Chapter 8

Summary and general discussion

Diabetes mellitus affects approximately 1 million people in The Netherlands (1). Diabetes is characterized by an absolute or relative deficiency in insulin secretion from β-cells, leading to an impaired glucose homeostasis. For these patients, therapies that restore, maintain or prevent loss of functional β-cells are needed. Therefore, it is critical to understand how the β-cell mass is regulated. When the demand for insulin is chronically increased by physiological or pathological changes, the endocrine pancreas can adapt by increasing insulin secretion via an enhanced β-cell function and/or by increasing β-cell mass in order to maintain glucose homeostasis. Both obesity and pregnancy lead to insulin resistance and multiple studies have associated these conditions with an increased β-cell mass in humans (2–9). Inadequate β-cell adaptation leads to the development of hyperglycemia and eventually diabetes mellitus.

β- And α-cell adaptation are topologically heterogeneous

The pancreas is a regionally heterogeneous organ. During embryonic development the pancreas develops from two epithelial buds. The ventral bud gives rise to the posterior part of the head and the uncinate process and the dorsal bud to the anterior part of the head, body and tail of the mature pancreas (10, 11). Pancreatic islets from the ventral bud contain more cells producing pancreatic polypeptide (PP), whereas islets from the dorsal bud contain more α-cells and secrete more insulin upon glucose stimulation (12, 13). Also, several studies show that the relative area of islets in the tail-region of the pancreas is higher compared to the head and body region in humans (6, 14, 15). However, it is unknown whether β-cell adaptation to an increased insulin demand occurs homogeneously throughout the pancreas.

In **chapter 2** we describe a study in mice, which were fed a high-fat diet (HFD) to induce insulin resistance or a control diet. The pancreas was divided in a duodenal, gastric and splenic region (corresponding to the head, body and tail-region of the human pancreas, respectively) (Figure 1) and β-cell mass, β-cell proliferation and insulin secretory function of islets were studied. After 6 weeks of diet no change in β-cell mass was apparent yet, however, β-cell proliferation and glucose-induced insulin secretion were significantly higher in islets derived from the splenic region compared to islets derived from the duodenal and gastric region of the pancreas (Table 1). We therefore conclude that β-cell adaptation is topologically heterogeneous in response to HFD in mice. Also, α-cell mass was found to be decreased in the splenic region only after 6 weeks HFD (data not shown).

Next, we assessed whether β-cell adaptation is topologically heterogeneous in a different animal model of insulin resistance. Glucocorticoid-induced insulin resistance occurs within 5 days of treatment (16) and is therefore an acute stimulus for β-cell adaptation. We investigated β-cell adaptation throughout the pancreas in glucocorticoid-induced insulin resistance in rats (**chapter 3**). After 6 weeks, the β-cell area was significantly increased in DXM-treated rats, and this increase

mainly occurred in the splenic region of the pancreas. This increase was associated with an enlarged β-cell cluster size while no change in β-cell proliferation was observed after 3 and 6 weeks of treatment.

Subsequently, we wondered whether β-cell mass adaptation in humans would be topologically heterogeneous as well. **Chapter 4** describes a study in which we examined the β-cell mass and glucagon-producing α-cell mass of 15 non-diabetic obese and 15 lean age-matched human subjects in the head (excluding regions that were rich in PP cells), body and tail region. Both β- and α-cell area were the highest in the tail-region of the pancreas (Table 1). In obese subjects β- and α-cell mass were increased and both β- and α-cell area were significantly higher in the head-region of the pancreas compared to lean controls, whereas islet density was significantly increased in the tail-region. The α- to β-cell ratio was similar throughout the pancreas and preserved following adaptation in non-diabetic obese subjects. Altogether these data show that in obese human subjects β- and α-cell mass adaptation is topologically heterogeneous.

	Model	Head / DR	Body / GR	Tail / SR
Insulin secretory function	Mice	=	=	↑
β-Cell mass	Mice	=	=	↑
	Rats*	=	=	↑
	Humans*	↑	=	=
α-Cell mass	Mice	=	=	↓
	Humans*	↑	=	=

Table 1. Changes in insulin secretory function, β-cell area and α-cell area of different pancreatic regions compared in HFD-fed vs. control mice (Mice), DXM-treated vs. control rats (Rats) and obese vs. lean human subjects (Humans). *β-cell area. DR = duodenal region, GR = gastric region, SR = splenic region.

Human versus rodents

In this thesis we show for the first time that β-cell adaptation to an increased insulin demand is topologically heterogeneous throughout the pancreas of mice, rats and humans (Table 1, **chapters 2 - 4**). In rodents, islets derived from the splenic region of the pancreas are involved in the first line of response in β- and α-cell adaptation. In obese humans the islet density was mostly increased in the tail-region of the pancreas. Nevertheless, the most prominent increase in β-cell area was observed in the head-region of the pancreas of obese human donors; which also appeared to be the region showing a preferential loss of β-cells in patients with type 2 diabetes (17). Dissimilarities observed between the study results from humans versus rodents can obviously be attributed to differences in species. Several differences between rodents and human endocrine pancreas have been observed (18). The gross morphology of the mature pancreas is different between rodents and humans (Fig. 1A, B). The rodent pancreas has a lobular structure of loosely connected tissue that aligns the spleen, stomach and upper part of the intestine whereas the human pancreas is a single, compact organ surrounded by a fibrous stroma. In rodents, the

majority of the islets consists of β-cells that are surrounded by a single layer of α-cells, whereas in humans endocrine cells are more mixed throughout the islets resulting in more heterologous contacts between α- and β-cells (Fig. 1 C, D) (19, 20). Also, the proportion of β-cells in humans islets is on average 55% versus 77% in mice, whereas about 38% of the human islet is composed of α-cells compared to 18% of the mouse islet (19). In humans, we show that the β-cell area is the highest in the tail-region of the pancreas (**chapter 4**); which is in line with previous observations in human donor pancreases (6, 14, 15). However, the β-cell area was similar throughout the pancreas of mice fed a control diet for 12 weeks (**chapter 2**). Hornblad et al. (21) reported that the β-cell area was the highest in the head-region of the pancreas of 8 weeks old mice. Together these studies indicate that the head-region of the pancreas in humans may not necessarily correlate to the head-region of the mouse pancreas.

Furthermore, in the splenic region of the pancreas in HFD-fed mice we observed an increase in β-cell proliferation (BrdU labeling for 7 days), whereas β-cell proliferation (identified by Ki67 as a proliferation marker) was not changed in DXM-treated rats after 3 and 6 weeks of treatment. Previous studies have observed increased β-cell proliferation after 3 days of DXM-treatment (22–24), suggesting that the peak in β-cell proliferation induced by DXM-treatment in our study occurred within the first 3 weeks. β-Cell proliferation was rarely observed in the human pancreas donors and was not different between regions. It should be noted that the animals in our studies (mice and rats were ~8 weeks old at the start of the study) could be considered young adults, whereas the average age in our study of human pancreas donors was approximately 50 years. As it is well known that β-cell proliferation and adaptation are negatively affected by ageing (25, 26), this may have contribute to observed differences as well.

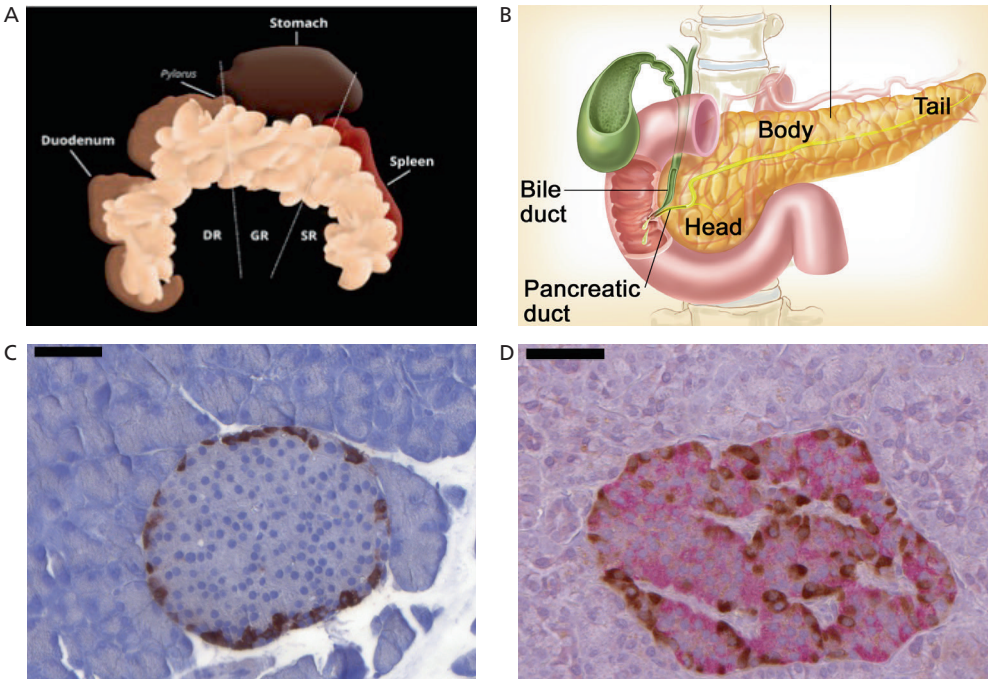

Figure 1. Mouse versus human pancreas and islets. A. Gross anatomy of mouse pancreas, DR = duodenal region, GR = gastric region, SR = splenic region. B. Gross anatomy of human pancreas. *Reprinted with permission from Terese Winslow*. C. Representative image of a mouse islet, with α-cells (brown) surrounding β-cells (center of the islet). Scale bar = 50 μm. D. Representative image of a human islet, α-cells (brown) are intermingled with β–cells (red). Scale bar = 50 μm.

Mechanisms and stimuli of topologically heterogeneous β-cell adaptation

The observed regional heterogeneity in β-cell adaptation in response to a HFD stimulus, DXM-treatment or in obese humans could be explained in two ways: the islets from different pancreatic regions are intrinsically different or, the islets receive distinct extrinsic signals from their microenvironment within the pancreas. In **chapter 2** we assessed this latter hypothesis, by transplantation of untreated mouse islets from the three pancreatic regions to an extrapancreatic location in diabetic mice, in which the increased demand for insulin will stimulate β-cell regeneration. After 10 days, no difference between islets isolated from different regions was found. These results suggest that the observed topological heterogeneity of β cell adaptation in HFD-fed mice is most likely the result of distinct extrinsic signals present in the microenvironment of the islet within the pancreas.

Stimuli that have been identified to affect β-cell proliferation comprise several growth factors and hormones. These proteins are often produced by other organs than the pancreas, such as the liver, adipose tissue and the intestine, and released in the vasculature. Islets are highly vascularized to enable efficient secretion of insulin and glucagon into the circulation; they receive

per unit weight about 20 times more arterial blood compared to the exocrine pancreas in rats (27, 28). Differences in vascular density between pancreatic regions could result in heterogeneous exposure to growth factors and hormones. By using *in vivo* labeling methods, Lau et al. (29) characterized a subpopulation of mouse islets (5%) with a greater blood perfusion and vascular density that were associated with increased β-cell function and proliferation. Recently, intravital blood vessel labeling revealed that the islet vascular supply increases during insulin resistance by dilation of preexisting vessels in mice (30). In addition, this same study showed that islets of insulin resistant mice have increased global islet innervation visualized by the labeling of the neuronal marker neuronal class II β-tubulin (TUJ1). Islets are densely innervated by the autonomic nervous system (31) and it was reported that obesity-induced β-cell mass expansion is regulated through neuronal signals from the liver (32).

It remains unclear whether changes in vascular supply or innervation are the cause or consequence of β-cell mass adaptation. Islet cells produce angiogenic factors including vascular endothelial growth factor (VEGF)-A, which is one of the principal regulators of vascular homeostasis (33). Interestingly, VEGF-A expression was increased in the subpopulation of islets having a greater blood perfusion (29). Also, it has been reported that α-cells of human islets provide cholinergic signals to neighboring β-cells, thereby priming β-cell function in a paracrine way (34).

Furthermore, islets are structurally and functionally closely related to the exocrine pancreas. This is referred to as the islet-acinar axis, in which exocrine functions are regulated by insulin and somatostatin (35). The content of one of the main enzymes produced by acinar cells, amylase, was found to be higher in the dorsal region compared to the ventral region of the pancreas in rats (36). Also, in the field of regenerative studies there appears to be a strong link between exocrine acinar cells and β-cells, which share their endodermal origin. One of the first studies showing that adult cells can be reprogrammed into another adult cell type, without reversion to a pluripotent stem cell state, showed the conversion of adult exocrine cells to β-cells by expressing three transcription factors (*Ngn3*, *Pdx1* and *MafA*) in mice (37). Recently it was shown that the β-cell mass of alloxan-induced diabetic mice was regenerated by acinar-to-β-cell reprogramming, without genetic modification, through transient cytokine exposure (38). In addition to demonstrating a potential source for de novo β-cell generation, these studies illustrate the intimate relation between islets and its exocrine environment. Future research should clarify whether the exocrine tissue surrounding islets is involved in the regulation of β-cell adaptation.

Implications for future research

Most importantly, the results in **chapters 2 - 4** imply that quantification of the β- and α-cell mass in animal or human pancreases should be based on representative samples throughout the entire organ. In most histological studies of α- and/or β-cell adaptation the head-region of the human pancreas was not included (5–7, 39, 40), which may have led to an incorrect estimation of actual changes in these studies. Furthermore, comparison of regional differences in β-cell adaptation may lead to the identification of novel factors involved in β-cell mass growth and function.

β-Cell adaptation in response to different metabolic stimuli

In this thesis we studied β-cell adaptation in response to different metabolic changes (Table 2). One of the main stimuli for β-cell adaptation is insulin resistance. In **chapters 2 – 4** we show that insulin resistance is associated with an increased β-cell mass in different species. High-fat diet induced insulin resistance in mice led to an increased β-cell function, β-cell proliferation and β-cell mass as a compensatory response to the increased demand for insulin (**chapter 2**). Also in human obesity, which is associated with insulin resistance, an increased β-cell mass was observed (**chapter 4**). In **chapter 3** we show that DXM treatment results in an increased insulin secretory response after a glucose load that is associated with an increased β-cell mass after 3 weeks.

One of the most potent hormones that can enhance both β-cell function and β-cell proliferation is the incretin glucagon-like peptide-1 (GLP-1). In animal models of diabetes, GLP-1 receptor agonist (GLP-1RA) treatment increases the β-cell mass (41–43). GLP-1 based therapies improve glycemic control in patients with type 2 diabetes and are associated with reduced blood pressure, improved lipid profiles and improved endothelial function (44, 45). Therefore, these compounds have also been evaluated in non-diabetic individuals with obesity and cardiovascular disease (46–49). However, their effect on β-cell mass in these normoglycemic conditions, in which there is no increased demand for insulin, is not clear. In **chapter 5** we studied the effects of the GLP-1RA liraglutide on β-cell mass and function in normoglycemic mice. Mice were treated with liraglutide or PBS and fed a control or HFD for 1 or 6 weeks. Treatment with liraglutide for 6 weeks led to increased insulin sensitivity and attenuation of HFD-induced insulin resistance. After 6 weeks of treatment a reduction in β-cell mass was observed in liraglutide-treated control and HFD-fed mice. This was associated with a lower β-cell proliferation rate after 1 week of treatment. Islets isolated from liraglutide-treated control mice showed an enhancement of glucose-induced insulin secretion. Together these data show that GLP-1RA treatment in normoglycemic mice leads to increases in insulin sensitivity and β-cell function that are associated with a reduction in β-cell mass in order to maintain normoglycemia.

Nutrients like glucose and free fatty acids can modulate β-cell mass growth and function (50–54). In many popular weight loss diets the amount of fat is substantially increased at the expense of carbohydrates. Such diets force the body to use fats instead of carbohydrates as primary source of energy. However, the long-term effects of these high-fat low-carbohydrate ketogenic diets (KD) on pancreatic endocrine cells are unknown. We hypothesized that a long-term KD creates a metabolic environment in which there is a decreased demand for insulin and an increased demand for glucagon to stimulate gluconeogenesis. **Chapter 6** describes a study in which mice were fed a KD for 22 weeks. Despite an initial weight loss, KD did not result in weight loss after 22 weeks. Long-term KD resulted in glucose intolerance that was associated with insufficient insulin secretion from β-cells. After 22 weeks, the β-cell mass was found to be reduced in KD-fed mice compared to controls. Together our data show that long-term KD causes dyslipidemia,

a proinflammatory state, signs of hepatic steatosis, glucose intolerance, and a reduction in β-cell mass, but no weight loss. This indicates that long-term KD leads to features that are also associated with the metabolic syndrome and an increased risk for type 2 diabetes in humans.

Model	Species	Insulin secretory function	β-Cell mass
Obesity	Humans		↑
DXM	Rats	↑	↑
HFD	Mice	↑	↑
GLP-1RA	Mice	↑	↓
HFD + GLP-1RA	Mice	↑	↓
KD	Mice	↓	↓

Table 2. Changes in insulin secretory function and β-cell mass in obese vs. lean human subjects (Obesity), DXM-treated vs. control rats (DMX), HFD-fed vs. control mice (HFD), liraglutide-treated vs. control mice (GLP-1RA), liraglutide-treated HFD-fed vs. control mice (HFD + GLP-1RA), ketogenic diet-fed vs. control mice (KD). DXM = dexamethasone, HFD = high-fat diet, GLP-1RA = glucagon-like peptide 1 receptor agonist, KD = ketogenic diet.

Mechanisms of β-cell adaptation in response to different metabolic stimuli

GLP-1RA can lead to different effects on insulin secretion and β-cell proliferation under normoglycemic conditions

The effect of GLP-1-based therapies on insulin secretion from β-cells has been reported to be glucose-dependent (55). No insulin secretory response was observed from isolated perfused rat pancreas to GLP-1 stimulation at a glucose concentration of 2.8 mM, whereas insulin secretion was increased when glucose concentrations were raised to 6.6 and 16.7 mM (51). We show in **chapter 5** that sustained GLP-1RA treatment during normoglycemic conditions is associated with increased insulin secretion from isolated islets, in the absence of direct GLP-1RA stimulation. These results imply that GLP-1RA treatment during normoglycemic conditions enhances insulin secretion. In contrast, GLP-1RA treatment during normoglycemic conditions did not enhance β-cell mass in mice. Moreover, the increased insulin sensitivity and enhancement of insulin secretion resulted in a decreased need for new β-cells, resulting in a decrease in β-cell proliferation and a reduced β-cell mass in GLP-1RA treated normoglycemic mice. This is in line with the observation by Porat et al. that glucose-driven glycolysis is one of the key drivers for β-cell proliferation (56).
In our study, GLP-1RA treatment during normoglycemia resulted in increased insulin secretion whereas β-cell proliferation was reduced. Activation of the GLP-1R on β-cells leads to an increase in cyclic AMP (cAMP) concentrations, which is a key messenger in β-cells (Fig. 2) (55, 57). Activation of its signaling pathways has been reported to stimulate insulin secretion and β-cell proliferation (58). It is unknown how GLP-1RA treatment regulates these two different effects in

the β-cell. cAMP signals are transduced via two pathways in the β-cells, the cAMP-dependent protein kinase A (PKA) and the exchange protein activated by cAMP (EPAC). Both PKA and EPAC have been implicated in transducing the beneficial effects on β-cell function and the protection of β-cell mass (58, 59). Recent studies have shown the predominant role for PKA-dependent signaling for β-cell function *in vivo* (60, 61). PKA activity is transduced either to transcriptional events through PKA phosphorylation of the transcription factor cAMP response element-binding protein or by the formation of complexes with A-kinase anchoring proteins (AKAPs). AKAPs are a family of intracellular-signaling adaptor proteins that direct PKA to locations within the cell where it can exert specific effects (57, 62). Future research should elucidate which AKAP complexes are involved in regulation of insulin secretion or β-cell proliferation to understand the mechanisms by which cAMP/PKA signaling is regulating both β-cell function and mass.

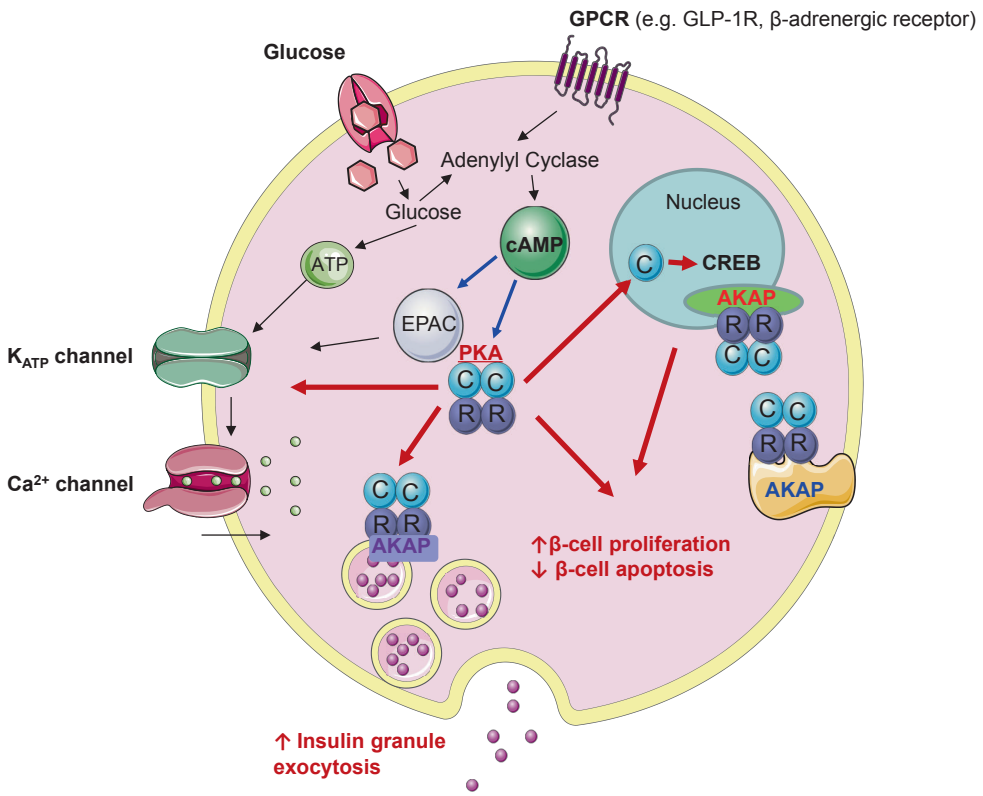

Figure 2. Schematic overview of cAMP/PKA signaling pathway in the β-cell. Red arrows indicate the potential different pathways by which cAMP regulates β-cell function and survival. GPCR=G-protein coupled receptor; GLP-1R=Glucagon-like peptide-1 receptor; cAMP=cyclic AMP; PKA=cAMP-dependent protein kinase A; EPAC=exchange protein activated by cAMP; CREB=cAMP response element-binding protein; AKAP=A-kinase anchoring proteins; R=regulatory subunit; C=catalytic subunit.

Long-term KD leads to a reduced β-cell mass and an insulin secretory defect

In **chapter 6** we show that a long-term ketogenic diet results in glucose intolerance most likely because of β-cell dysfunction and a reduction in β-cell number that result in inadequate insulin secretion. Already after 5 weeks of KD diet, mice show the first signs of glucose intolerance. The insulin secretory response is not increased to compensate for this increased demand for insulin. Ultimately insulin secretory function and the number of β-cells in mice fed a KD was reduced. This strongly suggests that the β-cell adaptive response and secretory function have become dysfunctional as a result of the long-term KD feeding. Similar to patients with type 2 diabetes (63), the reduction in β-cell mass was most prominent in the DR of the pancreas (data not shown). Long-term KD results in dyslipidemia, which can lead to β-cell dysfunction due to lipotoxicity. Chronic exposure of β-cells to increased concentrations of free fatty acids (FFA), reduces insulin secretion and induces β-cell apoptosis (64, 65). Excess FFAs can promote the expression of pro-inflammatory factors in islets, such as Il-1β (66). Il-1β, which was increased after long-term KD in our study, is a master regulator of inflammation and can inhibit insulin secretion and stimulate β-cell death (66, 67). Altogether, long-term KD leads to dyslipidemia and a pro-inflammatory state, which are associated with an impaired adaptive response of β-cell function and mass to KD-induced changes in glucose metabolism.

α-cell adaptation in response to different metabolic stimuli

Adaptation of α-cell mass in human obesity (**chapter 4**) and in normoglycemic mice receiving incretin therapy (**chapter 5**) was similar to changes in the β-cell mass and resulted in maintenance of the α- to β-cell ratio (Table 3). It has been reported that human islets prefer heterologous contacts between β- and α-cells (20). Also, insulin secretion from individual human β-cells is enhanced when they are coupled to an α-cell (68). This functional connection between β- and α-cells may explain the maintenance of the α- to β-cell ratio following adaptation to metabolic stimuli.

In contrast, the α- to β-cell ratio in long-term KD mice was decreased due to the considerable reduction of α-cell mass. This change can be a direct consequence of KD or a response to counteract glucose intolerance. During ketosis, glucagon stimulates hepatic glucose production and lipolysis to generate energy. In **chapter 6** we show that long-term KD results in a reduced insulin-stimulated glucose uptake. This could result in a negative feedback to glucagon-producing α-cells resulting in less gluconeogenesis and no further worsening of the blood glucose concentration. This is supported by the observation that circulating glucagon concentrations were decreased after 5 weeks of KD in mice (69). In order to maintain glucose homeostasis, the rate of glucose entering the circulation should be balanced by the removal of glucose out of the circulation. In this process both insulin and glucagon play a major role. Past research has shown that the insulin producing β-cell mass can adapt to changing metabolic demands (reviewed in **chapter 1**). Little

is known about the involvement of the α-cell mass in this process. In **chapters 4 - 6** we show that, in addition to adaptation of the β-cell mass, metabolic changes also affect the α-cell mass. Interestingly, in **chapter 5** we show changes in α-cell mass preceding adaptation of the β-cell mass in HFD-fed mice, which is in line previous observations in mice and non-human primates (70, 71). An imbalance between glucagon and insulin characterizes both type 1 and type 2 diabetes (72, 73). This suggests that failure of both β- and α-cell adaptation can contribute to the development of diabetes. Future research on β-cell adaptation should therefore also study changes in α-cell mass and function.

Model	Species	α-cell mass	Ratio α- to β-cells
Obesity	Humans	↑	=
HFD	Mice	↓	=
GLP-1RA	Mice	↓	=
HFD + GLP-1RA	Mice	↓	=
KD	Mice	↓	↓

Table 3. Changes in α-cell mass and the ratio α- to β-cells in obese vs. lean human subjects (Obesity), HFD-fed vs. control mice (HFD), liraglutide-treated vs. control mice (GLP-1RA), liraglutide-treated HFD-fed vs. control mice (HFD + GLP-1RA), ketogenic diet-fed vs. control mice (KD). HFD = high-fat diet, GLP-1RA = glucagon-like peptide 1 receptor agonist, KD = ketogenic diet.

Mechanistic studies of human islet adaptation

For studying β- and α-cell adaption in human islets, we currently depend on histological analyses of biopsies taken at autopsy or after pancreatectomy generating a static picture. Animal models can provide more mechanistic insight because β- and α-cell adaptation in response to metabolic changes can be studied in a controlled setting at different time points. In addition, *in vitro* biotechnology platforms can be a powerful tool to assess the influence of different metabolic stimuli and factors on human islet function and survival in order to identify new mechanisms involved in β- and α-cell adaptation. Since no assay platforms for human islet adaptation studies were available, we developed three high-throughput culture platforms for primary human islets to assess β-cell function in **chapter 7**: intact human islets, and cells from dispersed human islets cultured either in monolayer on extracellular matrix coated plates or reaggregated into islet-cell clusters. Dispersed islet cells can be efficiently transduced using adeno- and lentivirus. Activation of cAMP/EPAC-2 signal transduction and inhibition of K-channels enhanced glucose-induced insulin secretion in intact islets and islet cell aggregates, but not in monolayer culture. This shows that human islet cells behave most similar to intact human islets when cells are clustered three-dimensionally. Furthermore, these systems can also be used to study other aspects of human islet adaptation, such as α-cell function, and β- or α-cell proliferation and survival. These three culture platforms can be used in future studies for the screening of viral shRNA or small compound libraries to identify new mechanisms involved in β- and α-cell adaption of human islets.

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