptation can be modulated by a shift in the receptor expression.

α-Cell mass adaptation

When in 1921 Frederick Banting and Charles Best tested their first crude pancreatic extract in a pancreatectomized dog, which would lead to the Nobel Prize awarded discovery of insulin, they noticed mild hyperglycemia preceding the insulin-induced hypoglycemia. This was attributed to the presence of a substance that mobilized glucose, therefore named 'glucagon' (9, 162). Until recently, most research has focused on how β-cells adapt to physiological and pathophysiological changes in the glucose metabolism. Therefore, little is known about α-cell adaptation during changing metabolic demands. Both type 1 and type 2 diabetes are characterized by a disrupted glucagon-insulin balance due to an absolute or relative hypoinsulinemia leading to insufficient suppression of glucagon secretion (9, 163). The subsequent (relative) hyperglucagonemia aggravates the consequences of hypoinsulinaemia because of an increased glucose output from the liver (9). This is illustrated by the observation that glucagon receptor knockout mice are protected against the development of streptozotocin-induced diabetes in mice (164). Also, GLP-1RA decreases glucagon secretion, which is one of the mechanisms by which this therapy improves glucose homeostasis in patients with type 2 diabetes (165). In non-human primates that spontaneously develop insulin resistance associated with obesity and type 2 diabetes, an increased islet amyloid deposition is associated with increased α-cell proliferation leading to an imbalance in the α- to β-cell ratio (166). Recently this same group showed that in overweight insulin-resistant baboons the α-cell volume was significantly increased, even preceding changes in β-cell volume (167). Also in mice fed a high-fat diet for 8 weeks, α-cell mass was increased in the absence changes in β-cell mass (168). In patients with type 2 diabetes a higher α- to β-cell ratio has been reported, that is due to a decrease in β-cell mass rather than an increase in α-cell

mass (169). In this study the α- to β-cell ratio did not change when comparing obese versus non-obese individuals. This suggests that the extent of α-cell mass adaptation is similar to β-cell mass adaptation.

Aims and structure of this thesis

The aim of the research described in this thesis was to investigate β- and α-cell adaptation in response to different metabolic changes.

Although it has been recognized for a long time that the pancreas is a regionally heterogeneous organ, it is unknown whether β-cell adaptation also occurs heterogeneously throughout the pancreas. **Chapters 2 – 4** describe studies in which we assessed whether β-cell adaptation is topologically homogenous throughout the pancreas in response to an increased demand for insulin in different species. In **chapter 2**, we examined early events of β-cell adaptation in different regions of the pancreas of high-fat diet induced insulin resistant mice. **Chapter 3** describes β-cell adaptation throughout the pancreas in dexamethasone-induced insulin resistant rats. Glucocorticoid-induced insulin resistance occurs within 5 days of treatment (170) and is therefore an acute stimulus for β-cell adaptation. In **chapter 4**, findings from rodent studies are translated to humans. In this chapter we examined β- and α-cell adaptation in different regions of the pancreas from lean and obese human donors.

In **chapter 5**, we studied the effect of one of the most potent stimuli involved in the regulation of β-cell mass and function, GLP-1R activation, on β- and α-cell adaptation under normoglycemic conditions in mice. In animal models of diabetes, incretin-based therapies increase β-cell mass. GLP-1R agonist treatment is also associated with a reduced blood pressure, improved lipid profiles and endothelial function and may therefore also be of benefit for non-diabetic individuals with obesity or cardiovascular disease (171–174). However, the effect of GLP-1R agonist under normoglycemic conditions on β- and α-cells is unclear.

Chapter 6 describes a study in which the influence of a long-term high-fat low-carbohydrate ketogenic diet on glucose tolerance and β- and α-cell adaptation in mice was assessed. Nutrition plays an important role in the development of diabetes and can directly affect β-cell growth and function (98, 115, 116, 145, 147). In many popular diets the amount of fat is substantially increased at the cost of carbohydrates. Thereby the body is forced to use fats instead of carbohydrates as a primary source of energy. We investigated whether these changes in glucose metabolism are associated with changes in β- and α-cells on the long term.

Diabetes mellitus results from an absolute or relative deficiency of functional β-cells leading to an impaired glucose homeostasis. For patients with diabetes, therapies are needed that restore, maintain or prevent loss of functional β-cells. Insight in mechanisms relevant for the protection or improvement of β-cell function is therefore important. Currently, there is no robust technique

available to measure the β-cell mass or function of humans *in vivo*. Also, existing *in vitro* assay platforms are mostly using rodent-derived cell lines and are set up to assess insulin gene expression or protein content (175–178). Following glucose stimulation only a fraction of the total insulin content is secreted from β-cells, which makes these existing read-outs poor indicators of secretory function (14). Therefore, there is a strong need for a robust assay platform using human islets to study β-cell function in order to find novel mechanisms involved in human β-cell function and adaptation to changing metabolic demands. In **chapter 7** we describe three culture platforms using primary human islets in which β-cell function can be assessed. These platforms can be used for high-throughput screening assays to identify novel mechanisms involved in β- and α-cell adaptation.

Chapter 8 summarizes the findings and aims to place various aspects of this thesis in the context of current literature about β- and α-cell adaptation.

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