Search for molecular and metabolic mechanisms contributing to impaired β-cell function

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"Science is like sex: sometimes something useful comes out, but that is not the reason we are doing it."

Richard P. Feynman (1918-1988)

TABLE OF CONTENT

LIST OF PUBLICATIONS

Scientific papers included in this thesis

I. **Malmgren S**, Nicholls DG, Taneera J, Bacos K, Koeck T, Tamaddon A, Wibom R, Groop L, Ling C, Mulder H, Sharoyko VV. *Tight coupling between glucose and mitochondrial metabolism in clonal beta-cells is required for robust insulin secretion*. J Biol Chem. 2009 Nov 20; 284 (47): 32395-404

II. Spégel P, **Malmgren S**, Sharoyko VV, Newsholme P, Koeck T, Mulder H. *Metabolomic analyses reveal profound differences in glycolytic and tricarboxylic acid cycle metabolism in glucose-responsive and -unresponsive clonal β-cell lines*. Biochem J. 2011 Apr 1; 435 (1): 277-84

III. **Malmgren S**, Spégel P, Danielsson A, Nagorny C, Andersson A, Dekker Nitert M, Ridderstråle M, Mulder H, Ling C. *Coordinate changes in histone modifications, mRNA levels and metabolite profiles in clonal INS-1 832/13 β-cells accompany functional adaptations to lipotoxicity.* Submitted to J Biol Chem 2012 Nov

Scientific papers not included in this thesis

I. Yang BT, Dayeh TA, Volkov PA, Kirkpatrick CL, **Malmgren S**, Jing X, Renström E, Wollheim CB, Nitert MD, Ling C. *Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes.* Mol Endocrinol. 2012 Jul; 26 (7): 1203-12

II. Stamenkovic JA, Olsson AH, Nagorny CL, **Malmgren S**, Dekker-Nitert M, Ling C, Mulder H. *Regulation of core clock genes in human islets.* Metabolism. 2012 Jul; 61 (7): 978-85

III. Spégel P, Sharoyko VV, Goehring I, Danielsson A, **Malmgren S**, Nagorny C, Koeck T, Sharp G, Straub S, Wollheim C, and Mulder H. *Time-resolved metabolomics analysis of ß-cells implicates the pentose phosphate pathway in the control of insulin release.* Resubmitted to Biochem J. 2012

ABBREVIATIONS

AA	Amino acid
Ab	antibody
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	analysis of variance
ATP	Adenosine triphosphate
BMI	Body mass index
CE	coupling efficiency
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
FA	Fatty acids
$FADH_2$	Flavin adenine dinucleotide
GC/MS	Gas chromatography/mass spectrometry
GK	Glucokinase
GSIS	Glucose-stimulated insulin secretion
GWAS	Genome-wide association studies
HAT	Histone acetyltransferase
HbA_{1c}	Glycated hemoglobin
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor-1α
LC-CoA	Long chain acyl-CoA
LDA	Low density array
LDH	Lactate dehydrogenase

LDL	Low density lipoprotein
ME	Malic enzyme
MODY	Maturity-onset diabetes of the young
NADH	Nicotine amide adenine dinucleotide
NADPH	Nicotine amide adenine dinucleotide phosphate
OCR	Oxygen consumption rate
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PLS	Partial least squares
qPCR	Quantitative polymerase chain reaction (Real-time-PCR)
RCR	Respiratory control ratio
RNA	Ribonucleic acid
SEM	Standard error of the mean
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
SREBP1c	Sterol regulatory element-binding protein-1c
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TCA	Tricarboxylic acid
TSS	Transcription start site
UCP-2	Uncoupling protein 2
WHO	Word health organization

INTRODUCTION

Type 2 Diabetes (T2D) is a multifactorial disease where genetic, epigenetic and environmental factors interact. This result in an illness characterized by the combination of peripheral insulin resistance and impaired insulin secretion. It is becoming an increasing socio-economic burden in multiple nations and is often referred to as a pandemic. In the last five years, several gene variants have been linked to increased disease susceptibility, vastly increasing our knowledge of T2D. However, the relationship between the genetic and the environmental aspects of the disease remains to be revealed. Also, the mechanism underlying insulin secretion is still not fully understood. This thesis addresses metabolic regulatory pathways involved in the control of fuel stimulated insulin secretion as well as potential casual relations between epigenetic mechanisms, gene expression and metabolic regulation in β-cell function and insulin secretion.

Diabetes Mellitus

Diabetes Mellitus is an endocrine disease characterized by chronic high plasma glucose concentrations (hyperglycemia). The Word Health Organization (WHO) definition of Diabetes Mellitus is a fasting plasma glucose of $\geq$7.0 mmol/L or a plasma glucose of $\geq$11.0 mmol/L 2 hours after an oral glucose tolerance test ((WHO) August 2011). Recently, an $HbA_{1c} \geq 6.5\%$ was recommended as an additional diagnostic criterion for diabetes. However, an HbA_{1c} value less than 6.5% does not exclude diabetes (World Health Organization 2011). Diabetes Mellitus is actually the common name for a group of disorders. There are two main types of Diabetes Mellitus, Type 1 Diabetes (T1D) and T2D. In T1D, the insulin-producing β-cells of the pancreas are destroyed due to autoimmune mechanisms resulting in absolute insulin deficiency. Patients with T1D are therefore dependent on exogenous insulin to survive. T2D, however, is a multifactorial polygenic disease, resulting from the combination of decreased insulin secretion from β-cells and insulin resistance in target tissues.

The prevalence of diabetes is steadily increasing in the world and is now considered a major threat to world health. In 2011, it was estimated that 366 million people, 8.3% of individuals 20-79 years, had diabetes mellitus and this

number is expected to increase world-wide to an estimated 552 million people, 9.9% of individuals 20-79 years by 2030 (Fig. 1). Out of the total number of diabetes cases in 2010, 90% is accounted for by T2D ((IDF) 2011). This number is likely to increase even further to epidemic proportions due to an ageing population, changes in diet and physical inactivity.

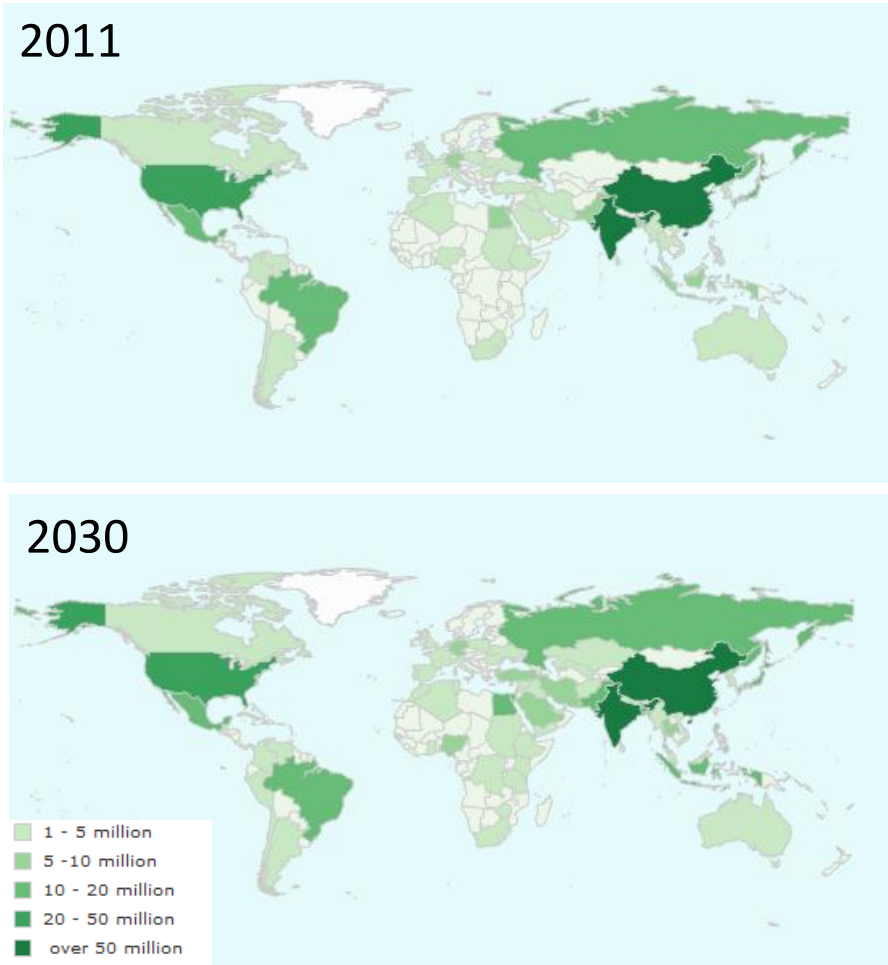

FIGURE 1: Map showing the estimated number of people with diabetes per country in 2011 and 2030, respectively.
The estimated number of adults living with diabetes in 2011 is 366 million, representing 8.3% of the global adult population. This number is projected to increase to 552 million people by 2030, or 9.9% of adults, which equates to approximately three more people with diabetes every 10 seconds. Map derived from IDF diabetes atlas, 5th edition (http://www.idf.org/diabetesaltlas.)

Type 2 Diabetes

T2D, the most prevalent form of diabetes mellitus, typically manifests later in life than T1D, however, the prevalence among younger subjects is increasing (Zimmet 2003). The risk of developing T2D is determined by the combination of genetic, epigenetic and environmental factors (Ling and Groop 2009). Environmental factors stemming from life style changes resulting in physical inactivity and obesity are thought to underlie the great increase of the world-wide prevalence of T2D (Shaw, Sicree et al. 2010).

Diabetes is the leading cause of blindness, non-traumatic lower-limb amputation (diabetic foot), and chronic kidney disease in the United States (Ismail-Beigi 2012), largely due to the micro- and macro-vascular complications. Thus, the disease is becoming a great socio-economic burden in many parts of the world ((IDF) 2011).

Impaired insulin release

T2D is characterized by a combination of insufficient insulin secretion, deriving from β-cell failure to meet whole body insulin demands, and insulin resistance in target tissues. Insulin resistance increases the demand of insulin. An insulin resistant subject can maintain healthy plasma glucose levels as long as the pancreatic β-cells can compensate for the increasing insulin demands. When this compensation fails however, the subject's plasma glucose levels will rise and the insulin resistant subject will become diabetic (Fig. 2). A progressive decrease in β-cell mass that results from an increase in β-cell apoptosis have been proposed to accompany the onset of T2D and contribute to decreased β-cell function (Kjems, Kirby et al. 2001; Prentki and Nolan 2006; Rahier, Guiot et al. 2008; Ma, Zhao et al. 2012).

There has been some debate regarding whether insulin resistance or insufficient insulin release is the hen or the egg in the pathogenesis of T2D. It has been suggested that the β-cell, being forced to hypersecrete insulin by peripheral impaired glucose tolerance (IGT), would eventually "wear out", resulting in β-cell failure but the causal mechanism behind this remains unknown (Defronzo 2009)

Instead, a large body of evidence now points towards insufficient insulin release being the determining mechanism in T2D development (Ashcroft and Rorsman 2012). Secondary effects of insulin resistance, such as increased fatty acid (FA) disposition in β-cells (Unger and Zhou 2001) and hypersecretion of islet amyloid polypeptide (Johnson, O'Brien et al. 1989; Eriksson, Nakazato et al. 1992), have been suggested to link insulin resistance to β-cell failure. However, the mechanism

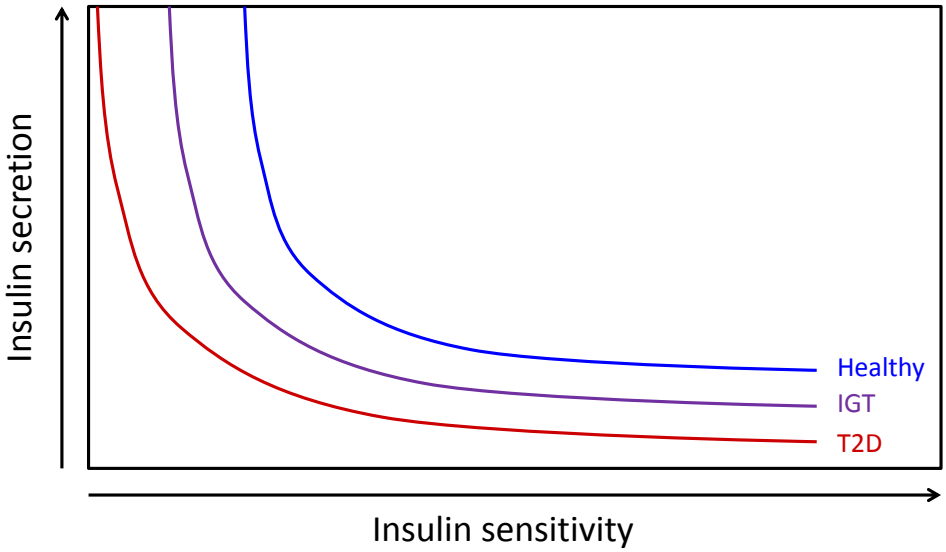

FIGURE 2: Hyperbolic relationship between insulin secretion and insulin sensitivity in the development from healthy to T2D.
In a subject with impaired glucose tolerance, normoglycemia can be maintained through increased insulin output, which compensates for decreased insulin sensitivity. Eventually, the β-cell can no longer increase insulin secretion to meet the demand and hyperglycemia and T2D develop. IGT, impaired glucose tolerance; T2D, type 2 diabetes.

behind the β-cells' developing an incapacity to produce and release sufficient amounts of insulin is to date not fully understood.

Pathogenesis

T2D is a chronic and, most often, progressive metabolic disease where lack of metabolic control results in chronic hyperglycemia. Abnormalities commonly associated with T2D include hypertension, dyslipidemia and inflammation. Insulin resistance is typically present for some time before T2D onset, leading to a progressive decrease of insulin-stimulated glucose uptake by muscle and adipose tissue and inadequate suppression of glucose production in the liver by insulin (Fig. 3).

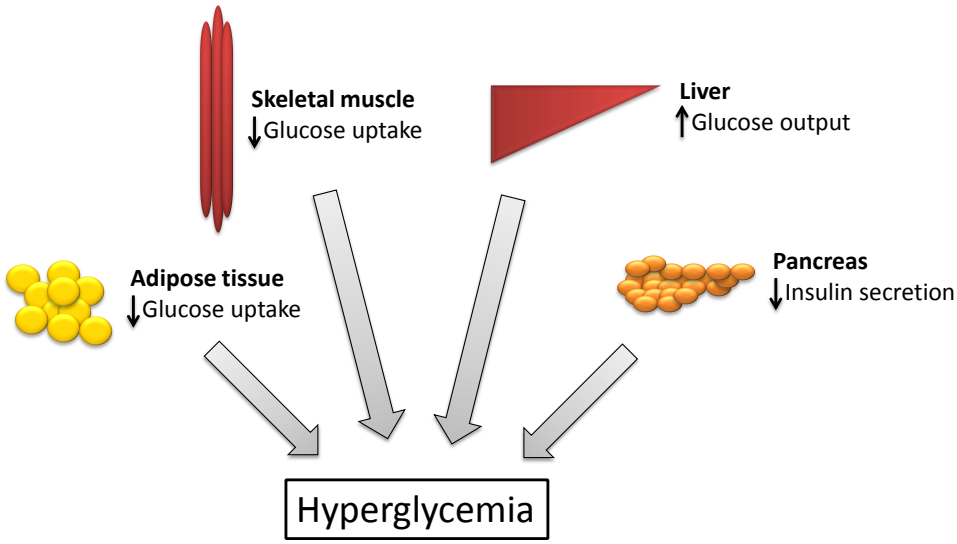

FIGURE 3: Mechanisms of hyperglycemia in T2D
T2D is caused by the combination of impaired insulin secretion from the pancreas and disturbed insulin action in target tissues. This results in decreased glucose uptake by skeletal muscle, adipose tissue and liver and increased glucose synthesis and output from the liver. The end result is elevated blood glucose, hyperglycemia.

T2D is often preceded by a pre-diabetic state, defined by insulin resistance and IGT. Studies have shown that already at this state, β-cell function is reduced by 50-80% (Defronzo 2009). With further impairments of β-cell function, these IGT subjects might progress to T2D (Fig. 2).

Age

Increased age is a risk-factor for T2D. There is a documented decline in β-cell function and increase in T2D with advancing age (Muller, Elahi et al. 1996).

Insulin resistance

There is a vicious circle in which insulin resistance and β-cell failure interact. Since skeletal muscle accounts for around 75% of the insulin stimulated glucose uptake in the whole body (Lin and Sun 2010), insulin resistance in this tissue will drive plasma glucose to increase unless the beta-cell can increase insulin output to meet demands. Obesity and physical inactivity have both been found to correlate with insulin resistance (Ingelsson, Arnlov et al. 2009). Increased triglyceride content in muscle (Krssak, Falk Petersen et al. 1999) and hepatocytes (Fabbrini, Magkos et al. 2009) have been linked to increased insulin resistance in these tissues.

Chronically elevated levels of plasma glucose impair β-cell function (Glucotoxicity) (Poitout and Robertson 2008). In adipose tissue, insulin resistance decreases glucose absorption as well but it also raises secretion of free FAs into the bloodstream (Lin and Sun 2010). Elevation of plasma free FAs impairs insulin secretion (Lipotoxicity), particularly in genetically predisposed individuals (Kashyap, Belfort et al. 2003)

Genetic factors

The effects of the genetic component of T2D have been analyzed by comparing the risk of developing T2D during a lifetime of people with a family history of T2D compared to the risk of the rest of the population. This risk is 40% with one diabetic parent and almost 70% with two diabetic parents (Groop, Forsblom et al. 1996). This clearly shows that genetic predisposition is a major factor in the development of T2D. When studying twin pairs, it was found that the concordance rate of T2D in monozygotic twins is 70% while dizygotic twins only have a 20-30% concordance (Kaprio, Tuomilehto et al. 1992).

Monogenic diabetes, where the disease can be traced back to a mutation in a single gene, accounts for 1-2% of all diabetes cases although it is commonly misdiagnosed as either T1D or T2D. Traditionally, monogenic diabetes has been classified either as neonatal diabetes or maturity-onset diabetes of the young (MODY) depending on the age of diabetes onset. However, the age of onset, the severity of hyperglycemia and risk of complications vary greatly between the different mutations. Diabetes diagnosed before 6 months of age is usually associated with mutations in *KCNJ11*, *SUR1* (encoding the sulfonylurea receptor 1), or with abnormalities in chromosome 6q24. Mild fasting hyperglycemia has been associated with the gene encoding for glucokinase (*GCK*; MODY2) mutations and familial, young-onset diabetes has been associated with the HNF1 homeobox A gene (*HNF1A*; MODY3) or HNF4 homeobox A gene (*HNF4A*; MODY1). Mutations in the gene encoding for HNF1 homeobox B (*HNF1B*; MODY5) have been associated with diabetes with extrapancreatic features (Murphy, Ellard et al. 2008).

Mitochondrial diabetes is another form of monogenetic diabetes that affects up to 1% of patients with diabetes. Maternally inherited diabetes and deafness (MIDD) resulting from the mutation 3243A>G (Goto, Nonaka et al. 1990) of the mitochondrial DNA is the most frequent mutation causing mitochondrial diabetes. Since this is a mutation in the mitochondrial DNA, it is maternally inherited. The mutation causes impaired activity of complexes I and IV of the respiratory chain. The resulting energy deficit in MIDD is manifest within tissues with high metabolic activity, such as the endocrine pancreas and the cochlea, causing a diabetic phenotype and deafness (Murphy, Turnbull et al. 2008).

An important breakthrough in T2D genetics happened in 2007 when the results of several genome-wide association studies (GWAS), genotyping large case control cohorts and comparing the frequency of single nucleotide polymorphisms (SNPs) in patients with T2D to healthy controls, were first published (Saxena, Voight et al. 2007; Scott, Mohlke et al. 2007; Sladek, Rocheleau et al. 2007; Zeggini, Weedon et al. 2007) These GWAS were performed using arrays that genotyped over 500 000 SNPs per subject. SNPs are the most common form of variation in the human genome. It is estimated that approximately 10 million sites vary in such a way that the frequency of the variation exceeds 1%, which is one in approximately every 300 base pairs. Theses 10 million SNPs constitute 90% of the genetic variation in the population (Int. Hapmap Consortium 2003). To this day, approximately 60 SNPs have been associated with T2D or glycemic traits (Ahlqvist, Ahluwalia et al. 2011). Most of the T2D risk variants found in GWAS are thought to be associated with impaired β-cell function (Mussig, Staiger et al. 2010), further highlighting the importance of insulin secretion in the development of T2D. We have come a long way in identifying the biological mechanisms underlying impaired insulin secretion in T2D, however, the full picture remains yet to be revealed. Even though much is known about the genetic component of T2D, the known genetic variants still account for less than 10% of the overall estimated genetic contribution to T2D predisposition (Voight, Scott et al. 2010).

Environmental factors

When considering the rapid increase in T2D prevalence worldwide it becomes evident that there is more to it than genetic factors. The world we live in is changing much more rapidly than our genes ever could. The change towards a more sedentary life style with great access to calorie-rich food that has occurred during the last decade has started a worldwide epidemic in the prevalence of T2D, obesity and other metabolic conditions. The body mass index (BMI) is used to define obesity: a BMI of >25 kg/m^2 is considered as overweight while subjects with a BMI of >30 qualify as obese. It is estimated that 44% of diabetes, 23% of ischemic heart disease and between 7% and 41% of some cancers are attributable to overweight and obesity ((WHO) August 2011). However, the onset of T2D can be delayed or even prevented through lifestyle modification, including healthy diet and increased physical activity (Knowler, Barrett-Connor et al. 2002; Garber 2012). In fact, reducing weight by caloric restriction may even reverse manifest diabetes (Lim, Hollingsworth et al. 2011).

Our collective knowledge about the link between environmental factors and T2D is still limited. Nevertheless, epigenetic mechanisms have emerged as possible agents in the complex interplay between environment, gene regulation and T2D.

Epigenetics and T2D

Epigenetics define chemical modifications, which can be inherited through cell division, and that may alter gene expression without affecting the DNA code itself. They may be affected by environmental factors (Fradin and Bougneres 2011). Epigenetic mechanisms have recently been highlighted in many other fields such as inflammation, obesity and T2D.

The most extensively studied epigenetic modification with respect to T2D is DNA methylation, where a methyl group is added to the DNA on the 5´ position of a cytosine base in CG-dinucleotides (Ooi, O'Donnell et al. 2009). Increased methylation of the promoter regions of *PPARGC1A*, *PDX-1* and insulin can be seen in pancreatic islets from patients with T2D; this has been associated with decreased gene expression and to correlate with glycemic control assessed as HbA_{1c} levels (Ling, Del Guerra et al. 2008; Yang, Dayeh et al. 2011; Volkmar, Dedeurwaerder et al. 2012; Yang, Dayeh et al. 2012). Also, most forms of MODY are caused by mutations in genes that code for transcription factors, some of which exert their function through the recruitment of histone acetylases (HAT) (Ban, Yamada et al. 2002) or histone deacetylases (HDAC) (Mosley and Ozcan 2004) to the promoter of their target genes (Ling and Groop 2009).

The linking of environmental factors to impaired β-cell function is crucial for understanding T2D development. Also, since drugs targeting epigenetic processes are already being used to treat cancer (Mann, Johnson et al. 2007), such drugs may also provide a potential, novel therapeutic strategy to treat T2D. In light of this, it is important to study epigenetic mechanisms in β-cells.

Pancreatic islets

The pancreas is a gland located in the abdominal cavity of vertebrates. It functions both as an endocrine organ, producing several hormones, and as a digestive one, secreting enzymes into the small intestine that enable digestion and absorption of micronutrients.

The endocrine function of the pancreas is conducted by a small, round organ called the Islet of Langerhans after Paul Langerhans, who first described them in 1869 (Langerhans 1869). The human pancreas contains about 1 000 000 islets; they are crucial for whole body glucose homeostasis. Islets typically consist of five types of secretory cells, the insulin-producing β-cells, the glucagon-producing α-cells, the somatostatin-producing δ-cells, the pancreatic polypeptide-producing (PP) cells and ghrelin cells. In rodent islets, the β-cells outnumber all other cell types and generally cluster in the core of the islet, surrounded by a mantle of α-, δ-, and PP-cells. Human islets contain proportionally fewer β-cells and more α-cells

than rodent islets. Also, the secretory cells are scattered throughout the islet in no particular order. The islets composition in human islets is ~50% β-cells, 35% α-cells, 15% δ cells and 1% ghrelin cells (Wierup, Svensson et al. 2002), where β-cells still constitute the vast majority of cells (Cabrera, Berman et al. 2006).

The β-cell

As the most abundant cell in the islet of Langerhans, the β-cell regulates whole body glucose homeostasis by secreting insulin, a key metabolic peptide hormone that exerts anabolic functions in several tissues, including muscle, liver and adipose tissue. β-cells secrete insulin in a biphasic manner; secretion is stimulated mainly by glucose but other nutrients present in the plasma, such as amino acids and lipids, might also evoke a response through stimulating metabolic pathways (Newsholme, Brennan et al. 2005). Other factors, such as autonomic neuronal regulation, hormones and neuropeptides also regulate secretion of this important hormone (Ahren 2000).

Fuel-stimulated insulin secretion

Metabolites in plasma stimulate β-cells to release insulin by increasing β-cell metabolism. An increase in plasma glucose concentration results in increased glycolytic and mitochondrial metabolism and the generation of ATP from ADP, which stimulates insulin secretion. In the β-cell, more than 95% of ATP is produced by oxygen-consuming respiration in the mitochondria, and this ATP is of primary importance for insulin release (Tarasov, Dusonchet et al. 2004). The tight coupling of glycolysis to mitochondrial respiration in the β-cell is crucial for proper β-cell function. This high rate of mitochondrial respiration in the β-cell is largely due the unusually low expression of lactate dehydrogenase (LDH) (Pullen, Khan et al. 2010). LDH produces ATP from ADP by converting pyruvate into lactate, preventing pyruvate from entering the TCA cycle in the mitochondrion thus disturbing the coupling between glucose concentrations and ATP production via mitochondrial oxidative pathway. Accordingly, over-expression of LDH perturbs β-cell mitochondrial metabolism and, in the end, insulin secretion (Ainscow, Zhao et al. 2000).

Uncoupling proteins could induce a proton leak over the inner mitochondrial membrane, resulting in impaired ATP production. This would affect the ATP:ADP ratio and in the end insulin secretion. In agreement with this, rodent islets overexpressing uncoupling protein 2 (UCP-2) exhibit impaired insulin secretion (Chan, MacDonald et al. 1999). However, these results have not been unequivocally supported by studies of UCP-2 knockout mice (Krauss, Zhang et al. 2002; Pi, Bai et al. 2009).

Glucose

The β-cell secretes insulin in proportion to extracellular glucose concentrations. Secretion in humans is induced when plasma glucose rises above 5 mmol/Glucose is transported into the β-cell via GLUT1 (in humans, GLUT2 in rodents), a glucose transporter with high capacity and low affinity for glucose. Once within the cell, glucose is phosphorylated by glucokinase (GK), the main glucose-sensing enzyme. The glucose concentration at which the enzyme is most sensitive to alterations of the glucose levels is approximately 3.5 mmol/L for GK. This enables GK to increase its activity and phosphorylate glucose at a rate relative to glucose concentration within a physiological range (Matschinsky 2005). Once glucose is phosphorylated by GK, it enters glycolysis where it is further metabolized into pyruvate. Pyruvate then enters the tricarboxylic acid (TCA) cycle, where its oxidization yields the electron carriers NADH and $FADH_2$. These reduced electron carrier molecules are then oxidized by the electron transport chain (ETC) of the inner mitochondrial membrane to yield ATP. This mitochondrial ATP is produced at the expense of ADP, thus increasing the ATP:ADP ratio (Jitrapakdee, Wutthisathapornchai et al.). This in turn closes ATP-sensitive K^+ channels (K_{ATP} channels), hereby preventing the K^+ efflux through these channels that serves to maintain the resting membrane potential at a hyperpolarized level of around -70 mV at non-stimulatory glucose levels. The closing of K_{ATP} channels leads to plasma membrane depolarization, which in turn leads to the opening of voltage-gated Ca^{2+} channels in the plasma membrane. Ca^{2+} flows in, increasing the cytoplasmic Ca^{2+}concentration, which cause insulin- containing granules in the cytoplasm to fuse with the plasma membrane, thus releasing insulin into the blood stream (Fig. 4) (Tarasov, Dusonchet et al. 2004).

Insulin secretion through closure of K_{ATP} channels is called the triggering pathway and it causes exocytosis of a readily-releasable pool of insulin granules. These readily-releasable granules are already docked to the plasma membrane and constitute ~10% of the entire pool of insulin granules. A first surge of insulin from the immediately releasable pool constitutes the first phase of the biphasic insulin secretion. The second phase of glucose-stimulated insulin secretion (GSIS) is accounted for by the K_{ATP} channel-independent pathways in synergy with the triggering pathway (Straub and Sharp 2002; Jensen, Joseph et al. 2008).

Although the triggering pathway is needed for GSIS to occur, second messengers, thought to derive from mitochondrial metabolic processes may sustain secretion by the amplifying pathway. Such second messengers include GTP, ATP, cAMP, NADPH, glutamate, malonyl-CoA and long chain acyl-CoA (LC-CoA) (Henquin 2000; Maechler 2002). Although these amplifying signals cannot evoke a secretory response by themselves, they have been estimated to account for as much as 70% of the total GSIS response (Henquin, Ravier et al. 2003).

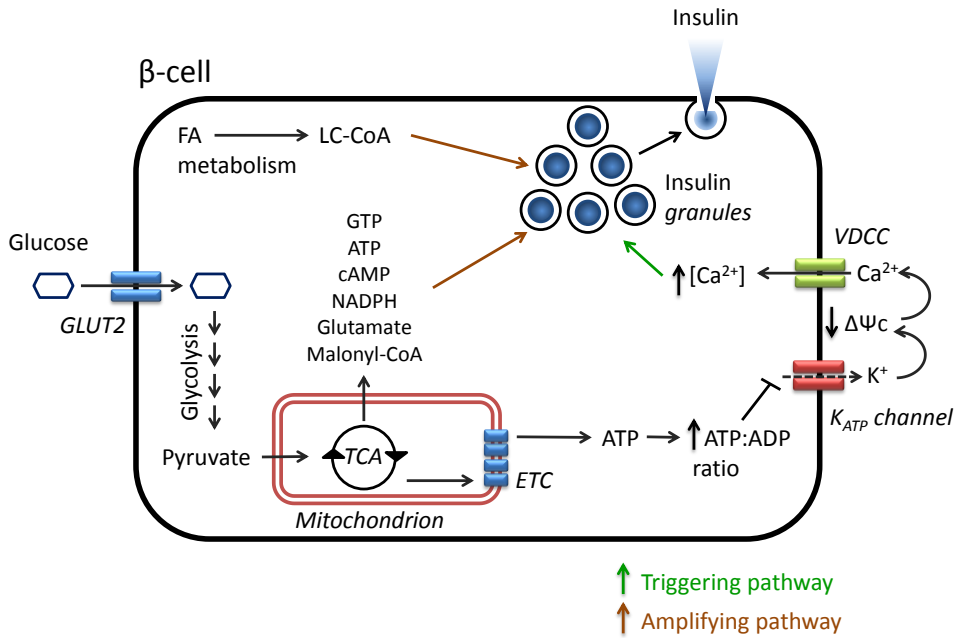

FIGURE 4: Triggering and amplifying pathway of glucose-stimulated insulin secretion.
Glucose enters the cell trough active transport by the glucose transporter GLUT2 (rodent β-cells). Glucose is metabolized into pyruvate through glycolysis and pyruvate enters the mitochondrion where it is further metabolized in the TCA cycle resulting in reducing equivalents. These enter the electron transport chain (ETC) resulting in ATP production. The increase in the cytosolic ATP:ADP ratio triggers closure of K_{ATP} channels in the plasma membrane. When K+ ceases to flow into the cell, the plasma membrane potential (ΔΨc) collapses leading to depolarization of the plasma membrane. This opens VDCC leading to Ca2+ influx causing insulin granules to fuse to the membrane and release insulin into the blood stream.
In addition to this triggering pathway, there is an amplifying pathway constituted by coupling factors that stimulate insulin secretion independent of K_{ATP} channels. TCA, tricarboxylic acid cycle; ETC, electron transport chain; VDCC, voltage-dependent calcium channels.

Fatty Acids

FAs are mainly oxidized in the mitochondrion and generate reducing equivalents, resulting in ATP production through the ETC. With shorter exposure, FAs will potentiate insulin secretion (Itoh, Kawamata et al. 2003). With increased glucose concentrations, metabolic flux increases in the β-cell and the level of the TCA cycle intermediate citrate will rise. When citrate is abundant, it will escape the mitochondrion through a process called cataplerosis and be transformed into

malonyl-CoA. This acts as a ”signal of plenty” to the cell and inhibits FA oxidation by interacting with carnitine palmitoyltransferase I (CPT-1), excluding FAs from the mitochondrion (McGarry and Brown 1997). Consequently, LC-CoA accumulates in the cytoplasm, and may stimulate insulin release. Removal of malonyl-CoA from the β-cell using malonyl-CoA decarboxylase, however, has no effect in β-cells in the absence of FA (Mulder, Lu et al. 2001). In contrast, malonyl-CoA decarboxylase overexpression in the presence of FA reduces GSIS in β-cell lines and islets (Roduit, Nolan et al. 2004).

After chronic exposure, high levels of FAs are deleterious to β-cell function. In humans as well as in animals, obesity and T2D are associated with hypertriglyceridemia and elevated free FA levels. Elevated free FA levels have been shown to predict diabetes (Charles, Eschwege et al. 1997). Chronic exposure of pancreatic islets and β-cells to elevated levels of FAs leads to a disrupted glucose-induced insulin secretion and, in some cases, even β-cell death (Prentki, Joly et al. 2002).

Amino acids

Some amino acids (AA) are known to stimulate insulin secretion from primary islets and insulin-secreting β-cell lines alike (Smith, Sakura et al. 1997; Dixon, Nolan et al. 2003). The combination of the AAs leucine and glutamine is a potent stimulator of insulin secretion (Sener, Somers et al. 1981). This is believed to be the end result of several metabolic effects. First, leucine allosterically activates glutamate dehydrogenase (GDH), an enzyme converting glutamate to α-ketoglutarate, one of the metabolites in the TCA cycle. Secondly, leucine can be metabolized into α-ketoisoaproic acid (α-KIC) and further into acetyl-CoA, providing fuel for the TCA cycle. Thirdly, glutamine provides a surplus of glutamate, further potentiating the stimulatory effects of leucine. The end result is increased insulin secretion through an increased flux through the TCA cycle and subsequently ATP production (Gao, Young et al. 2003; Li, Najafi et al. 2003).

Metabolic regulation

The metabolism of β-cells regulates insulin secretion and through that whole body metabolism of glucose but also other micro-nutrients such as fat, cholesterol and AAs. Needless to say, the regulatory mechanisms of each and every β-cell ultimately control the functionality of the entire organism. These regulatory mechanisms can roughly be divided into short-term regulation, such as phosphorylation of enzymes or allosteric regulation by metabolic intermediates, and long-term regulation, such as regulation of the transcriptional machinery (Harris 2002).

Allosteric regulation

Allosteric regulation is the quick, self-regulating effect exerted by metabolic intermediates. These may inhibit enzymes determining rate the metabolic flux (Gibson 2002). Glycolysis produces pyruvate that enters the TCA cycle, ultimately leading to the production of ATP from ADP and AMP. A low ATP:ADP/AMP ratio increases glycolytic flux while citrate, a TCA cycle intermediate, diminishes it.

FA oxidation is allosterically regulated by malonyl-CoA formation. It inhibits carnitine palmitoyl transferase I (CPT-1), thus limiting the entry of LC-CoA into the mitochondrion for oxidation. Citrate from the TCA cycle promotes malonyl-CoA formation and, ultimately, FA synthesis (Nolan, Madiraju et al. 2006).

Mitochondrial metabolism

The mitochondrion is an organelle situated in the cytosol of mammalian cells. It is unique in that it has a double membrane as well as its own circular DNA containing genes crucial for cellular function. The mitochondrial part of glucose metabolism is the main coupling signal for β-cell insulin secretion, both directly via ATP production but also through different metabolic intermediates acting as coupling factors that affect insulin secretion through the amplifying pathway (Jitrapakdee, Wutthisathapornchai et al. 2010).

Pyruvate, the end product of glycolysis, is shuttled into the mitochondrion via two different routes, both feeding into the TCA cycle. The predominant way of pyruvate entry into the TCA cycle in most cells is through conversion into acetyl-CoA by pyruvate dehydrogenase (PDH). In β-cells, however, carboxylation by pyruvate carboxylase (PC) to oxaloacetate is also a quantitatively and functionally essential pathway (Khan, Ling et al. 1996): it is called anaplerosis from the Greek word for "filling up". This pathway increases the number of TCA cycle intermediates, enabling a higher TCA cycle flux; the inhibition of this enzyme decreases insulin secretion in primary islets as well as in β-cell lines (Liu, Jetton et al. 2002; Lu, Mulder et al. 2002; Fransson, Rosengren et al. 2006).

Pyruvate cycling

Three different pyruvate cycles, shuttling pyruvate in and out of the mitochondrion, occur in β-cells: the pyruvate/malate, pyruvate/citrate, and

pyruvate/isocitrate cycles (Jensen, Joseph et al. 2008). These shuttles all generate NADPH, a putative coupling factor of insulin secretion (Fig. 5) (Maechler 2002).

The first cycle involves the conversion of oxaloacetate to malate by mitochondrial malate dehydrogenase; the export of malate to the cytosol and conversion of malate to pyruvate is catalyzed by cytosolic malic enzyme (ME). There are several pieces of evidence linking pyruvate/malate shuttling and ME expression to GSIS in β-cell lines (Guay, Madiraju et al. 2007; Pongratz, Kibbey et al. 2007) but these effects have not been confirmed in primary islets (Ronnebaum, Jensen et al. 2008).

The second cycle involves the conversion of oxaloacetate to citrate, which is exported to the cytosol, cleaved to oxaloacetate and acetyl-CoA by ATP-citrate lyase (CL). Oxaloacetate is recycled to pyruvate via malate and ME. Acetyl-CoA, the other product of the CL reaction, is converted to malonyl-CoA, which functions as a substrate for lipogenesis as well as an allosteric inhibitor of CPT-1. PC-mediated anaplerosis is believed to be linked to control of insulin secretion via pyruvate/citrate cycling and increases in the levels of malonyl-CoA and LC-CoA deriving from this cycle (Prentki, Vischer et al. 1992; Farfari, Schulz et al. 2000) and by potentiating FA effects in β-cell lines and rat islets (Roduit, Nolan et al. 2004).

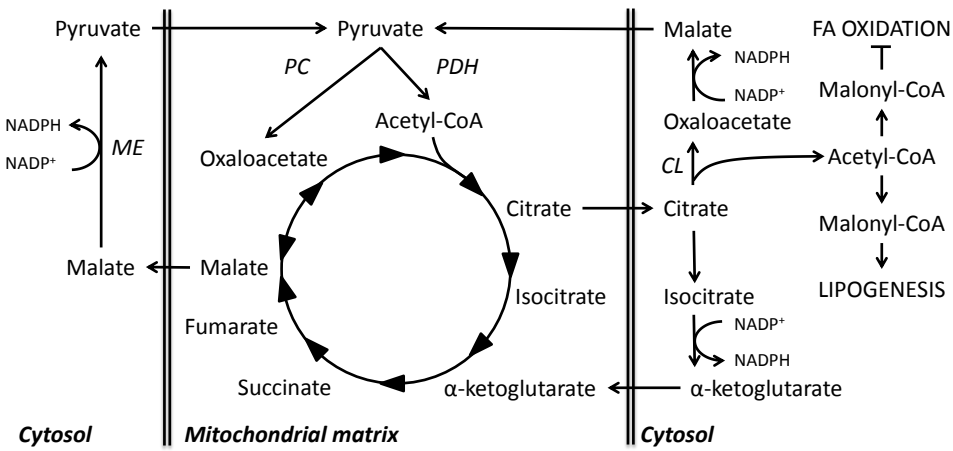

FIGURE 5: Mitochondrial metabolism and cycling pathways.
Pyruvate from glycolysis is shuttled into the mitochondrion via two different routes both feeding into the TCA cycle: carboxylation to oxaloacetate by carboxylase (PC; anaplerosis) and conversion into acetyl-CoA by pyruvate dehydrogenase (PDH). Additionally, three different pyruvate cycles shuttle pyruvate between the cytosol and the mitochondrion, generating NADPH in the process. These are the pyruvate/malate, pyruvate/citrate, and pyruvate/isocitrate cycles. ME, Malic enzyme; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase.

The third cycle, known as the "pyruvate/isocitrate cycle", involves export of citrate and isocitrate from the mitochondria, which is converted to α-ketoglutarate by a cytosolic NADP-dependent isocitrate dehydrogenase. Knockdown of key enzymes of the pyruvate/isocitrate cycle impairs GSIS in rat islets (Joseph, Jensen et al. 2006; Ronnebaum, Ilkayeva et al. 2006).

Oxidative phosphorylation

In the mitochondrion, the reducing equivalents NADH and $FADH_2$ are used to produce ATP through oxidative phosphorylation. It is the end point of glucose, AA and FA metabolic pathways.

The mitochondrion produces ATP in an O_2-dependent manner through the shuttling of electrons (e^-) through a chain of trans-membrane protein complexes, situated in the inner mitochondrial membrane. The underlying force driving these reactions is the Gibbs free energy of the reactants derived by cell metabolism and the products of the oxidative phosphorylation. The oxidative phosphorylation system requires a number of protein complexes called the ETC and ATP synthase. They are unique in that they require the coordinate expression of both mitochondrial and nuclear genes (Scarpulla 2008).

The ETC is constituted by four complexes, referred to as complex I – IV. The reducing potential of NADH enters the ETC through complex I while succinate from the TCA cycle reduces FAD to $FADH_2$ at complex II. $FADH_2$ is oxidized back to FAD by complex II. In each case, an electron is transferred from the reducing equivalent to the complex. Both complexes transfer their electron to ubiquinone (coenzyme Q), which passes electrons to complex III. Complex III passes the electrons, via cytochrome c, to complex IV that uses it to reduce O_2 and H^+ to H_2O. Complexes, I, III and IV pump protons (H^+) across the mitochondrial inner membrane, producing a proton-gradient. In each case, the drop in redox potential of the e^- passing through the complex is coupled to the extrusion of H^+ from the mitochondrial matrix, i.e., the space between the inner and the outer mitochondrial membrane (Brand and Nicholls 2011). Finally, ATP synthase, sometimes referred to as complex V, uses the energy potential of the proton gradient to phosphorylate ADP to ATP by use of the energy released when protons flow back into the matrix through the ATP synthase down the proton potential generated by the ETC (Fig. 6).

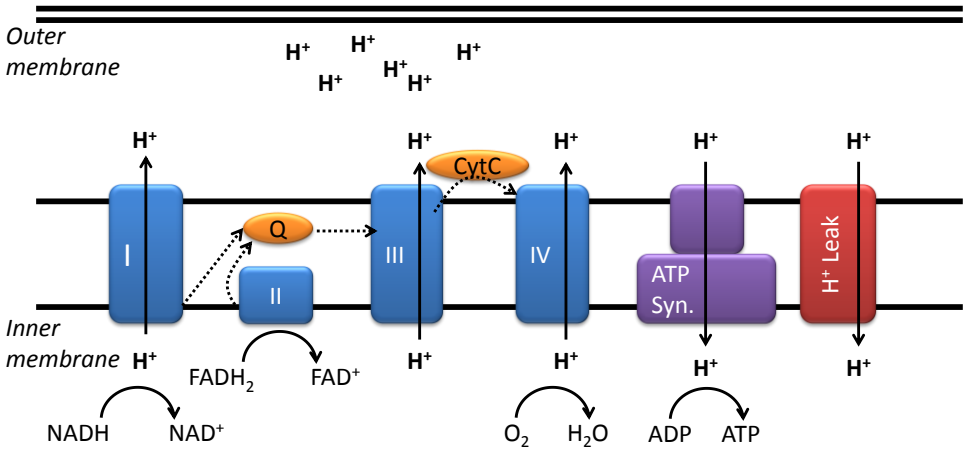

FIGURE 6: Oxidative phosphorylation is the main source of ATP in the β-cell
The oxidative phosphorylation system is constituted by a number of protein complexes (I – IV) called the electrode transfer chain (ETC) and ATP synthase. The energy from the electron donated either from NADH to complex I or by $FADH_2$ to complex II flows through the ETC as the dotted line indicates. Along its way, the complexes I, III and IV uses the energy released to pump protons (H^+) across the inner mitochondrial membrane. This results in a proton gradient across the membrane, something that allows the ATP synthase to convert ADP into the high-energy molecule ATP by using the energy releases when a H^+ flows back through the protein complex in the direction of the H^+ gradient. In addition to proton re-entry through the ATP synthase, all mitochondria possess an endogenous proton leak that allows H^+ to enter the matrix without oxidative phosphorylation Q, coenzyme Q; CytC, cytochrome C; I, complex I; II, complex II; III, complex III; IV, complex IV; ATP Syn. ATP synthase; H^+ Leak, proton leak.

In addition to proton re-entry through the ATP synthase, all mitochondria possess an endogenous proton leak that allows H^+ to enter the matrix without oxidative phosphorylation to occur, generating nothing but heat. Exogenous uncouplers, such as carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) or endogenous ones, such as uncoupling proteins, introduce such a leak, thus decreasing ATP production. UCP-2, which is stimulated by reactive oxygen species (ROS) production, has been suggested as a link between obesity and impaired GSIS since the ob/ob mice, which are obese and hyperglycemic, exhibit increased levels of UCP-2 in their pancreatic islets. Ob/ob mice lacking UCP-2, however, exhibit increased serum insulin levels and less hyperglycemia (Zhang, Baffy et al. 2001).

Cellular stress

Impaired insulin secretion coupled to T2D is believed to be caused by several abnormalities which impact β-cell function and viability. These include glucotoxicity, lipotoxicity and increased oxidative stress. The underlying concept is that in the pre-diabetic state, hyperglycemia and hyperlipidemia ensue and exert additional damaging or toxic effects on the β-cell resulting in further deterioration of β-cell function and perhaps, ultimately, β-cell death (Prentki and Nolan 2006).

Lipotoxicity

Prolonged exposure of β-cells to FAs *in vitro* decreases insulin secretion. This decrease is constituted both by an increased basal insulin release and an inhibited GSIS (Zhou and Grill 1995), a phenomenon that has also been observed *in vivo* in rats and humans (Paolisso, Gambardella et al. 1995; Mason, Goh et al. 1999; Carpentier, Mittelman et al. 2000). This effect is referred to as lipotoxicity (Unger 1995). The mechanisms underlying the impaired secretory response are to this date not completely clear.(Busch, Cordery et al. 2002; Gehrmann, Elsner et al. 2010)

UCP-2 has been brought forward as a potential link between lipotoxicity and impaired insulin secretion. UCP-2 is increased in rodent β-cells after FA exposure *in vitro* (Briaud, Kelpe et al. 2002; Li, Skorpen et al. 2002). This, together with the fact that islets from UCP-2 knock-out mice are protected from lipitoxicity (Joseph, Koshkin et al. 2004), suggest a possible role of increased proton leak over the inner mitochondrial membrane as one pathogenetic mechanism in lipotoxicity.

Furthermore, palmitate has been shown to inhibit insulin gene expression, probably through direct inhibition of glucose-induced insulin promoter activity in primary islets and altered PDX-1 nuclear localization (Kelpe, Moore et al. 2003; Hagman, Hays et al. 2005). Since palmitate serves as a substrate for *de novo* ceramide synthesis, ceramide generation has been suggested to mediate part of palmitate inhibition of insulin gene expression. Palmitate exposure is associated with an increase in ceramide content in isolated islets. When this is blocked by inhibitors of ceramide synthesis, it prevents the decrease in insulin mRNA expression (Kelpe, Moore et al. 2003). β-cell apoptosis induced by increased FA in Zucker diabetic fatty rats have been suggested to do so via *de novo* ceramide formation and increased NO production (Shimabukuro, Zhou et al. 1998).

Finally, a role for intracellular cholesterol metabolism in lipotoxicity has been proposed (Poitout and Robertson 2008). Intracellular cholesterol accumulation leads to islet dysfunction and impaired GSIS. The role of essential cholesterol modulators like the ATP-binding cassette transporter A1, the LDL receptor and

sterol regulatory element-binding protein-1c (SREBP1c), also believed to activate the UCP-2 promoter (Medvedev, Robidoux et al. 2002), have emerged as possible regulators of insulin secretion.

Metabolic stress

Fuel surplus caused by hyperlipidemia (lipotoxicity), hyperglycemia (glucotoxicity) or by the combination of the two (glycolipotoxicity) in the β-cell is likely to lead to increased metabolism through mitochondrial oxidation. This will result in an increased mitochondrial membrane potential and possibly superoxide production. Obesity, hyperglycemia and T2D are all associated with increased ROS production in human islets (Anello, Lupi et al. 2005).

Since the β-cell has unusually low levels of antioxidant enzymes compared to other tissues (Tiedge, Lortz et al. 1997), it has been suggested to be at particular risk for oxidative stress.

ROS molecules include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (.HO). The ETC continually generates small amounts of ROS, principally through complexes I and III, but these are normally removed by Mn^{2+}-superoxide dismutase; it produces H_2O_2 subsequently reduced to H_2O at the expense of glutathione, an anti-oxidant. If they are not rapidly eliminated, ROS can injure mitochondria by promoting DNA fragmentation, protein crosslinking, and peroxidation of membrane phospholipids as well as by activating a series of stress pathways (Prentki and Nolan 2006; Ma, Zhao et al. 2012). Indeed, β-cell mitochondria in islets from T2D subjects have been found to have morphologic abnormalities that include hypertrophy and higher density, something which parallels increased UCP-2 expression and increased oxidative stress (Anello, Lupi et al. 2005).

ROS might also directly induce β-cell death if they accumulate in high enough concentrations. Under conditions of a heavy metabolic load, causing overwhelming oxidative stress, mitochondrial ROS production might increase to the extent that protective, anti-oxidative mechanisms fail to eliminate it. When this occurs, cardiolipin, an essential part of the mitochondrial inner membrane structure, is oxidized. This destabilizes cytochrome c, and leads to its release into the cytosol. This triggers the induction of apoptosis, which culminates in β-cell failure and the development of T2D (Kagan, Tyurin et al. 2005; Ma, Zhao et al. 2012).

Mitochondrial ROS production might not be the only system causing oxidative stress in the β-cell. Peroxisomally generated hydrogen peroxide has also been suggested to generate ROS causing β-cell dysfunction in lipotoxicity, rather than mitochondrial ROS (Gehrmann, Elsner et al. 2010).

Pseudohypoxic stress

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that regulates cellular responses to hypoxic stress. Under normoxic conditions, HIF-1α interacts with the von Hippel-Lindau (VHL) protein, leading to proteosomal degradation (Cantley, Grey et al. 2010). Nevertheless, HIF-1α can also be induced in normoxia by growth factors, oncogene products, and mediators of inflammation. Primary cancer cells and carcinoma cell lines have been known to show increased expression of HIF-1α (Manolescu, Oprea et al. 2009).

Target genes of HIF-1α are involved in cell survival, proliferation, and energy metabolism (Manolescu, Oprea et al. 2009). HIF-1α activation results in impaired GSIS, probably by altered gene expression inducing a switch from aerobic glucose metabolism to anaerobic glycolysis, thus disrupting the triggering pathway of GSIS. Several recent studies suggest that HIF-1α is required for normal β-cell function and that its dysregulation may contribute to the pathogenesis of T2D (Cantley, Grey et al. 2010; Cheng, Ho et al. 2010; Choi, Cai et al. 2011).

Epigenetic regulation

As previously stated, epigenetics include chemical modifications that may alter gene expression without affecting the DNA code itself and that can be affected by environmental factors (Fradin and Bougneres 2011). Epigenetic modifications acts to fine tune gene expression and is involved in development by silencing imprinted genes and inactivating the X chromosome in females (Reik, Dean et al. 2001). It is also involved in cell differentiation and the reversal of epigenetic modifications have been shown to change fully differentiated cells back to pluripotent stem cells (Mohtat and Susztak 2010). Epigenetic mechanisms include DNA methylations, histone modifications and microRNAs. The great difference between a worker bee and a queen, which share the same genetic material but differ in epigenetic marks introduced by diet during development (Kucharski, Maleszka et al. 2008), highlights the power of epigenetic mechanisms.

DNA methylation

DNA methylation is an epigenetic modification that occurs when a methyl group is enzymatically transferred to the nucleotides that constitute the DNA sequence. In differentiated mammalian cells, this occurs primarily on the 5’ position of a cytosine followed by a guanine. This is referred to as a CpG-site and more than half of the genes in vertebrate genomes contain short CpG-rich regions of approximately 1 kb known as CpG islands (Ooi, O'Donnell et al. 2009). DNA

methyltransferases (DNMTs) regulate this process. Different DNMTs are responsible for the maintenance of methylation during replication and *de novo* methylation. DNA methylation of gene promoters is generally associated with transcriptional repression, either by obstructing transcription factors to bind to the promoter or by attracting other transcriptional repressors such as HDACs (Jones 2012).

Histone modifications

The total length of the DNA packaged into the nucleus of each eukaryotic cell is almost 2 meters long. This, along with the fact that the DNA is continuously transcribed and repaired poses no small demand on its structure and storage. This structure is obtained by the assembly of DNA into chromatin. Chromatin is built up by nucleosomes, which each contains ~147 bp of DNA wrapped around a protein octamer of histones. The histone octamer is composed of a central heterotetramer of histones H3 and H4, flanked by two heterodimers of histones H2A and H2B (Peterson and Laniel 2004; Ling and Groop 2009). Each histone also has an N-terminal segment that extends from the surface of the nucleosome, commonly referred to as a histone tail. These histone tails are sites of many post-translational modifications. They do not change the structure of individual nucleosomes or their stability, but they play an essential role in regulating high-order chromatin structures (Peterson and Laniel 2004). These post-translational modifications include acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serines (S) and threonines (T), ubiquitylation and sumoylation of lysines, as well as ribosylation.

Histone acetylation

A key step towards understanding the role of histone modifications was the discovery of nuclear HAT (Brownell, Zhou et al. 1996) and its antagonist the HDAC (Taunton, Hassig et al. 1996). They were found to function as co-activators and co-repressors of transcription, respectively. They exert their function by adding (HAT) or removing (HDAC) acetyl groups to lysine residues of histone H3 or H4 (Choi and Friso 2010) . Acetylation of histones is generally associated with activation of gene transcription (Fig. 7). A possible function in DNA repair has also been proposed for acetylation of certain lysines on histones H3 and H4 (Peterson and Laniel 2004). HDAC inhibitors have been recognized as a novel cancer treatment since they induce cell cycle arrest and apoptosis (Song, Han et al. 2011).

There seems to be a relationship between histone hyperacetylation and gene expression under diabetic conditions since primary human endothelial cells exposed to hyperglycemic conditions display increased expression and enrichment

of the HAT p300 (Chen, Feng et al. 2010). In addition to this, PDX1, a β-cell specific transcription factor, has been shown to interact with p300 to mediate glucose-induced expression of insulin (Mosley, Corbett et al. 2004).

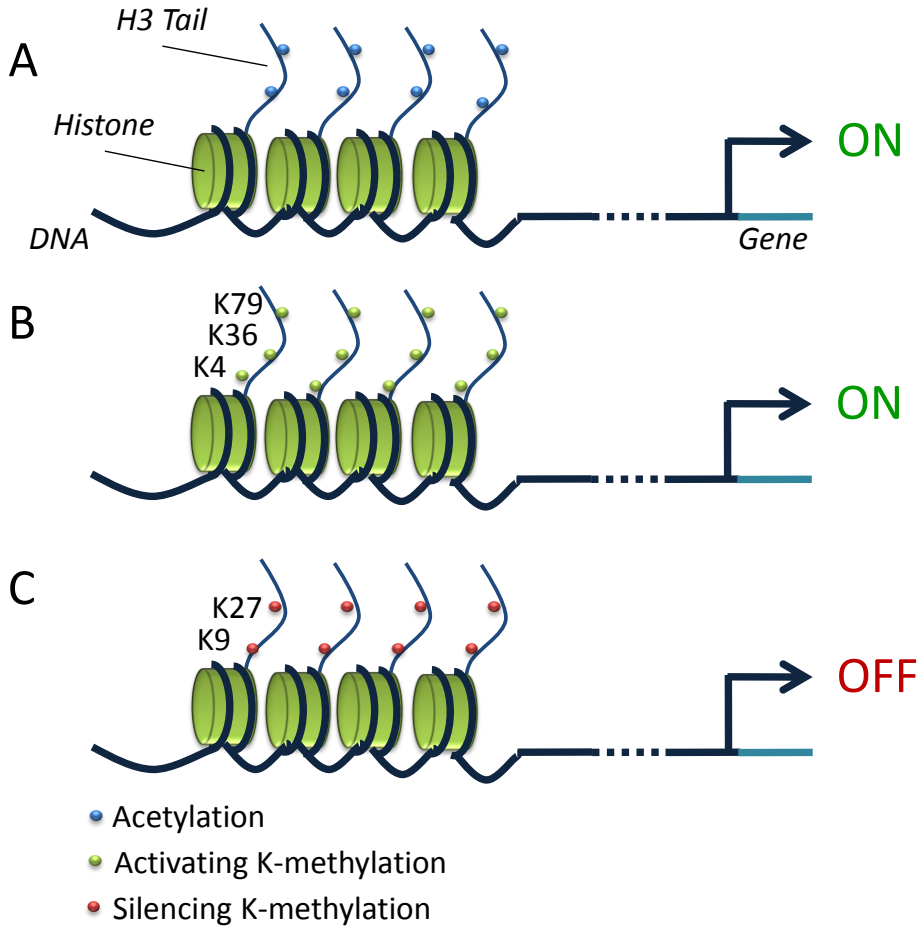

FIGURE 7: Histone modifications in gene promoter regions and regulation of gene transcription.
A. Histone H3 acetylation of the gene promoter region is generally associated with activation of gene transcription. B. Methylation of lysine (K) 4, 36 and 79 of histone H3 is generally associated with activation of gene transcription C. Methylation of lysine (K) 9 and 27 of histone H3 is generally associated with repressed gene transcription

Histone methylation

Unlike histone acetylations that are almost exclusively associated to transcriptional activation, histone methylations are more complicated and diverse modifications. A particular modification or set of modifications can have different or even opposite biological consequences depending on their locations. Adding to the complexity is the fact that each lysine residue can be mono-, di- or even tri-methylated (Martin and Zhang 2005), and arginine residues can be either mono- or di-methylated (Di Lorenzo and Bedford 2011). Over 20 mammalian histone lysine methyltransferase enzymes have been identified to date. These enzymes act on different lysine residues and might mono-, di- or tri- methylate them, or all three (Keating and El-Osta 2012).

However, methylation of lysine residues 4, 36 and 79 of histone H3 are generally associated with active genes and an open chromatin structure, while methylation of lysine residues 9 and 27 of histone H3 are generally associated with silenced genes or inactive chromatin (Fig. 7) (Martin and Zhang 2005).

Histone H3 lysine 4 monomethylase Set 7/9 has been shown to be necessary for the maintenance of glucose-regulated genes in islets (Deering, Ogihara et al. 2009; Ogihara, Vanderford et al. 2009). It has also been linked to the transcriptional regulation of the insulin gene in β-cells (Chakrabarti, Francis et al. 2003), an effect that seems to be dependent on PDX-1 (Francis, Chakrabarti et al. 2005). In a streptozotocin diabetes mouse model, TNF-α-induced recruitment of Set7/9 is increased in macrophages, implying a role for set 7/9 in inflammation and diabetes (Li, Reddy et al. 2008).

Cross talk

There is cross talk between epigenetic modifications; the presence or absence of one epigenetic modification might influence the other (Suganuma and Workman 2011). One example of this is the repression of transcription exerted by DNA methylations. Part of it is due to obstruction of transcription factors but also attraction of methyl-CpG binding proteins that further may recruit HDACs. The HDACs de-acetylate histone tails in the promoter region, further altering chromatin structure and inactivating genes (Clouaire and Stancheva 2008).

Another example of epigenetic cross talk occurs in *C. elegans*, where a demethylase acting on H3 lysine 9 and 27 recognizes and binds H3K4me3. This results in removal of repressing modifications in chromatin regions with increased amount of the activating modification H3K4me3 (Yang, Hu et al. 2010).

Also, Set7-mediated H3K4 methylation inhibits H3K9 methylation and facilitates acetylation of both H3 and H4 by the HAT p300 (Zegerman, Canas et al. 2002).

In all of these examples, epigenetic modifications influence other epigenetic modifications with an opposite effect on transcription, thus amplifying their own transcriptional effects.

Some modifications, such as methylation of histone H3 lysine 9 (H3K9) are considered to be bivalent marks signaling either repression or transcription depending on context. It appears that this modification might have a different readout depending on its localization on the chromosome as well as the combinations of proximate modifications, as well as what enzyme is involved in establishing the particular modification (Zhang and Reinberg 2001).

Clonal β-cells

The use of primary cells for research is restricted both by availability and by their limited proliferative potential. Normal somatic cells divide a finite number of times before permanently becoming post-mitotic and senescent. Cells belonging to an immortalized cell line, having lost these limitations, can divide an infinite number of times and survive as long as nutrition access in not exhausted. However, their usability is to some extent limited since they are normally transformed and cancerous, they may become de-differentiated with time, losing some of the characteristics of primary cells. Also, serial passage of cells can further cause genotypic and phenotypic variation and genetic drift can cause heterogeneity in the cell culture (Kaur and Dufour 2012).

Immortalized cell lines

Tumor cell culture methods were developed in the 1930s and the first immortalized cell line was reported in the 1940s (Stacey 2006). The most famous example of an immortal cell-line is perhaps the HeLa cell line derived from a cervical cancer biopsy taken from a woman named Henrietta Lacks in 1951. Henrietta died from her cancer later that same year. Her cells, however, lives to this day and has helped scientists to study a wide variety of human conditions (Skloot 2010). Many cell lines, such as the HeLas, are derived from tumors and there are now cell lines available from a large variety of species and tissues. For those studying β-cells, there are several cell lines available, such as the INS-1 derived cell lines from rat (Asfari, Janjic et al. 1992; Hohmeier, Mulder et al. 2000), MIN-6 from mouse (Ishihara, Tsuneoka et al. 1993) and the newly engineered EndoC-βH1 from human cells (Ravassard, Hazhouz et al. 2011).

INS-1-derived cell lines

The original INS-1 rat β-cell line was widely used to study β-cell function; however, with time it generally exhibited only a 2-4-fold increase in insulin secretion when stimulated with glucose. This is far less than that from freshly isolated islets, where the response is usually around 15-fold. It was originally derived from an x-ray-induced rat β-cell carcinoma (Asfari, Janjic et al. 1992). After stable expression of the human insulin gene and clonal expansion of these cells a number of subclones were obtained with different secretory abilities. The most strongly responsive cell line, the INS-1 832/13 β-cell line, showed a glucose-stimulated insulin response of >10-fold as well as the presence of a K_{ATP}-channel-independent pathway of glucose sensing. One of the poorly secreting clonal lines, INS-1 832/2, had a comparable insulin content to the INS-1 832/13 β-cells but a considerably weaker glucose-stimulated insulin response (<2-fold) (Hohmeier, Mulder et al. 2000). The comparisons between these two cell lines that share the same parental cell line but have vastly different secretory abilities provide an interesting model of functional and dysfunctional β-cells.

Another INS-1 derived β-cell line is the INS-1E. Here, novel sublines from parental INS-1 cells were established by high dilution of the cells and the INS-1E cells were selected based on their higher insulin secretion responses to glucose (Asfari, Janjic et al. 1992).

Even though the INS-1 832/13 β-cell is a cancer cell, it remained highly differentiated, exhibiting a robust β-cell phenotype with respect to production and secretion of insulin and metabolic function.

AIMS

The main aim of this thesis is to find molecular and metabolic mechanisms contributing to impaired β-cell function. To reach this goal, this thesis combines comprehensive approaches to study metabolic, transcriptional and epigenetic events linked to impaired GSIS in the pancreatic β-cell.

The specific aims were:

- To compare INS-1-derived clonal β-cell lines with different glucose responsiveness to identify metabolic disturbances that impairs GSIS (Study I & II**).**

- To look for causative mechanisms underlying a metabolic shift that perturbs GSIS and to study the relevance of these mechanisms in human T2D (Study I & II).

- To study cellular mechanisms underlying the β-cell response to lipotoxicity-induced metabolic stress (Study III).

- To study the link between histone modifications, transcriptional control and altered function in lipotoxic β-cells (Study III)

MODELS AND METHODS

Clonal β-cell lines

Clonal β-cell lines were used for the vast majority of the experiments in this thesis. Cell lines used were INS-1 and three cell lines derived from it, INS-1 832/1, INS-1 832/2 and INS-1 832/13.

As previously mentioned, the INS-1 rat β-cell line was established in 1992 (Asfari, Janjic et al. 1992) from an x-ray-induced rat insulinoma (RIN) (Chick, Warren et al. 1977). The INS-1 832 daughter cell lines were established after stable expression of the human insulin gene and clonal expansion of several cell lines (Hohmeier, Mulder et al. 2000). These clonal daughter cell lines exhibit different abilities to secrete insulin in response to high glucose ranging from strongly responsive clones such as the INS-1 832/13 (>10-fold) to poorly responsive clones such as INS-1 832/1 and INS-1 832/2 (<2-fold). This cannot be attributed to insulin content of the β-cells since this is comparable in all three clones.

Because these cell lines are sister clones, yet still exhibit such profound differences in their ability to secrete insulin, a comparison of the cells might provide important clues to what mechanisms are essential for proper β-cell function. Also, the INS-1 832/13 cell line serves as a useful model of normal β-cell function.

Human pancreatic islets

Human pancreatic islets were obtained from the Nordic Network for Clinical Islet Transplantation by courtesy of Professor Olle Korsgren at Uppsala University. Clinical information regarding the donors as well as information regarding islet purity was available. The islets were subsequently processed by the Human Tissue Lab within EXODIAB. At the Human Tissue Lab, GSIS was determined and RNA was extracted from the islets. Using this RNA, gene expression was examined by the Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA).

In study I, pancreatic islets from 23 non-diabetic deceased donors (13 females, 10 males) 26-73 years old with BMI ranging from 17.6 to 29.0 kg/m^2 were used. In

study II, pancreatic islets from 55 non-diabetic (26 females, 29 males) and nine T2D (four female, five male) deceased donors were used. The non-diabetic donors had an average age of 56.7 years, an average BMI of 25.9 kg/m^2 and an average HbA_{1c} of 5.7%. The T2D donors had an average age of 57.0 years, an average BMI of 28.5 kg/m^2 and an average HbA_{1c} of 7.3%.

A lipotoxicity model

To study β-cell function in the prediabetic state, we induced lipotoxicity in the cells by chronic exposure to high levels of FAs. Lipotoxicity perturbs both GSIS and insulin synthesis in β-cells (Sako and Grill 1990; Carlsson, Borg et al. 1999; Karaskov, Scott et al. 2006). To focus on cellular effects of lipotoxicity and to exclude effects mediated via impaired viability, a two-level full factorial design with insulin secretion and viability as responses was created.

Development of a lipotoxic *in vitro* model

To define an optimal lipotoxic setting, three partial least squares (PLS) models were calculated for the factors basal insulin secretion, stimulated insulin secretion and fold-change of insulin secretion (Fig. 8). These models estimate synergetic effects of time, glucose and palmitate concentrations, and can be used to distinguish between them.

Interactions between palmitate concentrations and time of treatment were observed in these three models. Basal insulin secretion was increased by the synergy of palmitate and time, whereas GSIS was decreased by the same factor interaction. Judging from these models, 0.5 mM palmitate for 48h resulted in a lipotoxic condition where both increased basal insulin secretion and decreased stimulated insulin secretion were observed.

To assess the number of viable cells, an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was used. This assay measures cellular metabolic activity, reflecting the number of viable and proliferating cells.

Counting viable cells using the MTS assay revealed no decreased cell viability/proliferation with 0.5 mM palmitate for 48h.

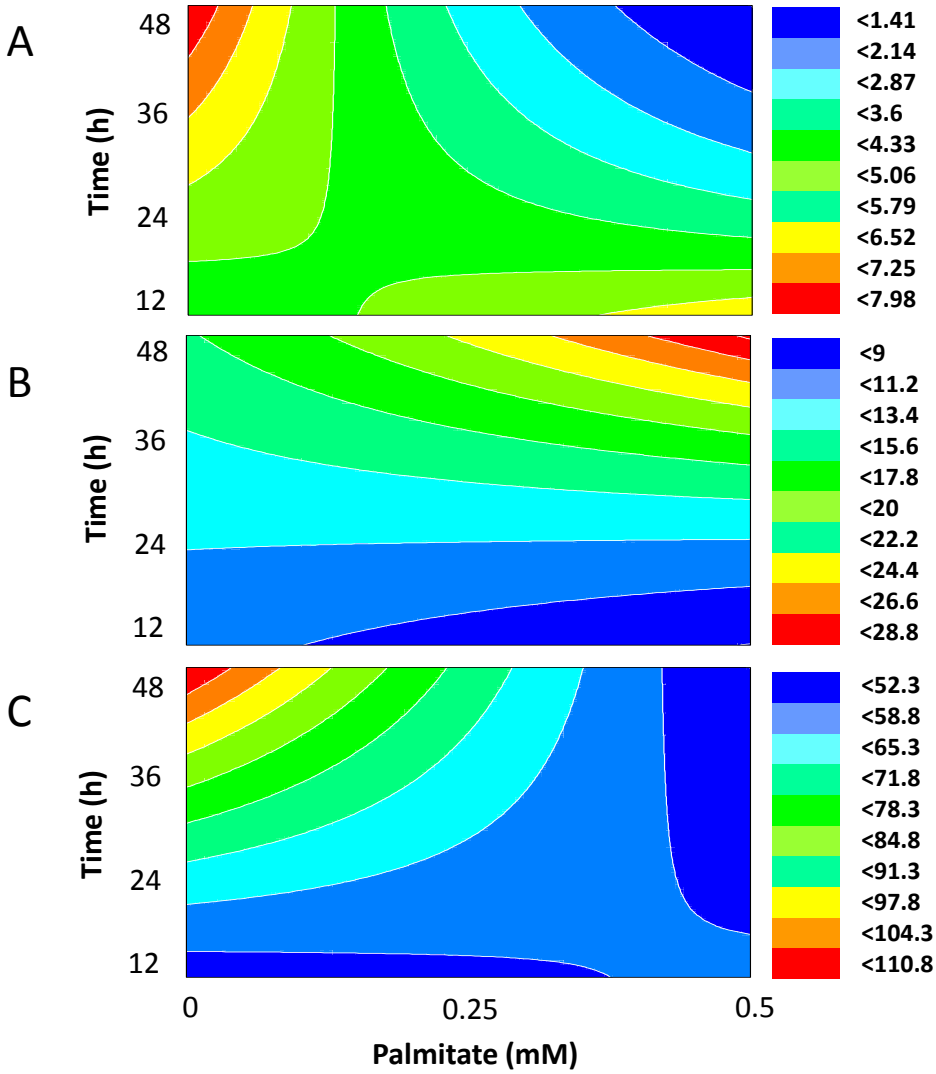

FIGURE 8: The insulin secretion response of the β-cell to palmitate concentrations and time of treatment identifies a lipotoxic condition with impaired GSIS. Response surfaces for palmitate concentrations ranging from 0 to 0.5 mM at 11.1 mM glucose for (a) fold-change of insulin secretion, (b) basal insulin secretion at 2.8 mM glucose, and (c) stimulated insulin secretion at 16.7 mM glucose. In this model, 0.5 mM palmitate for 48h defines lipotoxic conditions with increased basal insulin secretion and decreased stimulated and fold change insulin secretion.

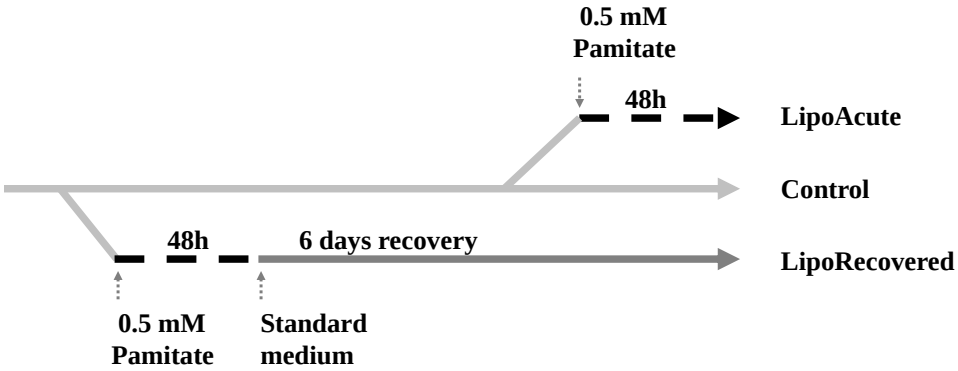

FIGURE 9: Lipotoxic model.
Clonal INS-1 832/13 β-cells were cultured for 8 days prior to experiments in Control (8 days standard medium) and LipoAcute (6 days standard medium – 48 h in 0.5 mM palmitate) conditions. To examine recovery from lipotoxicity and the possibility of a "metabolic memory", a third condition, LipoRecovered (48 h in 0.5 mM palmitate – 6 days standard medium), was created.

To study the recovery and a possible "metabolic memory" of lipotoxicity (LipoRecovered), cells were allowed to recover for 6 days in normal medium following the 48 h exposure to 0.5 mM palmitate (Fig. 9).

Oxygen consumption rate

Since the β-cell produces most of its ATP via oxygen-dependent mitochondrial respiration, extracellular measurement of the rate of oxygen consumption is a way of determining this metabolic process essential to β-cell function. Extracellular oxygen consumption rate (OCR) measurements were performed, using the Seahorse Extracellular Flux Analyzer XF24 (Seahorse Bioscience, Billerica, MA) (Fig. 10).

This instrument measures OCR in real time in intact cells grown in mono-layers. Through the addition of the chemical compounds oligomycin, FCCP (Carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone) and rotenone, it is possible to dissect the total OCR into specific parts of the total respiratory capacity.

The functions of these compounds are as follows:

Oligomycin: inhibits ATP synthase by blocking its proton channel, thus blocking the production of ATP through oxidative phosphorylation.

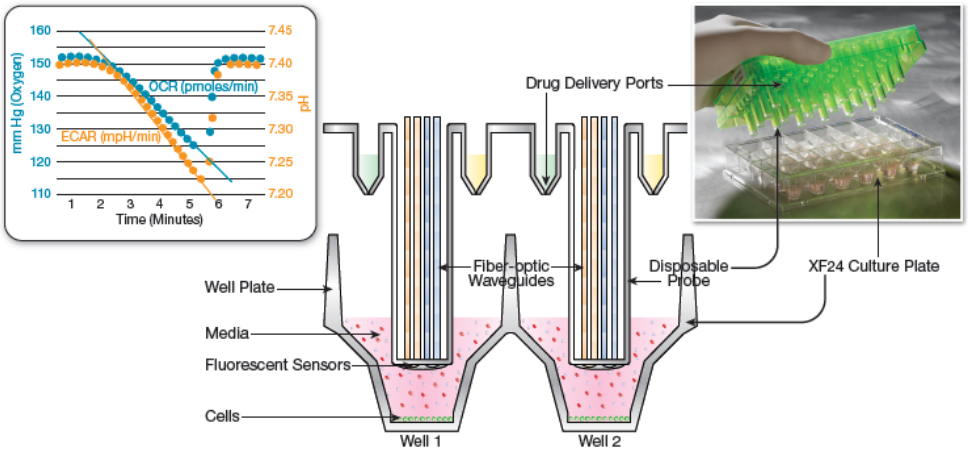

FIGURE 10: How the Seahorse XF instrument works.
Cells are grown in monolayers and oxygen consumption in these is measured in real time. The fluorescent sensor measures [O_2] in a small volume (7 µL) at each measurement time point. Four delivery ports allow injections of compounds to manipulate respiration during the run. The slope of the curve of [O_2] enables assessment of the oxygen consumption rat (OCR).
Figure from Seahorse bioscience image library

FCCP: introduces a proton leak over the inner mitochondrial membrane, to give uncoupled respiration.

Rotenone: Inhibitor of mitochondrial electron transport by interfering with the transfer of electrons between complex I and ubiquinone.

This is an example of a seahorse experiment workflow:

105 min	Pre-incubation at 2.8 mM glucose
↓	*Start reading plate*
15 min	Measure: Basal OCR at 2.8 mM glucose
↓	*Fuel addition*
60 min	Measure: Stimulated OCR at 16.8 mM glucose (or other stimulatory secretagogues)
↓	*Oligomycin addition*
15 min	Measure: Proton Leak, OCR after blocking of ATP synthase
↓	*FCCP addition*

15 min	Measure: Maximum respiratory capacity
↓	*Rotenone addition*
15 min	Measure: Non-mitochondrial OCR
↓	
STOP	

From an experiment such as this, a number of respiratory parameters can be assessed (Fig. 11). Respiratory control ratio (RCR; Maximal respiration/Proton leak) and coupling efficiency (CE; Total respiratory ATP/Basal OCR) can be calculated as previously described (Brand and Nicholls). The RCR assesses the coupling of state3 (uncoupled state)/state4 (oligomycin state) respiration and the CE assesses the coupling of ATP production to oxygen consumption.

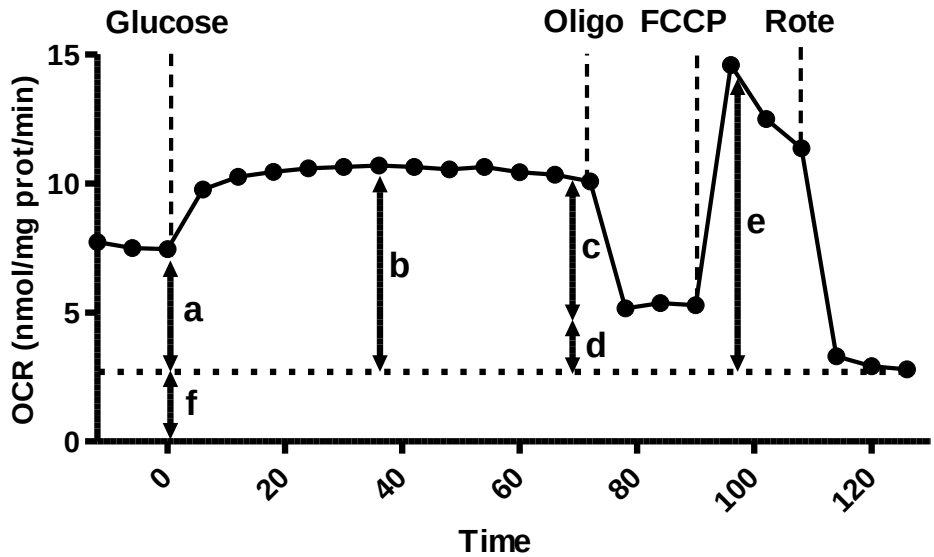

FIGURE 11: Control experiment measuring OCR.
Clonal INS-1 832/13 β-cells in the presence of 2.8 mM glucose were exposed sequentially to: 16.7 mM glucose, 4 μg/ml oligomycin (oligo), 4 μM FCCP and 1 μM rotenone (Rote). Non-mitochondrial respiration after the final addition (f) was subtracted from the other values: a, basal respiration; b, glucose-stimulated respiration; c, oligomycin-sensitive respiration; d, proton leak (oligomycin-insensitive respiration) e, maximal respiration in the presence of FCCP. Respiratory indexes derived from this data were: respiratory control ratio (RCR; e/d) and coupling efficiency (CE; c/b).

Metabolic profiling

Since β-cell metabolism regulates fuel-stimulated insulin secretion, assessing alterations in metabolite levels in the cell are of importance.

Metabolic profiling using gas chromatography (GC)/mass spectrometry (MS) provides a snap shot of whole cell metabolite levels at a given condition. Increased concentrations of metabolites in any given metabolic pathway may be caused either by increased metabolic flux or by decreased flux in pathways downstream, causing accumulation of metabolites. Therefore, the use of metabolomics, gives a comprehensive pattern of whole cell metabolic alterations.

For metabolic profiling, cells are stimulated using glucose (or other cellular fuels) followed by addition of -80°C methanol, which quenches metabolic processes. After this, internal standards that allow identification of metabolite masses are added. Next, metabolites are extracted from the samples using one-phase liquid extraction, derivatized and analyzed using GC/MS. Identification of the metabolites is obtained by comparing the peaks to those of the internal standards; the relative amount of a certain metabolite is calculated from the peak area.

Gene expression

To identify changes in gene expression, the levels of messenger RNA (mRNA) were measured using three different techniques, Taqman quantitative PCR (qPCR; study I and III), SYBR Green qPCR (study III) and Affymetrix microarray (study I, II and III). To enable detection of sample mRNA levels, the mRNA strands were first converted into complementary DNA (cDNA) using reverse transcription.

Taqman qPCR

The Taqman probe technique relies on the 5´ to 3´ exonuclease activity of Taq polymerase and the fact that when two DNA strands are made up by nucleotide bases that can form non-covalent hydrogen-bonds, i.e., are complementary, they will bind tightly to each other (hybridize). Taq polymerase cleaves a labeled probe, losing a quencher in the process, during hybridization to its complementary target sequence. The resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the qPCR. The Taqman probe increases the specificity of this detection significantly compared to other qPCR methods (Fig. 12). The quantity of the gene of interest is then normalized to the expression of another, abundantly and stably expressed gene, a so called housekeeping gene and may be quantified using the ΔΔCt

method. In study I and III, hypoxanthine phosphoribosyltransferase (*hprt*) was used as the housekeeping gene.

Normfinder (Andersen, Jensen et al. 2004), an algorithm for identifying the optimal housekeeping gene**,** was used to test the stability of the selected housekeeping gene expression compared to that of the genes of interest as well as an additional housekeeping gene, cyclophilin A (*Ppia*).

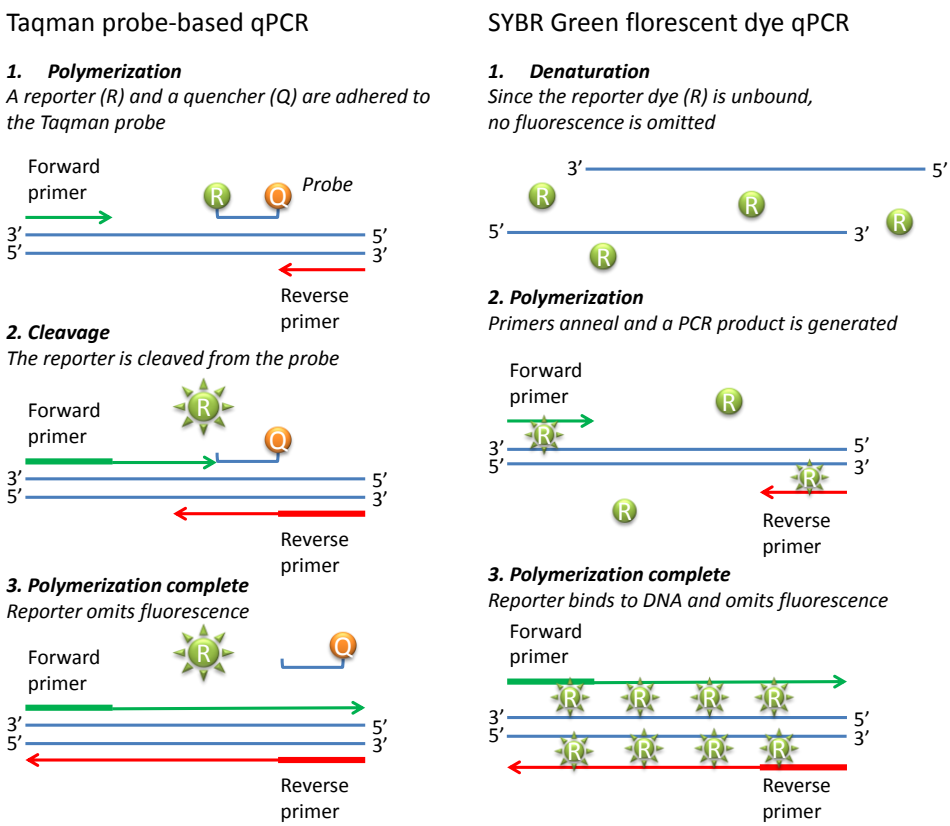

FIGURE 12: Comparison of Taqman and SYBR Green qPCR
In the Taqman probe-based qPCR, the reporter (R) is bound to a gene-specific probe together with a quencher (Q). When the sequence corresponding to the probe is transcribed, the reporter is released and emits fluorescence in proportion to the amount of transcripts produced. In the SYBR Green dye based qPCR, the dye (R) emits fluorescence when bound to double-strand DNA (dsDNA) in proportion to dsDNA amounts. Figure was modified from www.invitrogen.com
The low density array (LDA) used in study I is an array with Taqman gene expression assays. It was designed to target 46 essential genes in glycolysis, the TCA cycle, and the respiratory chain.

SYBR Green qPCR

SYBR Green is a cyanine dye that binds to double-strand DNA (dsDNA) and emits fluorescence in proportion to the amount of dsDNA present (Fig. 12). This qPCR system is somewhat less specific than the Taqman system since it is not based on specific probes but detects any dsDNA, hence increasing the risk of false positive signals. It is however, considerably more cost-efficient and easier to work with when using primers of your own design. In study III, SYBR Green in combination with primer design was used to map the spatial distribution of fold enrichment of histone modifications.

See Figure 12 for a comparison between methods.

Affymetrix microarray

A microarray is a collection of microscopic DNA spots attached to a solid surface, a "chip". The principle behind microarrays is that two DNA strands that are complementary will bind tightly to each other. The larger the number of bonds, the stronger two sequences will be bound to each other. This binding is referred to as hybridization. Microarray analysis of gene expression allows you to measure thousands of genes at the time with multiple probes per gene.

To measure gene expression by microarray, the mRNA is converted to cDNA through reverse transcription; the cDNA is then labeled with fluorescent probes before being hybridized to the microarray chip. After washing, only the strongest bound cDNA strands will remain on the chip. The fluorescent labeling generates a signal from each spot and the strength of this signal depends on the amount of the target sequence that is present in the sample.

In study I and II, expression data were extracted from gene expression analysis of human islet donors, using the Human Gene 1.0 ST array.

In study III, a comprehensive gene expression analysis of clonal β-cells exposed to lipotoxicity was performed. The Rat Gene 1.0 ST Array gene chip (Affymetrix, Santa Clara, CA) that was used in study III analyzes the expression of 27,342 genes using 722,254 distinct probes. That means that each of the genes is represented on the array by approximately 26 probes (Affymetrix 2007). The location of these probes is spread out across the exons of the genes. Background is estimated using a set of approximately 17,000 generic background probes. Background correction, normalization and probe summarization were performed by using the Robust Multichip Average (RMA) method (Irizarry, Hobbs et al. 2003) implemented in the Expression Console software Version (v) 1.0 (Affymetrix).

Chromatin immunoprecipitation (ChIP)

To study the prevalence of specific histone modifications on certain genes we applied a strategy called ChIP where a histone modification is being immunoprecipitated (IP), using specific antibodies (Ab's). The comparison of the enrichment of a specific gene in the ChIP DNA to input control (total DNA before IP), using qPCR is a measure of how prevalent the histone modification is on the gene targeted by the primers used in the qPCR.

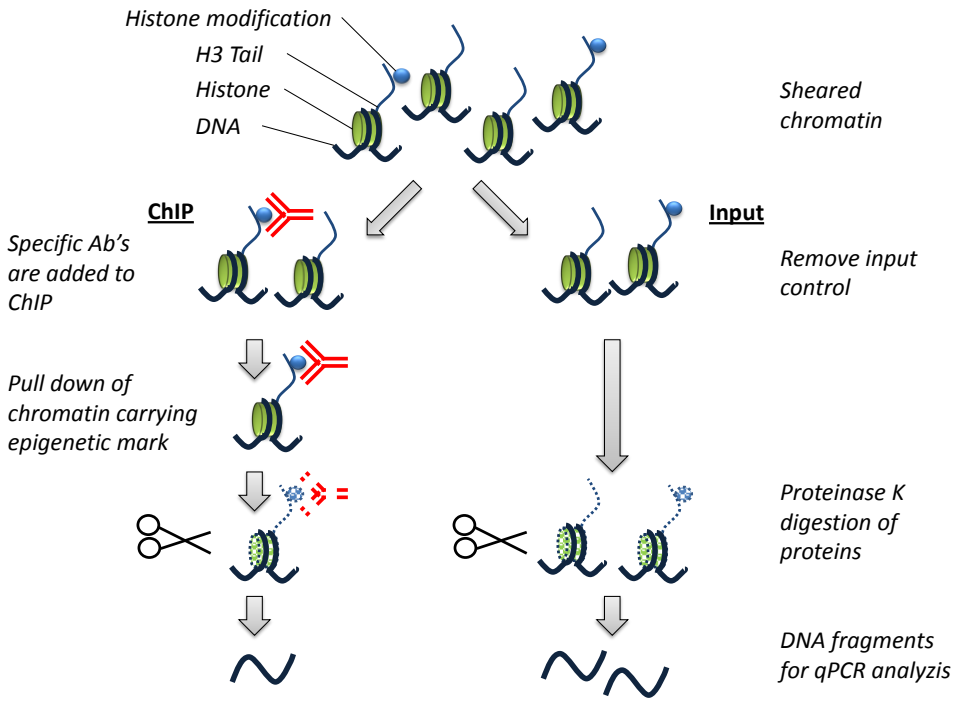

FIGURE 13: Chromatin immunoprecipitation (ChIP) of cross-linked chromatin Cells were cross-linked and sonicated to shear chromatin into 200 – 800 bp size and incubated together with specific antibodies (Ab's) to immunoprecipitate (IP) chromatin carrying the histone modifications that the Ab used is specific for. Before IP, 10% of the samples were removed as input control. After IP, total DNA was eluted and proteins digested using proteinase K to produce two fractions, one enriched for a certain histone modification (ChIP) and one virgin (input). Comparison of these two fractions reveals to what degree the ChIP has enriched a sample for a certain histone mark. The fold enrichment of the histone modification is proportional to the amount of that histone modification present on the analyzed locus.

First, cells are cross-linked with 1% formaldehyde for 10 min. Cross-linking is a way of stabilizing the histone-DNA interactions before analysis. After the cells were put on ice, scraped off and sonicated in a lysis buffer. Sonication produced sheared chromatin with an approximate size of 800 to 200 base pairs (bp). Before IP, 10% of the sonicated chromatin was removed for input control. Following this, the sheared chromatin was incubated with specific antibodies (Ab's) against four different histone modifications; H3K4me3, H3K27me3, H9Ac and H3K79me2 or IgG control and second with protein A/G magnetic beads. Genomic DNA was eluted in combination with proteinase K treatment followed by phenol/chloroform extraction and ethanol precipitation (Fig. 13). Genomic DNA was genome-wide amplified. Amplified DNA was then used as input for SYGR Green qPCR using specific primers.

Primer design

qPCR was used to analyze the fold enrichment of the histone modifications immunoprecipitated in the ChIP and this was performed for 12 selected genes. 10 of these genes were selected based on their differential mRNA expression in clonal INS-1 832/13 β-cells exposed to lipotoxicity and two genes, *Gapdh* and *Ldha*, were selected as positive and negative control genes based on their high and low expression in clonal INS-1 832/13 β-cells, respectively.

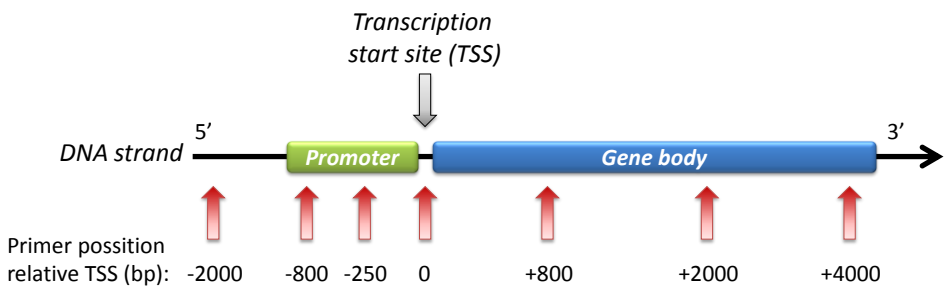

FIGURE 14: Primer design
PCR primers were designed at seven different locations per gene (-2000, -800, -250, 0, 800, 2000 and 4000 bp in relation to the transcription start site (TSS)) to assess spatial distribution of histone modifications. This was done using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Due to the size of the DNA fragments after sonication, the PCR product size was set to 80 – 120 pb. Generated primers were used in SYBR Green qPCR reactions.

PCR primers were designed to cover promoter and gene body regions at seven different approximate locations per gene, -2000, -800, -250, 0, 800, 2000 and 4000 bp in relation to the transcription start site (TSS), using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) as seen in Figure 14. The length of the transcript was set to be between 80 and 120 bp.

Statistical methods

All data are shown as mean ± standard error of the mean (SEM) for the number of experiments indicated in the figure legends. Statistical comparisons of the mean values of two groups were performed using a parametric Student's test (study I, II and III) or non-parametric Mann-Whitney *U* test (study II). When more than two groups were compared, a parametric one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test *post hoc* (study III), or a non-parametric Kruskal-Wallis test followed by Mann-Whitney in combination with Bonferronis' test (study II) was used. Microarray data were compared statistically using one-way ANOVA in combination with false discovery rate (FDR) where $q<0.01$ was considered significant. *Post hoc* analysis of between group differences was performed using Tukey's multiple comparison test. In addition to this, a pathway analysis was performed using DAVID Bioinformatics Resources 6.7 (david.abcc.ncifcrf.gov) (Dennis, Sherman et al. 2003). This analysis assesses gene set enrichment of significantly altered genes by assigning the genes to biological pathways and then examines whether any pathway has a higher representation than it would by chance.

The ChIP experiments were compared using a paired ANOVA followed by Bonferronis' test *post hoc.*

Correlations between two variables were analyzed using non-parametric Spearman's tests (study I and II) or a parametric Pearsons' test (study III).

Differences between mean values were considered significant when $p<0.05$ unless otherwise stated. The p-values were two-tailed.

Statistical analyses were performed using SPSS (SPSS, Chicago, IL), GraphPad Prism 5 (GraphPad Software, La Jolla, CA), MeV (TM4 Microarray Software Suite) (Saeed, Bhagabati et al. 2006) and R software (R Development Core Team 2010).

RESULTS AND DISCUSSION

The importance of tight coupling of metabolic flux in β-cells (Paper I and II)

The biochemical mechanisms underlying GSIS from pancreatic β-cells are to date not fully understood. The triggering pathway, directly coupling glucose-driven ATP production to insulin release, has been fairly well characterized. The mechanisms and signals constituting the amplifying pathway are more complex and less clear. Several metabolites mainly deriving from mitochondrial metabolism have been suggested to amplify GSIS in a K_{ATP}-channel independent manner (Fig. 4) (Henquin 2000; Maechler 2002). This, along with the fact that more than 95% of ATP in the β-cell is produced by oxygen-consuming respiration in the mitochondria (Tarasov, Dusonchet et al. 2004), highlights the importance of proper funneling of intermediates derived from cytosolic glucose metabolism into mitochondrial metabolic pathways. A disturbed stimulus-secretion coupling, caused by alterations of metabolic flux could potentially be involved in the pathogenesis of T2D. In **paper I and II**, we used several β-cell lines to examine the metabolic coupling in β-cells and the effects on GSIS when this coupling is perturbed. We also sought causal mechanisms behind these perturbations.

Tight coupling between glucose and mitochondrial metabolism in clonal β-cells is required for robust insulin secretion (Paper I)

Perturbations in the fuel-responsive alterations of metabolic flux may lead to impaired GSIS from the β-cell, the ultimate cause of T2D. To identify metabolic disturbances in β-cells that impair GSIS, we compared two INS-1-derived clonal β-cell lines, one that is glucose-responsive (INS-1 832/13) and one that is glucose-unresponsive (INS-1 832/2).

In **paper I**, we show that despite impaired GSIS, INS-1 832/2 β-cells exhibited a higher rate of glucose utilization, reflecting glycolysis. However, no glucose-induced increases in parameters in mitochondrial metabolism such as OCR, oligomycin-sensitive OCR and ATP production were observed in the glucose-unresponsive INS-1 832/2 β-cells. Activities of the respiratory chain complexes I, III, and IV were decreased as well (Fig. 15).

The importance of mitochondrial metabolism for GSIS and T2D development was recently emphasized by Koeck *et al.*, who showed that a risk variant of the human transcription factor B1 mitochondrial (*TFB1M*) gene associates with reduced GSIS, elevated postprandial glucose levels, and future risk of T2D. This was accompanied by decreased levels of proteins of complex I and III of the ETC. Knock down experiments in 832/13 β-cells confirmed the link between decreased ETC complex activity, decreased OCR and decreased GSIS (Koeck, Olsson et al. 2011). In addition, pancreatic islets from patients with T2D exhibit decreased expression of multiple genes encoding parts of the ETC, further supporting a role for mitochondrial dysfunction in T2D (Olsson, Yang et al. 2011).

When examining gene expression closer in INS-1 832/2 β-cells, it was found that genes coding for glycolytic enzymes were up-regulated, whereas mitochondria-related genes were down-regulated.

In particular, the genes hexokinase 1 (Hk1) and lactate dehydrogenase A (Ldha) were expressed only in the INS-1 832/2 and not in the 832/13 β-cells. Hexokinase 1 is a low K_m hexokinase isozyme that transports glucose already at very low concentrations and lactate dehydrogenase A forms NAD^+ from NADH to maintain glycolysis by converting pyruvate to lactate. This diverts pyruvate from the TCA-cycle, thus lowering total ATP production. This could account for the Warburg-like effect in the 832/2 β-cell clone, lacking in 832/13 β-cells as well as primary β-cells.

The glucose-responsive INS-1 832/13 β-cell did not release lactate. In contrast, the glucose-unresponsive INS-1 832/2 β-cells released lactate regardless of the glucose concentration in the medium. This is reminiscent of the Warburg effect seen in cancer cells. Such cells predominantly produce energy by a high glycolytic rate followed by lactate production in the cytosol, rather than by oxidation of pyruvate in the mitochondrion, as occurs in most other cell types (Warburg 1956). This is an adaptation to a hypoxic situation in tumors.

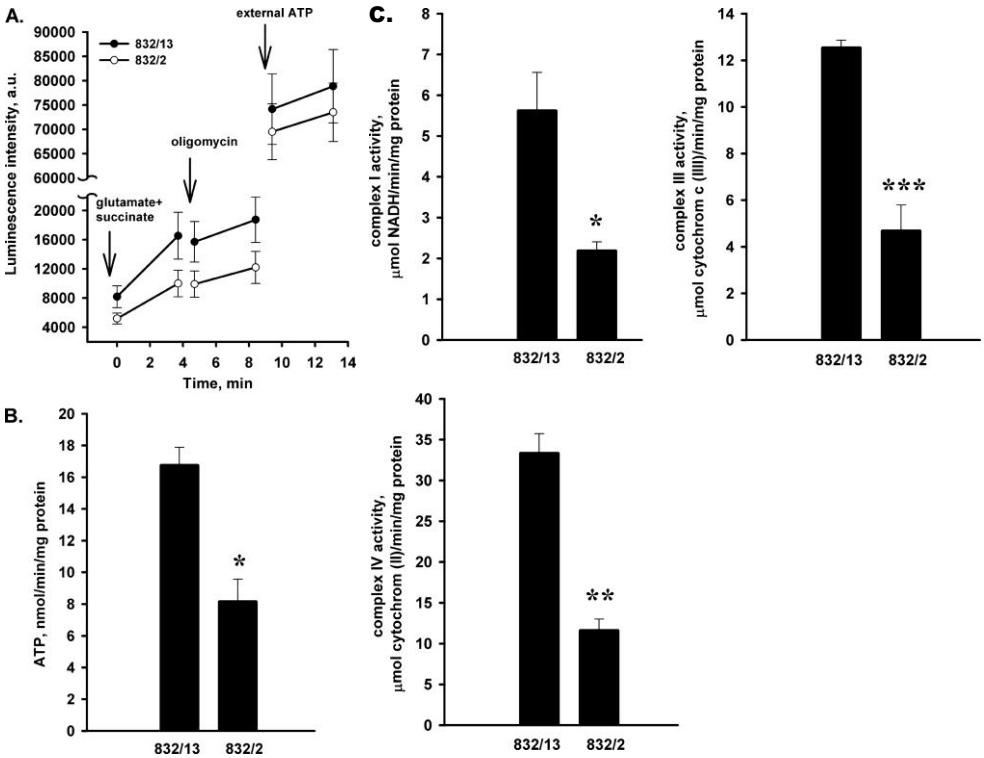

FIGURE 15: Oligomycin-dependent ATP production and Complex I, III and IV activity

A, ATP production in INS-1 832/13 and 832/2 β-cells. Glutamate-succinate-induced mitochondrial ATP production was calculated as the difference before and after oligomycin addition (n=9); **B**, oligomycin-sensitive ATP production is significantly lower in INS-1 832/2 β-cells compared with 832/13 β-cells. ; **C**, complex I, complex III and complex IV activity is significantly lower in 832/2 β-cells compared with 832/13 β-cells (n=3).

The data are presented as the mean ± SEM. (*, $p<0.05$; **, $p<0.01$;***, $p<0.001$)

Primary β-cells express Ldha at very low levels compared to other tissues (Sekine, Cirulli et al. 1994). It has been proposed as a "disallowed β-cell gene" (Quintens, Hendrickx et al. 2008), probably because it would disturb an exclusive role of glucose to stimulate mitochondrial metabolism and consequently insulin secretion. There is some controversy on the subject of whether this in itself is enough to attenuate GSIS since overexpression of Ldha alone had no effect on GSIS in INS-1 β-cells (Ishihara, Wang et al. 1999) while it has been shown to perturb mitochondrial metabolism and GSIS in MIN6 β-cells (Ainscow, Zhao et al. 2000). A possible explanation to this is that the expression of MCTs (encoding monocarboxylic acid transporters) allows lactate efflux from β-cells in the latter study.

Previous studies comparing INS-1 subclones showed that glucose responsiveness correlated with an increased pyruvate cycling through the anaplerotic pathway (Lu, Mulder et al. 2002). This is in agreement with later studies showing that an impaired anaplerosis attenuates GSIS in rat islets by decreasing the glucose-induced rise in the ATP:ADP ratio (Fransson, Rosengren et al. 2006). However, in the end, they are cell lines and even a well differentiated β-cell line could be driven partly by the needs for growth and replication, which involve anaerobic and anabolic glycolysis.

To examine whether the differential expression of said genes was relevant to β-cell function *in vivo*, we examined gene expression of these genes in human islets and correlated it to HbA_{1c} levels reflecting long term glucose homeostasis. In human islets, expression of lactate dehydrogenase A (*LDHA*), hexokinase I (*HK1*), triosephosphate isomarase 1 (*TPI1*) and citrate synthase (*CS*) correlated positively with HbA_{1c} levels. A high HbA_{1c} level is a sign of poor long term glucose homeostasis. The expression of genes for glucose transporter type 2 (*SLC2A2*), malic enzyme 1 (*ME1*), glycerol-3-phosphate dehydrogenase 2 (*GPD2*) and acetyl-CoA carboxylase α (*ACACA*) correlated negatively with HbA_{1c}. This suggests that these genes may be essential for good metabolic control (Fig. 16).

Since increased HbA_{1c} is reflecting prolonged elevated blood glucose, it is a sign of poor metabolic control. The fact that four genes found to be upregulated in INS-1 832/2 β-cell compared to INS-1 832/13 β-cells also correlated positively with HbA_{1c} suggested that subjects with increased levels of these genes also have worse long term glucose homeostasis. Consequently, the four genes that correlated negatively with HbA_{1c} might be involved in improving glucose homeostasis. It is hard to draw any conclusions regarding cause and effect of this since this is only a correlation. However, together with the fact that these genes differ between glucose-responsive and -unresponsive subclones suggests some possible involvement in mechanisms underlying disturbed GSIS.

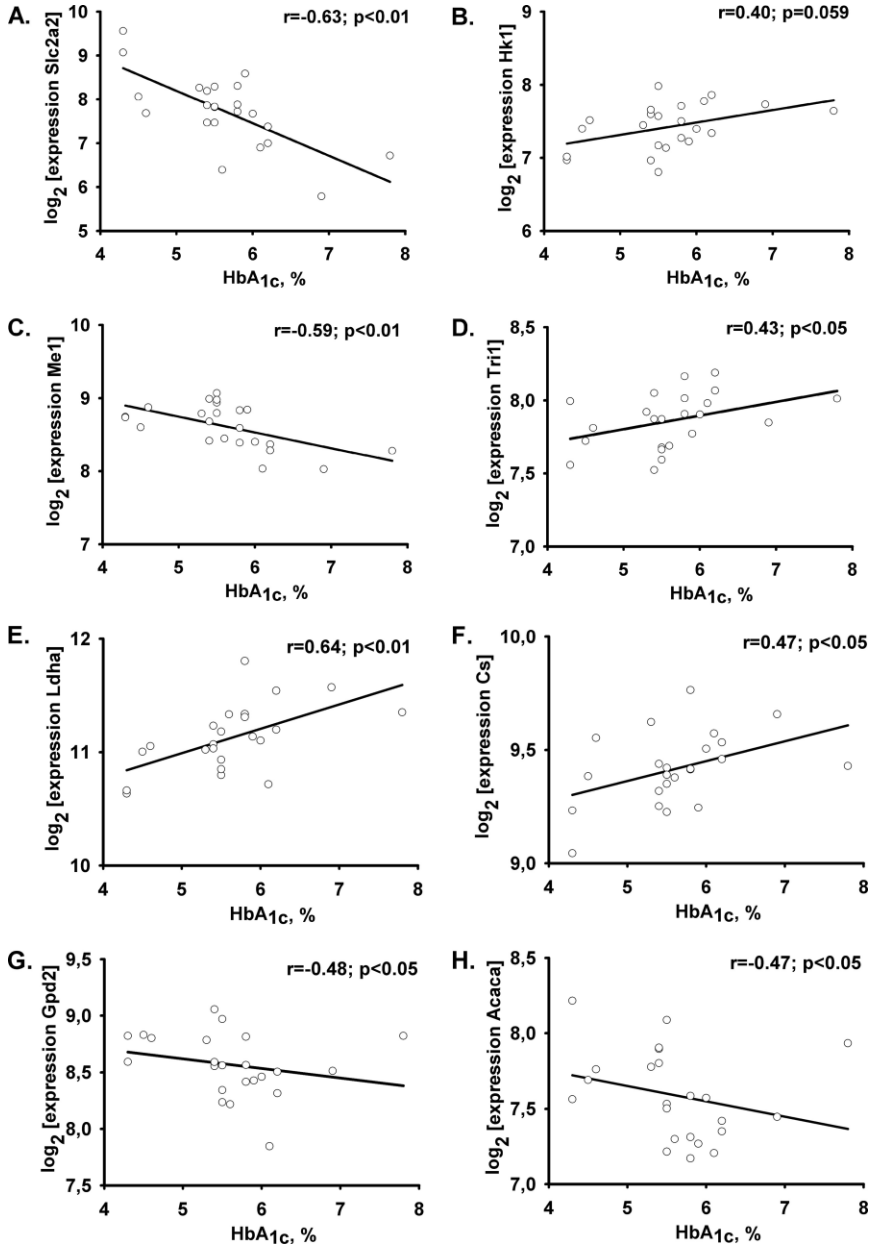

FIGURE 16: Log_2 [gene expression] in human islets plotted against plasma HbA_{1c} levels for eight genes altered in unresponsive INS-1 β-cells
A, Slc2a2 (r =0.63; p<0.01); **B**, Hk1 (r =0.40; p<0.059); **C**, Me1 (r =0.59; p<0.01); **D**, Tri1 (r =0.43; p<0.05); **E**, Ldha (r =0.64; p<0.01); **F**, Cs (r =0.47; p <0.05); **G**, Gpd2 (r =0.48; p <0.05); **H**, Acaca (r =0.47; p<0.05)

In conclusion, the metabolic regulation enhancing mitochondrial metabolism and restricting glycolysis in INS-1 832/13 β-cells is required for robust GSIS. In the INS-1 832/2 β-cells, this metabolic regulation is disturbed in a manner reminiscent of the Warburg-effect, perturbing GSIS in these cells. The expression pattern of four genes that were up-regulated in the 832/2 β-cells compared to the 832/13 β-cells was found to correlate positively with HbA_{1c} levels in humans, reflecting long term glucose control while four down-regulated genes were found to be negatively correlated with HbA_{1c}. This suggests that a similar metabolic control with tight coupling to mitochondrial metabolism may be required to maintain proper β-cell function in human islets. In all, this work supports the usefulness of INS-1 832 β-cell lines in examinations of metabolic perturbations that may be occurring in β-cells in T2D.

Metabolomic analyses reveal profound differences in glycolytic and tricarboxylic acid cycle metabolism in glucose-responsive and -unresponsive clonal β-cell lines (Paper II)

We previously showed that the two INS-1 β-cell lines 832/13 and 832/2 with robust and poor glucose responsiveness, respectably, differed profoundly in their metabolic regulation. In **paper II**, we further examined metabolic patterns by a comprehensive metabolomics approach. We looked for causative mechanisms behind this metabolic shift.

Metabolomics is a comprehensive and unbiased technique where one attempts to assess whole cell metabolome using GC/MS and multivariate statistics. Here, it was used in combination with glucose stimulation of glucose-responsive INS-1 832/13 β-cells and glucose-unresponsive INS-1 832/2 β-cells to increase our understanding of stimulus-secretion coupling and metabolic processes in β-cells. We found that the 832/2 β-cells were characterized by a lack of glucose response. This was reflected by the lack of glucose-stimulated increase in the levels of glycolytic and TCA-cycle metabolites. This confirms, to some extent, the lack of glucose responsiveness in metabolic flux that was observed through functional studies in **paper I.**

The importance of this metabolic coupling between glycolytic and TCA-cycle metabolism was reinforced by our observation that insulin secretion could be partially restored by stimulation of the cells with the mitochondrial fuels leucine and glutamine (Fig. 17). Stimulation with these AAs combined increases the flux through the TCA cycle directly (Gao, Young et al. 2003; Li, Najafi et al. 2003), bypassing glycolytic metabolism. This stimulating effect on insulin secretion was paralleled by stimulation of OCR, confirming an effect on mitochondrial metabolism (Fig. 17). The relevance of this finding for the pathogenesis of T2D is supported by studies in the GK rat, an animal model for T2D. In these animals, lactate production is increased (Ling, Efendic et al. 1998) and even though these animals exhibit a poor secretory response to glucose, leucine and glutamate stimulate insulin secretion (Giroix, Saulnier et al. 1999).

Metabolic and functional profiling of two additional β-cell lines, INS-1 and INS-1 832/1, confirmed the importance of tight coupling between glycolytic and TCA-cycle metabolism in stimulus-secretion coupling. Here, as with the 832/13 and 832/2 β-cells, the poorer responder of the two, the INS-1 832/1 β-cell, exhibited a lower glucose-induced response in the levels of some glycolytic and TCA-cycle intermediates.

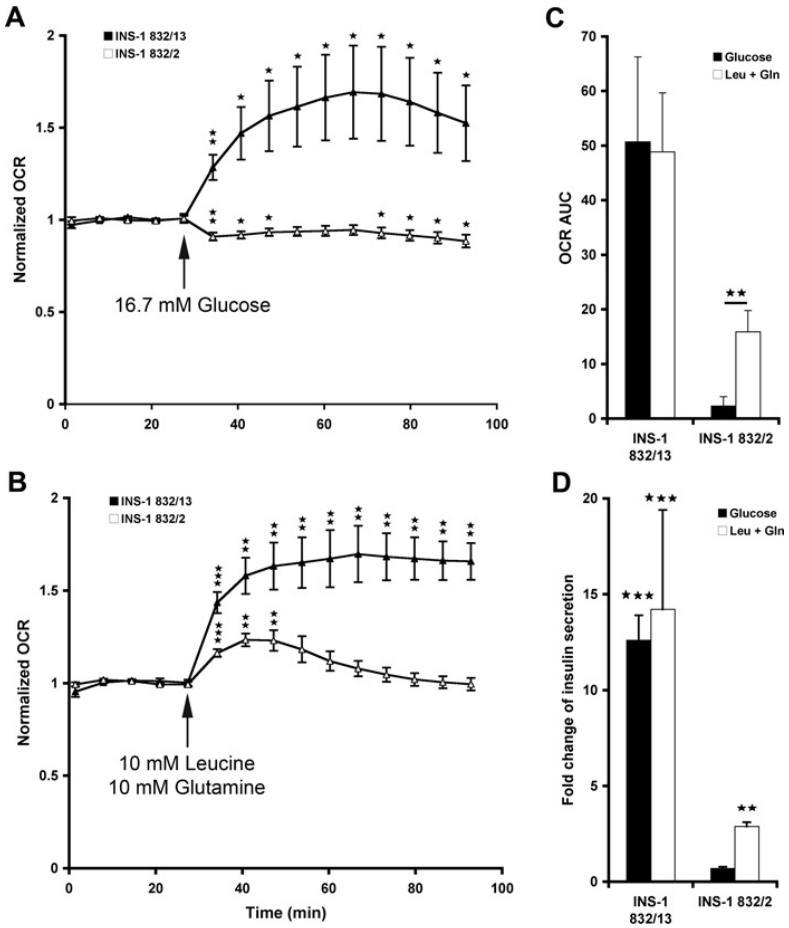

FIGURE 17: Respiration and insulin secretion in glucose-responsive 832/13 and glucose-unresponsive 832/2 β-cells
A and B, OCR in INS-1 832/13 (▲) and 832/2 (Δ) cells after addition of metabolic fuels: **A**, 16.7 mM glucose or **B**, 10 mM leucine+10 mM glutamine (n=6).
C, Fuel-stimulated OCR during the stimulation period normalized to protein content and basal OCR at 2.8 mM glucose; calculated based on an area under curve analysis.
D, Fold change of insulin secretion in INS-1 832/13 and INS-1 832/2 β-cells at 16.7 mM glucose and 10 mM leucine+10 mM glutamine relative 2.8 mM glucose (n=6 - 8).
Data are presented as mean ± SEM. (*p <0.05, **p <0.01, ***p <0.001)

The switch in the 832/2 β-cells from aerobic, mitochondrial metabolism to anaerobic, glycolytic metabolism, with lactate production, again resembled the Warburg effect (Warburg 1956). This, together with the fact that the INS-1 β-cell line is derived from a transplantable insulinoma (Asfari, Janjic et al. 1992), gave

us reason to believe that HIF-1α might be stabilized. It would be responsible for a pseudohypoxic, normoxic state in glucose-unresponsive β-cells (Fig. 18). Indeed, when measuring HIF-1α stabilization by Western blot, it became apparent that the dependence of the unresponsive clones on glycolytic metabolism was paralleled by its increased stabilization. Plotting HIF-1α levels against the citrate/pyruvate ratio (reflecting glycolysis to TCA-cycle coupling) and insulin secretion fold change revealed that a high level of HIF-1α coincided with a low citrate/pyruvate ratio and poor GSIS response.

Several recent studies have suggested that HIF-1α is required for normal β-cell function but also that its dysregulation may contribute to the pathogenesis of T2D (Cantley, Grey et al. 2010; Cheng, Ho et al. 2010; Choi, Cai et al. 2011). Since our model was a tumor-derived cell line, a certain degree of HIF-1α was, perhaps, to be expected.

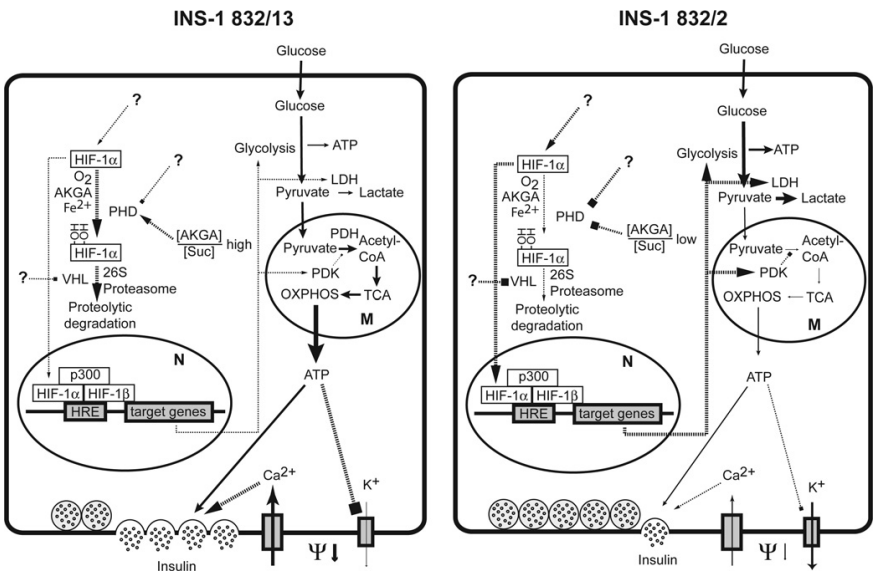

FIGURE 18: Suggested involvement of HIF-1α in β-cell stimulus-secretion coupling
The cartoons show the proposed metabolic regulation in the glucose-responsive and –unresponsive clonal β-cell lines. AKGA, α-ketoglutarate; HRE, hypoxia-response element; LDH, lactate dehydrogenase; M, mitochondrion; N, nucleus; PHD, proline hydroxylase; PDH, pyruvate dehydrogenase; PDK1, PDH kinase 1; OXPHOS, oxidative phosphorylation; Suc, succinate; VHL, von Hippel–Lindau factor.

To determine the relevance of these results for the development of T2D in humans, we wanted to look for possible involvement of HIF-1α in gene regulation in islets from healthy controls and T2D subjects.

Next, we examined expression of HIF-1α target genes in human islets from non-diabetic controls (HbA_{1c} 5.7±0.8%) and patients with T2D (HbA_{1c} 7.5±1.2%). Increased mRNA levels were found for hexokinase 2 (*HK2*), lactate dehydrogenase A (*LDHA*), and phosphofructokinase-2/fructose-2,6-bisphosphatase- 3 (*PFKFB3*). These genes are all HIF-1α target genes (Manolescu, Oprea et al. 2009). Increased transcription of these genes could be attributed to increased stabilization of HIF-1α. *HK2*, another low Km hexokinase isozyme like *HK1*, together with *LDHA* were upregulation in T2D islets compared to non-diabetic controls support the findings from study I where Hk1 and Ldha were found to be upregulated in a glucose-unresponsive cell line.

There were minor, but significant, decreases in pyruvate dehydrogenase kinase-1 (*PDK1*) and enolase (*ENO2*) mRNA levels in human T2D islets as well. This was somewhat unexpected since these genes are HIF-1α target genes. Nonetheless, taken together the data suggest similar perturbations in islets from human T2D patients compared to non-diabetic controls as in the glucose unresponsive β-cell lines.

Since the human islets arrive to us after being cultured for some time, there might be HIF-1α stabilization occurring in the islets due to prolonged culture so time in culture could affect our results. Studies have shown that islet culture do induce hypoxia-markers such as HIF-1α (Maillard, Juszczak et al. 2011) and HIF-1α protein have been correlated to apoptosis in human islets exposed to hypoxia (Moritz, Meier et al. 2002). However, we have no reason to believe that the overall handling time between control islets and those from T2D donors would differ significantly.

Coordinate changes in histone modifications, mRNA levels and metabolite profiles in clonal INS-1 832/13 β-cells accompany functional adaptations to lipotoxicity (Paper III)

Adiposity and chronic high levels of circulating free FAs associate with T2D and β-cell dysfunction (Lee, Hirose et al. 1994). This effect, referred to as lipotoxicity, may also cause β-cell apoptosis (Lupi, Dotta et al. 2002; Kharroubi, Ladriere et al. 2004), further impairing functional β-cell mass in T2D islets. In **paper III**, we created an experimental lipotoxic model in clonal INS-1 832/13 β-cells by exposing them to palmitate, the most abundant saturated FA in the body, for 48 h. To study the recovery and a possible "metabolic memory" of lipotoxicity, β-cells were allowed to recover for 6 days in normal medium following the 48 h palmitate exposure. This enabled us to examine molecular mechanisms underlying lipotoxicity in β-cells.

Under lipotoxic conditions, we observed elevated basal insulin secretion but impaired GSIS, both contributing to a dramatic drop in fold change of insulin secretion from 12- to 2-fold. This is similar to observations from islets from patients with T2D (Porte 1991) and from primary islets and β-cell lines chronically exposed to FAs (Zhou and Grill 1994; Liang, Buettger et al. 1997; Segall, Lameloise et al. 1999).

Metabolic parameters measured in β-cells exposed to lipotoxicity were: glucose utilization, reflecting glycolytic flux, palmitate oxidation, reflecting β-oxidation and oxygen consumption, assessing ATP-production and proton leak. Glucose utilization was unchanged while palmitate oxidation increased, something previously seen in high fat diet-fed mice (Fex, Nitert et al. 2007). Basal and stimulated oxygen consumption rate (OCR) and oxygen consumed by mitochondrial ATP production were impaired. Our OCR data differ from previous studies showing an overall increased OCR with chronic palmitate treatment (Boucher, Lu et al. 2004; Koshkin, Dai et al. 2008). However, this may be due to the fact that the methodology used in these studies differs greatly from the one used in this study.

Pancreatic islets from rat exposed to lipotoxicity have a decreased ATP:ADP ratio when stimulated with glucose. This is seen together with a decreased GSIS (Patane, Anello et al. 2002), something that is to be expected since the ATP:ADP ratio is part of the triggering pathway of insulin secretion.

Cells allowed recovery for 6 days largely reversed the lipid-induced functional changes.

Next, we performed metabolic profiling of β-cells exposed to lipotoxicity. This revealed profound but reversible increases in cellular lipid levels. The glucose-induced increases in TCA-cycle intermediate levels were abrogated by exposure to palmitate; levels of metabolites constituting mitochondrial shuttles were reduced. Overall, a reversible metabolic shift towards a more FA- and less glucose-driven metabolism seemed to occur in β-cells exposed to lipotoxicity.

A previous study performing metabolic profiling in clonal β-cells exposed to glucotoxicity revealed an altered metabolome reflecting an altered glucose handling. This is accompanied by decreased insulin gene expression and GSIS (Goehring, Sauter et al. 2011). With lipotoxicity, we found increased cellular lipid levels, most likely resulting from increased lipid portioning. This has been indicated in lipid-induced β-cell dysfunction (Poitout and Robertson 2008), however, this model has been debated since dramatic lowering of cellular triglyceride stores does not improve GSIS in lipotoxic cells (Boucher, Lu et al. 2004). Instead, mitochondrial pyruvate cycling has been suggested to underlie the disturbances in GSIS (Boucher, Lu et al. 2004). Indeed, levels of metabolites constituting mitochondrial shuttles were found to be reduced in our study.

It has previously been shown that lipotoxicity induces profound changes in gene expression in β-cells (Busch, Cordery et al. 2002; Poitout and Robertson 2008; El-Assaad, Joly et al. 2010). Therefore, we carried out a comprehensive analysis of gene expression, using Affymetrix Rat 1.0 ST microarray. It showed increased expression of 982 genes and decreased expression of 1032 genes in β-cells after exposure to palmitate. Pathway analysis was performed on these groups of genes separately. It revealed increases in genes encoding pathways for steroid biosynthesis, cell cycle, fatty acid metabolism, DNA replication, and biosynthesis of unsaturated fatty acids. Decreased gene expression was only seen in the aminoacyl-tRNA-synthesis pathway.

Two previous studies have used an experimental model similar to ours when studying gene expression in β-cells exposed to lipotoxicity (Busch, Cordery et al. 2002; El-Assaad, Joly et al. 2010). In the first, 126 genes were found to be regulated with palmitate exposure, among them genes of steroid synthesis and lipid metabolism (Busch, Cordery et al. 2002). In the latter, increased expression of lipid partitioning genes suggested increased FA esterification and reduced lipid oxidation while changes in the expression of metabolic genes suggested that mitochondrial dysfunction might have been induced (El-Assaad, Joly et al. 2010). These results somewhat support our data in that chronic FA exposure seems to increase lipid metabolism while mitochondrial function is impaired. Differences in regulation on an individual gene level can be found when comparing these studies but since the studies differ in palmitate exposure time, type of cell line and both palmitate and glucose concentrations, direct comparisons are not possible.

Technical validation of the microarray data using qPCR confirmed regulation of *Insig1*, *Lss* and *Peci* expression by palmitate.

The increased FA metabolism that can be seen in our functional data, metabolic profiling and expression analysis might even be a cell protective mechanism since inhibition of mitochondrial β-oxidation through CPT1-inhibition at lipotoxic conditions accentuates palmitate-induced apoptosis in β-cell lines and in islets (Sargsyan and Bergsten 2011).

To our knowledge, it is not previously known if lipotoxicity affects histone modifications in β-cells. Ten of the genes most profoundly altered in expression in the microarray were chosen for further analysis of histone modifications using ChIP. These included eight up-regulated genes (*Insig1* (Insulin induced gene 1), *Lss* (Lanosterol synthase), *Peci* (Peroxisomal D3,D2-enoyl-CoA isomerase), *Idi1* (isopentyl-diphosphate delta isomerase 1), *Msmo1* (methylsterol monooxygenase 1), *Hmgcs1* (3-hydroxy-3-methylglutaryl-CoA synthase 1), *Me1* (malic enzyme 1) and *Rpa1* (replication protein A1)) and two genes that were down-regulated (*Casr* (calcium-sensing receptor) and *Rilpl1* (Rab interacting lysosomal protein-like 1) in lipotoxic β-cells. To analyze epigenetic mechanisms that might influence gene expression in INS-1 832/13 β-cells exposed to lipotoxicity, histone-modifying enzyme activity was also measured in these cells. The activities of histone-modifying enzymes HAT and H3K27 methyltransferase were altered by lipotoxicity. This is reminiscent of previous studies showing that a diabetic environment with hyperglycemia can increase expression of the most studied HAT, p300 (Chen, Feng et al. 2010). In addition to this, p300 mediates glucose-induced expression of insulin gene transcription (Mosley, Corbett et al. 2004). Another possible mechanism underlying increased HAT activity is that alterations in metabolism might directly affect histone acetylation. Availability of acetyl-CoA for histone acetyltransferases have been shown to influence global histone acetylation (Galdieri and Vancura 2012).

ChIP analysis of histone modifications of the ten differentially expressed genes showed reversible alterations caused by lipotoxicity. The overall spatial distribution profiles of the four histone modifications that were analyzed here were in agreement with previous studies (Bernstein, Kamal et al. 2005; Barski, Cuddapah et al. 2007; Heintzman, Stuart et al. 2007; Bhandare, Schug et al. ; Weishaupt and Attema). The differentially expressed genes *Insig1*, *Lss*, *Peci*, *Idi1*, *Hmgcs1* and *Casr* were subject to epigenetic regulation. Histone modifications associated with transcriptional activation (H3K9Ac, H3K79me2 and H3K4me3) correlated positively with gene expression while one associated with transcriptional repression (H3K27me3) correlated negatively with gene expression (Fig. 19)

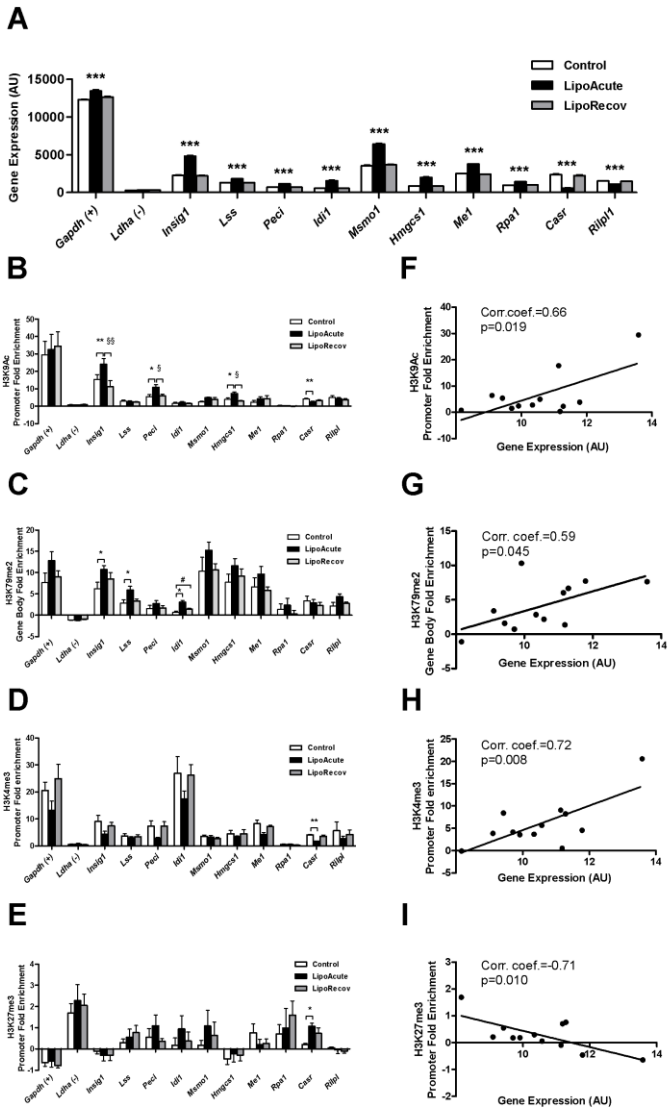

FIGURE 19: mRNA expression and Chromatin immunoprecipitation (ChIP) in clonal INS-1 832/13 β-cells subjected to lipotoxicity.

A, Gene expression of two control genes and 10 selected genes based on differential expression due to lipotoxicity in the microarray *** $p<0.001$ and $q<0.01$.

B – E, ChIP of four different histone modifications including xx. Bars represent mean ± SEM.

F-I Correlations between gene expressions derived from the microarray data and fold enrichment of four histone modifications (n=5). * $p<0.05$ LipoAcute compared to control, § $p<0.05$ LipoAcute compared to LipoRecov, * $p<0.05$, ** $p<0.01$

Epigenetic mechanisms, including histone modifications mediated by histone modifying enzymes, are a possible link between environmental factors and T2D (Ling and Groop 2009). We are not aware of any previous studies that have tested if lipotoxicity affects histone modifications in β-cells. However, studies of animals on a high fat diet show that dietary FA can affect the DNA methylation status of genes in brain tissue (Widiker, Karst et al. 2010). Also, short-term high fat overfeeding in healthy young men have been shown to alter the genome-wide DNA methylation pattern in skeletal muscle (Jacobsen, Brons et al. 2012). Together, these studies support a role for lipids in the regulation of epigenetic modifications.

Our data demonstrated that coordinate changes in histone modifications, mRNA levels and metabolite profiles accompanied functional adaptations of clonal β-cells to lipotoxicity. These changes are most likely underlying pathogenetic processes, such as the loss of glucose responsiveness and perturbed insulin secretion. Taken together, it proposes that epigenetic regulation of gene expression may serve as a link between a hyperlipidemia and impaired β-cell function.

SUMMARY AND GENERAL CONCLUSIONS

In only the last five years, over 60 new T2D risk genotypes have been found, vastly increasing the total body of knowledge in the diabetic field. However, the link between genotype and β-cell function is still being investigated. Since our genome cannot change fundamentally over just a few years, the vast increase in diabetes prevalence that has occurred is not due to an altered gene sequence. Instead, it is the environment surrounding us that has changed. Our genetic background makes us more or less susceptible to the deleterious effects of a diabetogenic environment on, primarily, β-cell function. I have devoted the work presented in this thesis to attempt to elucidate the coupling of β-cell metabolism to insulin secretion and to look for casual mechanisms underlying metabolic alterations disturbing secretion.

The major conclusions from this thesis are:

- Tight coupling between glycolysis and mitochondrial metabolism is required for robust insulin secretion from β-cells

- Transcription factors, such as HIF-1α, and metabolic enzymes, such as Ldha, may disturb GSIS in β-cells and islets by redirecting metabolism towards a more anaerobic one

- Metabolic patterns visualized through a comprehensive metabolomics approach are useful to dissect a pathological metabolic shift.

- β-cell stress introduced by lipotoxicity directs β-cell metabolism more towards metabolism of lipids than of glucose. This may explain the disrupted link of ambient glucose to intracellular metabolic flux, potentially disturbing GSIS.

- Changes in metabolic flux are accompanied by coordinate changes in histone-modifying enzyme activity, histone modifications and gene expression.

As the major socio-economic burden of T2D increases worldwide, so does the need to better understand the mechanisms underlying the disease. Since considerable evidence points towards insufficient insulin release being the main culprit in T2D development (Ashcroft and Rorsman 2012), it is crucial to elucidate the full secretory mechanism in healthy β-cells and to know what goes wrong when insulin secretion is disturbed. My hope is that the studies including in this thesis might be an ever so small piece in the larger puzzle of understanding T2D. However, much more work is needed to link these data to human T2D; in particular, it would be very interesting to conduct epigenetic studies on human material.

Epigenetic alterations of DNA allow us to adapt to our environment more rapidly than our rather rigid genetic code alone would. It also offers a possible link between environment, gene expression and β-cell function. Since epigenetic modifications can be reversed, they present a promising treatment strategy once the exact mechanisms linking life style-induced epigenetic modifications to T2D development. Since HDAC inhibitors are already in use as a cancer therapy, it would be interesting to see whether it might be used to reverse pathological epigenetic modifications in β-cells of T2D subjects.

Although much is known, much more remains yet to be discovered in the mechanisms underlying the complex disease of T2D. Further unraveling of the interplay between genetic, epigenetic and environmental factors would provide more insight into disease development.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Diabetes är en endokrin sjukdom som också kallas sockersjuka. Den kännetecknas av förhöjda mängder socker i blodet. Halten av socker i blodet, blodsockret, styrs av hormonet insulin, som produceras av β-celler i Langerhanska öar i bukspottskörteln. När insulin frisätts stimuleras muskler, fettväv och lever till att ta upp glukos ur blodet och lagra det. På så sätt sänks blodsockret.

Det finns två huvudsakliga former av diabetes, typ 1 och typ 2. Typ 1 diabetes drabbar främst barn och unga. Den orsakas av att det egna immunsystemet förstör de insulin-producerande β-cellerna i bukspottskörteln. Den andra sortens diabetes, typ 2, kallades förut åldersdiabetes. Denna beskrivning har nu frångåtts, delvis för att förekomst av typ 2 diabetes börjar krypa nedåt i åldrarna. Det höga blodsockret hos typ 2 diabetiker orsakas av en kombination av två mekanismer. Dels blir kroppens vävnader insulinresistenta, vilket innebär att de reagerar sämre på insulin. Fetma, inaktivitet och stigande ålder minskar insulinkänsligheten. β-cellerna blir även sämre på att skicka ut insulin när blodsockret är högt. Resultatet blir att den drabbades blodsocker stiger mer och mer tills diabetes slutligen utvecklas.

Förekomsten av typ 2 diabetes ökar just nu väldigt mycket över hela världen, så mycket att man nu talar om det som en hotande pandemi. Denna väldiga ökning diabetes beror paradoxalt nog på att levnadsstandarden blir högre i många delar av världen. Man talar ofta om en ”Western life style” vilket kännetecknas av ett stillasittande arbete, inaktivitet på fritiden och en kaloririk diet med mycket kolhydrater och fett. En sådan livsstil ökar risken för att drabbas av typ 2 diabetes.

Exakt vad som leder fram till att man utvecklar typ 2 diabetes är ännu inte helt klarlagt. De bakomliggande orsakerna är en kombination av ärftliga (genetiska) faktorer och miljöfaktorer, såsom diet och fysisk aktivitet. Generna är ritningarna till alla kroppens protein och de finns i vår genetiska kod, vårt DNA. När dessa ritningar kopieras för att proteiner skall kunna bildas säger man att generna uttrycks. De senaste fem åren har forskningen kring genetiska faktorer som ligger bakom typ 2 diabetes gjort stora framsteg. Man har identifierat 60 genetiska risktyper som går att koppla till ökad risk att utveckla diabetes. Men ännu finns mycket kvar att göra. Man vet till exempel att hur man lever kan påverka ens

gener genom så kallade epigenetiska förändringar. Dessa förändrar genutrycket utan att ändra den genetiska koden men man vet inte hur det styrs eller hur mycket det påverkar. Man vet ännu inte hur den minskade förmågan att frisätta insulin uppstår eller ens exakt vilka faktorer som styr frisättningen av insulin. I min avhandling utforskar jag de mekanismer som styr frisättningen av insulin och faktorer som kan ligga bakom en rubbad insulinfrisättning. Jag har också fördjupat mig i de effekter som en diabetesframkallande (diabetogen) miljö kan ha på β-cellen. I samband med det har jag utforskat förändringar i uttryck av gener samt epigenetiska förändringar som kan ligga bakom dessa.

I delarbete I har jag tittat närmare på mekanismer som styr insulinfrisättning, och på vilka störningar som kan ligga bakom en minskad förmåga att frisätta insulin genom att jämföra två β-cell linjer. Den ena av dessa linjer är mycket bra på att frisätta insulin när man stimmulerar den med socker. Det sker på ett sätt som liknar det som händer i kroppen när man precis ätit något sött. Den andra är dålig på detta trots att de båda cellinjerna är nära besläktade. Eftersom det är svårt att få tag på mänsklig vävnad och eftersom det är mycket lättare att kontrollera miljön fullständigt när man studerar celler är cellinjer en bra modell att arbeta med.

Resultaten från delarbete I visar att en ökad anaerob (syreoberoende) ämnesomsättning (metabolism) ligger bakom den dåliga insulinfrisättningen. Detta tycks vara kopplat till att gener som gör det lättare att metabolisera socker är mera aktiva. Från detta kan vi sluta oss till att det är viktigt för β-cellen att den anaeroba delen av metabolismen är direkt kopplad till den aeroba (syreberoende) delen. När detta störs blir känsligheten för socker lägre. Vi visade att detta sker tillsammans med ändrat genuttryck av vissa metabola gener och att vissa av dessa gener även påverkade blodsockerkontroll i människor.

I delarbete II utökade vi jämförelsen av bra och dåliga β-celler med att försöka undersöka samtliga molekyler i ämnesomsättningen (metabolomet). Metabolomet omfattar de metaboliter, det vill säga ämnen, som utgör metabola vägar i cellerna. I detta arbete mätte vi halterna av 164 metaboliter samtidigt. Det visade sig att de celler som var bra på att frisätta insulin när sockerhalten ökade även ökade sina halter av metaboliter vid högt socker. Detta var särskilt tydligt för metaboliter i aerob metabolism. Vi visade att en faktor som kunde ligga bakom detta var proteinet HIF1α; det uttrycktes och reglerades i våra celler. HIF1α får cellerna att tro att det råder syrebrist och slå om till en ämnesomsättning som inte kräver syre, en anaerob metabolism. Genom att jämföra gener som är uttryckta i mänskliga Langerhanska öar från friska och från typ 2 diabetiker kunde vi se att HIF1α även kan finnas i ökade mängder i β-celler hos människor med typ 2 diabetes.

I delarbete III använde vi den cellinje som var bäst på att frisätta insulin eftersom denna mest liknar en frisk β-cell. Sedan undersökte vi hur dessa celler reagerar på en diabetogen miljö. Diabetiker och människor, som är insulinresistenta, har förhöjda halter av fria fettsyror i blodet. Man har sett att höga halter av fria

fettsyror stör β-cellernas funktion. Det är fortfarande oklart vilka mekanismer som ligger bakom detta som slutligen kan tänkas leda till att typ 2 diabetes utvecklas. För att undersöka om en förhöjd halt av fettsyror kan vara en bidragande orsak studerade vi mekanismer som ligger bakom denna försämrade funktion. Vi utsatte celler för höga halter av fria fettsyror i 48 timmar; vi studerade också celler som fick återhämta sig i normalt medium efter att ha utsatts för fettsyror. Detta gjordes för att undersöka om cellerna ”kom ihåg” att de varit utsatta för fettsyror, det vill säga om de gett upphov till ett metabolt minne. Sedan mätte vi aktivitet i metabola vägar, metabolomet, uttryck av gener samt epigenetiska modifikationer.

Resultaten tydde på att celler som utsätts för fria fettsyror slår om sin metabolism från en som främst är inriktad på att behandla socker till en inriktad på att behandla fett. Detta gör att insulinfrisättningen vid ökade sockerhalter störs. Cellen åstadkommer detta med hjälp av ett ändrat uttryck av gener som reglerar metabola vägar. Dessa regleringar åtföljs av ändrade epigenetiska markeringar, vilket tyder på att epigenetisk reglering på grund av miljöpåverkan kan vara en faktor som ligger bakom försämrad insulinfrisättning. Vi hittade inga tecken på metabolt minne i cellerna.

Våra övergripande mål var således att undersöka mekanismer som styr insulinfrisättning från β-celler samt att undersöka vad som kan ligga bakom en försämrad frisättning. Vi visade att aerob metabolism är oumbärlig för en fungerande insulinfrisättning samt att mekanismer som ligger bakom försämringar i cellinjer även kan göra det i människor. Vi visade vidare att en diabetogen miljö med höga halter av fria fettsyror påverkar metabola vägar och att detta sker parallellt med ett ändrat genuttryck. Slutligen visade vi att epigenetiska mekanismer till viss del kan ligga bakom dessa förändringar. Jag hoppas de upptäckter vi har gjort på sikt kan leda till nya behandlingar av typ 2 diabetes.

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REFERENCES

(IDF), T. I. D. F. (2011). "IDF diabetes athlas, 5th edition." from http://www.idf,org/diabetesaltlas.

(WHO), W. h. o. (August 2011). "Fact sheet N°312." from http://www.who.int/diabetes.

Affymetrix (2007). "GeneChip® Gene 1.0 ST Array System for Human, Mouse and Rat."

Ahlqvist, E., T. S. Ahluwalia, et al. (2011). "Genetics of type 2 diabetes." Clin Chem **57**(2): 241-254.

Ahren, B. (2000). "Autonomic regulation of islet hormone secretion--implications for health and disease." Diabetologia **43**(4): 393-410.

Ainscow, E. K., C. Zhao, et al. (2000). "Acute overexpression of lactate dehydrogenase-A perturbs beta-cell mitochondrial metabolism and insulin secretion." Diabetes **49**(7): 1149-1155.

Andersen, C. L., J. L. Jensen, et al. (2004). "Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets." Cancer Res **64**(15): 5245-5250.

Anello, M., R. Lupi, et al. (2005). "Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients." Diabetologia **48**(2): 282-289.

Asfari, M., D. Janjic, et al. (1992). "Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines." Endocrinology **130**(1): 167-178.

Ashcroft, F. M. and P. Rorsman (2012). "Diabetes mellitus and the beta cell: the last ten years." Cell **148**(6): 1160-1171.

Ban, N., Y. Yamada, et al. (2002). "Hepatocyte nuclear factor-1alpha recruits the transcriptional co-activator p300 on the GLUT2 gene promoter." Diabetes **51**(5): 1409-1418.

Barski, A., S. Cuddapah, et al. (2007). "High-resolution profiling of histone methylations in the human genome." Cell **129**(4): 823-837.

Bernstein, B. E., M. Kamal, et al. (2005). "Genomic maps and comparative analysis of histone modifications in human and mouse." Cell **120**(2): 169-181.

Bhandare, R., J. Schug, et al. (2010). "Genome-wide analysis of histone modifications in human pancreatic islets." Genome Res **20**(4): 428-433.

Boucher, A., D. Lu, et al. (2004). "Biochemical mechanism of lipid-induced impairment of glucose-stimulated insulin secretion and reversal with a malate analogue." J Biol Chem **279**(26): 27263-27271.

Brand, M. D. and D. G. Nicholls (2011). "Assessing mitochondrial dysfunction in cells." Biochem J **435**(2): 297-312.

Briaud, I., C. L. Kelpe, et al. (2002). "Differential effects of hyperlipidemia on insulin secretion in islets of langerhans from hyperglycemic versus normoglycemic rats." Diabetes **51**(3): 662-668.

Brownell, J. E., J. Zhou, et al. (1996). "Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation." Cell **84**(6): 843-851.
Busch, A. K., D. Cordery, et al. (2002). "Expression profiling of palmitate- and oleate-regulated genes provides novel insights into the effects of chronic lipid exposure on pancreatic beta-cell function." Diabetes **51**(4): 977-987.
Cabrera, O., D. M. Berman, et al. (2006). "The unique cytoarchitecture of human pancreatic islets has implications for islet cell function." Proc Natl Acad Sci U S A **103**(7): 2334-2339.
Cantley, J., S. T. Grey, et al. (2010). "The hypoxia response pathway and beta-cell function." Diabetes Obes Metab **12 Suppl 2**: 159-167.
Carlsson, C., L. A. Borg, et al. (1999). "Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro." Endocrinology **140**(8): 3422-3428.
Carpentier, A., S. D. Mittelman, et al. (2000). "Prolonged elevation of plasma free fatty acids impairs pancreatic beta-cell function in obese nondiabetic humans but not in individuals with type 2 diabetes." Diabetes **49**(3): 399-408.
Chakrabarti, S. K., J. Francis, et al. (2003). "Covalent histone modifications underlie the developmental regulation of insulin gene transcription in pancreatic beta cells." J Biol Chem **278**(26): 23617-23623.
Chan, C. B., P. E. MacDonald, et al. (1999). "Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets." Diabetes **48**(7): 1482-1486.
Charles, M. A., E. Eschwege, et al. (1997). "The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris Prospective Study." Diabetologia **40**(9): 1101-1106.
Chen, S., B. Feng, et al. (2010). "Transcriptional coactivator p300 regulates glucose-induced gene expression in endothelial cells." Am J Physiol Endocrinol Metab **298**(1): E127-137.
Cheng, K., K. Ho, et al. (2010). "Hypoxia-inducible factor-1alpha regulates beta cell function in mouse and human islets." J Clin Invest **120**(6): 2171-2183.
Chick, W. L., S. Warren, et al. (1977). "A transplantable insulinoma in the rat." Proc Natl Acad Sci U S A **74**(2): 628-632.
Choi, D., E. P. Cai, et al. (2011). "Vhl is required for normal pancreatic beta cell function and the maintenance of beta cell mass with age in mice." Lab Invest **91**(4): 527-538.
Choi, S. W. and S. Friso (2010). "Epigenetics: A New Bridge between Nutrition and Health." Adv Nutr **1**(1): 8-16.
Clouaire, T. and I. Stancheva (2008). "Methyl-CpG binding proteins: specialized transcriptional repressors or structural components of chromatin?" Cell Mol Life Sci **65**(10): 1509-1522.
Deering, T. G., T. Ogihara, et al. (2009). "Methyltransferase Set7/9 maintains transcription and euchromatin structure at islet-enriched genes." Diabetes **58**(1): 185-193.
Defronzo, R. A. (2009). "Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus." Diabetes **58**(4): 773-795.
Dennis, G., Jr., B. T. Sherman, et al. (2003). "DAVID: Database for Annotation, Visualization, and Integrated Discovery." Genome Biol **4**(5): P3.

Di Lorenzo, A. and M. T. Bedford (2011). "Histone arginine methylation." FEBS Lett **585**(13): 2024-2031.
Dixon, G., J. Nolan, et al. (2003). "A comparative study of amino acid consumption by rat islet cells and the clonal beta-cell line BRIN-BD11 - the functional significance of L-alanine." J Endocrinol **179**(3): 447-454.
El-Assaad, W., E. Joly, et al. (2010). "Glucolipotoxicity alters lipid partitioning and causes mitochondrial dysfunction, cholesterol, and ceramide deposition and reactive oxygen species production in INS832/13 ss-cells." Endocrinology **151**(7): 3061-3073.
Eriksson, J., M. Nakazato, et al. (1992). "Islet amyloid polypeptide plasma concentrations in individuals at increased risk of developing type 2 (non-insulin-dependent) diabetes mellitus." Diabetologia **35**(3): 291-293.
Fabbrini, E., F. Magkos, et al. (2009). "Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity." Proc Natl Acad Sci U S A **106**(36): 15430-15435.
Farfari, S., V. Schulz, et al. (2000). "Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion." Diabetes **49**(5): 718-726.
Fex, M., M. D. Nitert, et al. (2007). "Enhanced mitochondrial metabolism may account for the adaptation to insulin resistance in islets from C57BL/6J mice fed a high-fat diet." Diabetologia **50**(1): 74-83.
Fradin, D. and P. Bougneres (2011). "T2DM: Why Epigenetics?" J Nutr Metab **2011**: 647514.
Francis, J., S. K. Chakrabarti, et al. (2005). "Pdx-1 links histone H3-Lys-4 methylation to RNA polymerase II elongation during activation of insulin transcription." J Biol Chem **280**(43): 36244-36253.
Fransson, U., A. H. Rosengren, et al. (2006). "Anaplerosis via pyruvate carboxylase is required for the fuel-induced rise in the ATP:ADP ratio in rat pancreatic islets." Diabetologia **49**(7): 1578-1586.
Galdieri, L. and A. Vancura (2012). "Acetyl-CoA carboxylase regulates global histone acetylation." J Biol Chem **287**(28): 23865-23876.
Gao, Z., R. A. Young, et al. (2003). "Distinguishing features of leucine and alpha-ketoisocaproate sensing in pancreatic beta-cells." Endocrinology **144**(5): 1949-1957.
Garber, A. J. (2012). "Current challenges in type 2 diabetes." Diabetes Obes Metab **14 Suppl 2**: 1-3.
Gehrmann, W., M. Elsner, et al. (2010). "Role of metabolically generated reactive oxygen species for lipotoxicity in pancreatic beta-cells." Diabetes Obes Metab **12 Suppl 2**: 149-158.
Gibson, D. M. H., Robert A. (2002). Metabolic Regulation in Mammals. London Taylor & Francis.
Giroix, M. H., C. Saulnier, et al. (1999). "Decreased pancreatic islet response to L-leucine in the spontaneously diabetic GK rat: enzymatic, metabolic and secretory data." Diabetologia **42**(8): 965-977.
Goehring, I., N. S. Sauter, et al. (2011). "Identification of an intracellular metabolic signature impairing beta cell function in the rat beta cell line INS-1E and human islets." Diabetologia **54**(10): 2584-2594.

Goto, Y., I. Nonaka, et al. (1990). "A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies." Nature **348**(6302): 651-653.
Groop, L., C. Forsblom, et al. (1996). "Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects." Diabetes **45**(11): 1585-1593.
Guay, C., S. R. Madiraju, et al. (2007). "A role for ATP-citrate lyase, malic enzyme, and pyruvate/citrate cycling in glucose-induced insulin secretion." J Biol Chem **282**(49): 35657-35665.
Hagman, D. K., L. B. Hays, et al. (2005). "Palmitate inhibits insulin gene expression by altering PDX-1 nuclear localization and reducing MafA expression in isolated rat islets of Langerhans." J Biol Chem **280**(37): 32413-32418.
Harris, D. M. G. R. A. (2002). Metabolic regulation in mammals, Taylor & Francis Inc.
Heintzman, N. D., R. K. Stuart, et al. (2007). "Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome." Nat Genet **39**(3): 311-318.
Henquin, J. C. (2000). "Triggering and amplifying pathways of regulation of insulin secretion by glucose." Diabetes **49**(11): 1751-1760.
Henquin, J. C., M. A. Ravier, et al. (2003). "Hierarchy of the beta-cell signals controlling insulin secretion." Eur J Clin Invest **33**(9): 742-750.
Hohmeier, H. E., H. Mulder, et al. (2000). "Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion." Diabetes **49**(3): 424-430.
Ingelsson, E., J. Arnlov, et al. (2009). "Relative importance and conjoint effects of obesity and physical inactivity for the development of insulin resistance." Eur J Cardiovasc Prev Rehabil **16**(1): 28-33.
International HapMap Consortium (2003). "The International HapMap Project." Nature **426**(6968): 789-796.
Irizarry, R. A., B. Hobbs, et al. (2003). "Exploration, normalization, and summaries of high density oligonucleotide array probe level data." Biostatistics **4**(2): 249-264.
Ishihara, H., K. Tsuneoka, et al. (1993). "Induction of the expression of the interleukin-1 beta gene in mouse spleen by ionizing radiation." Radiat Res **133**(3): 321-326.
Ishihara, H., H. Wang, et al. (1999). "Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory responses to pyruvate and lactate in beta cells." J Clin Invest **104**(11): 1621-1629.
Ismail-Beigi, F. (2012). "Clinical practice. Glycemic management of type 2 diabetes mellitus." N Engl J Med **366**(14): 1319-1327.
Itoh, Y., Y. Kawamata, et al. (2003). "Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40." Nature **422**(6928): 173-176.
Jacobsen, S. C., C. Brons, et al. (2012). "Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men." Diabetologia.
Jensen, M. V., J. W. Joseph, et al. (2008). "Metabolic cycling in control of glucose-stimulated insulin secretion." Am J Physiol Endocrinol Metab **295**(6): E1287-1297.
Jitrapakdee, S., A. Wutthisathapornchai, et al. (2010). "Regulation of insulin secretion: role of mitochondrial signalling." Diabetologia **53**(6): 1019-1032.

Johnson, K. H., T. D. O'Brien, et al. (1989). "Impaired glucose tolerance is associated with increased islet amyloid polypeptide (IAPP) immunoreactivity in pancreatic beta cells." Am J Pathol **135**(2): 245-250.
Jones, P. A. (2012). "Functions of DNA methylation: islands, start sites, gene bodies and beyond." Nat Rev Genet **13**(7): 484-492.
Joseph, J. W., M. V. Jensen, et al. (2006). "The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion." J Biol Chem **281**(47): 35624-35632.
Joseph, J. W., V. Koshkin, et al. (2004). "Free fatty acid-induced beta-cell defects are dependent on uncoupling protein 2 expression." J Biol Chem **279**(49): 51049-51056.
Kagan, V. E., V. A. Tyurin, et al. (2005). "Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors." Nat Chem Biol **1**(4): 223-232.
Kaprio, J., J. Tuomilehto, et al. (1992). "Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland." Diabetologia **35**(11): 1060-1067.
Karaskov, E., C. Scott, et al. (2006). "Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis." Endocrinology **147**(7): 3398-3407.
Kashyap, S., R. Belfort, et al. (2003). "A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes." Diabetes **52**(10): 2461-2474.
Kaur, G. and J. M. Dufour (2012). "Cell lines: Valuable tools or useless artifacts." Spermatogenesis **2**(1): 1-5.
Keating, S. T. and A. El-Osta (2012). "Chromatin modifications associated with diabetes." J Cardiovasc Transl Res **5**(4): 399-412.
Kelpe, C. L., P. C. Moore, et al. (2003). "Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis." J Biol Chem **278**(32): 30015-30021.
Khan, A., Z. C. Ling, et al. (1996). "Quantifying the carboxylation of pyruvate in pancreatic islets." J Biol Chem **271**(5): 2539-2542.
Kharroubi, I., L. Ladriere, et al. (2004). "Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress." Endocrinology **145**(11): 5087-5096.
Kjems, L. L., B. M. Kirby, et al. (2001). "Decrease in beta-cell mass leads to impaired pulsatile insulin secretion, reduced postprandial hepatic insulin clearance, and relative hyperglucagonemia in the minipig." Diabetes **50**(9): 2001-2012.
Knowler, W. C., E. Barrett-Connor, et al. (2002). "Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin." N Engl J Med **346**(6): 393-403.
Koeck, T., A. H. Olsson, et al. (2011). "A common variant in TFB1M is associated with reduced insulin secretion and increased future risk of type 2 diabetes." Cell Metab **13**(1): 80-91.
Koshkin, V., F. F. Dai, et al. (2008). "Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic beta-cells." J Biol Chem **283**(12): 7936-7948.
Krauss, S., C. Y. Zhang, et al. (2002). "A significant portion of mitochondrial proton leak in intact thymocytes depends on expression of UCP2." Proc Natl Acad Sci U S A **99**(1): 118-122.

Krssak, M., K. Falk Petersen, et al. (1999). "Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study." Diabetologia **42**(1): 113-116.
Kucharski, R., J. Maleszka, et al. (2008). "Nutritional control of reproductive status in honeybees via DNA methylation." Science **319**(5871): 1827-1830.
Langerhans, P. (1869). "Beitrage zur mikroscopischen anatomie der bauchspeichel druse". Inaugural-dissertation."
Lee, Y., H. Hirose, et al. (1994). "Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships." Proc Natl Acad Sci U S A **91**(23): 10878-10882.
Li, C., H. Najafi, et al. (2003). "Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets." J Biol Chem **278**(5): 2853-2858.
Li, L. X., F. Skorpen, et al. (2002). "Induction of uncoupling protein 2 mRNA in beta-cells is stimulated by oxidation of fatty acids but not by nutrient oversupply." Endocrinology **143**(4): 1371-1377.
Li, Y., M. A. Reddy, et al. (2008). "Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-kappaB-dependent inflammatory genes. Relevance to diabetes and inflammation." J Biol Chem **283**(39): 26771-26781.
Liang, Y., C. Buettger, et al. (1997). "Chronic effect of fatty acids on insulin release is not through the alteration of glucose metabolism in a pancreatic beta-cell line (beta HC9)." Diabetologia **40**(9): 1018-1027.
Lim, E. L., K. G. Hollingsworth, et al. (2011). "Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol." Diabetologia **54**(10): 2506-2514.
Lin, Y. and Z. Sun (2010). "Current views on type 2 diabetes." J Endocrinol **204**(1): 1-11.
Ling, C., S. Del Guerra, et al. (2008). "Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion." Diabetologia **51**(4): 615-622.
Ling, C. and L. Groop (2009). "Epigenetics: a molecular link between environmental factors and type 2 diabetes." Diabetes **58**(12): 2718-2725.
Ling, Z. C., S. Efendic, et al. (1998). "Glucose metabolism in Goto-Kakizaki rat islets." Endocrinology **139**(6): 2670-2675.
Liu, Y. Q., T. L. Jetton, et al. (2002). "beta-Cell adaptation to insulin resistance. Increased pyruvate carboxylase and malate-pyruvate shuttle activity in islets of nondiabetic Zucker fatty rats." J Biol Chem **277**(42): 39163-39168.
Lu, D., H. Mulder, et al. (2002). "13C NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS)." Proc Natl Acad Sci U S A **99**(5): 2708-2713.
Lupi, R., F. Dotta, et al. (2002). "Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated." Diabetes **51**(5): 1437-1442.
Ma, Z. A., Z. Zhao, et al. (2012). "Mitochondrial dysfunction and beta-cell failure in type 2 diabetes mellitus." Exp Diabetes Res **2012**: 703538.
Maechler, P. (2002). "Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic beta-cells." Cell Mol Life Sci **59**(11): 1803-1818.
Maillard, E., M. T. Juszczak, et al. (2011). "Perfluorodecalin-enriched fibrin matrix for human islet culture." Biomaterials **32**(35): 9282-9289.

Mann, B. S., J. R. Johnson, et al. (2007). "FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma." Oncologist **12**(10): 1247-1252.
Manolescu, B., E. Oprea, et al. (2009). "Natural compounds and the hypoxia-inducible factor (HIF) signalling pathway." Biochimie **91**(11-12): 1347-1358.
Martin, C. and Y. Zhang (2005). "The diverse functions of histone lysine methylation." Nat Rev Mol Cell Biol **6**(11): 838-849.
Mason, T. M., T. Goh, et al. (1999). "Prolonged elevation of plasma free fatty acids desensitizes the insulin secretory response to glucose in vivo in rats." Diabetes **48**(3): 524-530.
Matschinsky, F. M. (2005). "Glucokinase, glucose homeostasis, and diabetes mellitus." Curr Diab Rep **5**(3): 171-176.
McGarry, J. D. and N. F. Brown (1997). "The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis." Eur J Biochem **244**(1): 1-14.
Medvedev, A. V., J. Robidoux, et al. (2002). "Regulation of the uncoupling protein-2 gene in INS-1 beta-cells by oleic acid." J Biol Chem **277**(45): 42639-42644.
Mohtat, D. and K. Susztak (2010). "Fine tuning gene expression: the epigenome." Semin Nephrol **30**(5): 468-476.
Moritz, W., F. Meier, et al. (2002). "Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression." FASEB J **16**(7): 745-747.
Mosley, A. L., J. A. Corbett, et al. (2004). "Glucose regulation of insulin gene expression requires the recruitment of p300 by the beta-cell-specific transcription factor Pdx-1." Mol Endocrinol **18**(9): 2279-2290.
Mosley, A. L. and S. Ozcan (2004). "The pancreatic duodenal homeobox-1 protein (Pdx-1) interacts with histone deacetylases Hdac-1 and Hdac-2 on low levels of glucose." J Biol Chem **279**(52): 54241-54247.
Mulder, H., D. Lu, et al. (2001). "Overexpression of a modified human malonyl-CoA decarboxylase blocks the glucose-induced increase in malonyl-CoA level but has no impact on insulin secretion in INS-1-derived (832/13) beta-cells." J Biol Chem **276**(9): 6479-6484.
Muller, D. C., D. Elahi, et al. (1996). "Insulin response during the oral glucose tolerance test: the role of age, sex, body fat and the pattern of fat distribution." Aging (Milano) **8**(1): 13-21.
Murphy, R., S. Ellard, et al. (2008). "Clinical implications of a molecular genetic classification of monogenic beta-cell diabetes." Nat Clin Pract Endocrinol Metab **4**(4): 200-213.
Murphy, R., D. M. Turnbull, et al. (2008). "Clinical features, diagnosis and management of maternally inherited diabetes and deafness (MIDD) associated with the 3243A>G mitochondrial point mutation." Diabet Med **25**(4): 383-399.
Mussig, K., H. Staiger, et al. (2010). "Genetic variants affecting incretin sensitivity and incretin secretion." Diabetologia **53**(11): 2289-2297.
Newsholme, P., L. Brennan, et al. (2005). "New insights into amino acid metabolism, beta-cell function and diabetes." Clin Sci (Lond) **108**(3): 185-194.
Nolan, C. J., M. S. Madiraju, et al. (2006). "Fatty acid signaling in the beta-cell and insulin secretion." Diabetes **55 Suppl 2**: S16-23.
Ogihara, T., N. L. Vanderford, et al. (2009). "Expression and function of Set7/9 in pancreatic islets." Islets **1**(3): 269-272.

Olsson, A. H., B. T. Yang, et al. (2011). "Decreased expression of genes involved in oxidative phosphorylation in human pancreatic islets from patients with type 2 diabetes." Eur J Endocrinol **165**(4): 589-595.
Ooi, S. K., A. H. O'Donnell, et al. (2009). "Mammalian cytosine methylation at a glance." J Cell Sci **122**(Pt 16): 2787-2791.
Paolisso, G., A. Gambardella, et al. (1995). "Opposite effects of short- and long-term fatty acid infusion on insulin secretion in healthy subjects." Diabetologia **38**(11): 1295-1299.
Patane, G., M. Anello, et al. (2002). "Role of ATP production and uncoupling protein-2 in the insulin secretory defect induced by chronic exposure to high glucose or free fatty acids and effects of peroxisome proliferator-activated receptor-gamma inhibition." Diabetes **51**(9): 2749-2756.
Peterson, C. L. and M. A. Laniel (2004). "Histones and histone modifications." Curr Biol **14**(14): R546-551.
Pi, J., Y. Bai, et al. (2009). "Persistent oxidative stress due to absence of uncoupling protein 2 associated with impaired pancreatic beta-cell function." Endocrinology **150**(7): 3040-3048.
Poitout, V. and R. P. Robertson (2008). "Glucolipotoxicity: fuel excess and beta-cell dysfunction." Endocr Rev **29**(3): 351-366.
Pongratz, R. L., R. G. Kibbey, et al. (2007). "Cytosolic and mitochondrial malic enzyme isoforms differentially control insulin secretion." J Biol Chem **282**(1): 200-207.
Porte, D., Jr. (1991). "Banting lecture 1990. Beta-cells in type II diabetes mellitus." Diabetes **40**(2): 166-180.
Prentki, M., E. Joly, et al. (2002). "Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes." Diabetes **51 Suppl 3**: S405-413.
Prentki, M. and C. J. Nolan (2006). "Islet beta cell failure in type 2 diabetes." J Clin Invest **116**(7): 1802-1812.
Prentki, M., S. Vischer, et al. (1992). "Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion." J Biol Chem **267**(9): 5802-5810.
Pullen, T. J., A. M. Khan, et al. (2010). "Identification of genes selectively disallowed in the pancreatic islet." Islets **2**(2): 89-95.
Quintens, R., N. Hendrickx, et al. (2008). "Why expression of some genes is disallowed in beta-cells." Biochem Soc Trans **36**(Pt 3): 300-305.
R Development Core Team (2010). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing.
Rahier, J., Y. Guiot, et al. (2008). "Pancreatic beta-cell mass in European subjects with type 2 diabetes." Diabetes Obes Metab **10 Suppl 4**: 32-42.
Ravassard, P., Y. Hazhouz, et al. (2011). "A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion." J Clin Invest **121**(9): 3589-3597.
Reik, W., W. Dean, et al. (2001). "Epigenetic reprogramming in mammalian development." Science **293**(5532): 1089-1093.
Roduit, R., C. Nolan, et al. (2004). "A role for the malonyl-CoA/long-chain acyl-CoA pathway of lipid signaling in the regulation of insulin secretion in response to both fuel and nonfuel stimuli." Diabetes **53**(4): 1007-1019.

Ronnebaum, S. M., O. Ilkayeva, et al. (2006). "A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion." J Biol Chem **281**(41): 30593-30602.
Ronnebaum, S. M., M. V. Jensen, et al. (2008). "Silencing of cytosolic or mitochondrial isoforms of malic enzyme has no effect on glucose-stimulated insulin secretion from rodent islets." J Biol Chem **283**(43): 28909-28917.
Saeed, A. I., N. K. Bhagabati, et al. (2006). "TM4 microarray software suite." Methods Enzymol **411**: 134-193.
Sako, Y. and V. E. Grill (1990). "A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation." Endocrinology **127**(4): 1580-1589.
Sargsyan, E. and P. Bergsten (2011). "Lipotoxicity is glucose-dependent in INS-1E cells but not in human islets and MIN6 cells." Lipids Health Dis **10**: 115.
Saxena, R., B. F. Voight, et al. (2007). "Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels." Science **316**(5829): 1331-1336.
Scarpulla, R. C. (2008). "Transcriptional paradigms in mammalian mitochondrial biogenesis and function." Physiol Rev **88**(2): 611-638.
Scott, L. J., K. L. Mohlke, et al. (2007). "A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants." Science **316**(5829): 1341-1345.
Segall, L., N. Lameloise, et al. (1999). "Lipid rather than glucose metabolism is implicated in altered insulin secretion caused by oleate in INS-1 cells." Am J Physiol **277**(3 Pt 1): E521-528.
Sekine, N., V. Cirulli, et al. (1994). "Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells. Potential role in nutrient sensing." J Biol Chem **269**(7): 4895-4902.
Sener, A., G. Somers, et al. (1981). "The stimulus-secretion coupling of amino acid-induced insulin release. Biosynthetic and secretory responses of rat pancreatic islet to L-leucine and L-glutamine." Diabetologia **21**(2): 135-142.
Shaw, J. E., R. A. Sicree, et al. (2010). "Global estimates of the prevalence of diabetes for 2010 and 2030." Diabetes Res Clin Pract **87**(1): 4-14.
Shimabukuro, M., Y. T. Zhou, et al. (1998). "Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes." Proc Natl Acad Sci U S A **95**(5): 2498-2502.
Skloot, R. (2010). The immortal life of Henrietta Lacks, Writers House, New York.
Sladek, R., G. Rocheleau, et al. (2007). "A genome-wide association study identifies novel risk loci for type 2 diabetes." Nature **445**(7130): 881-885.
Smith, P. A., H. Sakura, et al. (1997). "Electrogenic arginine transport mediates stimulus-secretion coupling in mouse pancreatic beta-cells." J Physiol **499 (Pt 3)**: 625-635.
Song, S. H., S. W. Han, et al. (2011). "Epigenetic-based therapies in cancer: progress to date." Drugs **71**(18): 2391-2403.
Stacey, G. (2006). "Primary Cell Cultures and Immortal Cell Lines." eLS.
Straub, S. G. and G. W. Sharp (2002). "Glucose-stimulated signaling pathways in biphasic insulin secretion." Diabetes Metab Res Rev **18**(6): 451-463.
Suganuma, T. and J. L. Workman (2011). "Signals and combinatorial functions of histone modifications." Annu Rev Biochem **80**: 473-499.
Tarasov, A., J. Dusonchet, et al. (2004). "Metabolic regulation of the pancreatic beta-cell ATP-sensitive K+ channel: a pas de deux." Diabetes **53 Suppl 3**: S113-122.
Taunton, J., C. A. Hassig, et al. (1996). "A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p." Science **272**(5260): 408-411.

Tiedge, M., S. Lortz, et al. (1997). "Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells." Diabetes **46**(11): 1733-1742.
Unger, R. H. (1995). "Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications." Diabetes **44**(8): 863-870.
Unger, R. H. and Y. T. Zhou (2001). "Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover." Diabetes **50 Suppl 1**: S118-121.
Warburg, O. (1956). "On the origin of cancer cells." Science **123**(3191): 309-314.
Weishaupt, H. and J. L. Attema (2010). "A Method to Study the Epigenetic Chromatin States of Rare Hematopoietic Stem and Progenitor Cells; MiniChIP-Chip." Biol Proced Online **12**(1): 1-17.
Widiker, S., S. Karst, et al. (2010). "High-fat diet leads to a decreased methylation of the Mc4r gene in the obese BFMI and the lean B6 mouse lines." J Appl Genet **51**(2): 193-197.
Wierup, N., H. Svensson, et al. (2002). "The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas." Regul Pept **107**(1-3): 63-69.
Voight, B. F., L. J. Scott, et al. (2010). "Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis." Nat Genet **42**(7): 579-589.
Volkmar, M., S. Dedeurwaerder, et al. (2012). "DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients." EMBO J **31**(6): 1405-1426.
World Health Organization (2011). Use of Glycated Haemoglobin (HbA1c) in the Diagnosis of Diabetes Mellitus.
Yang, B. T., T. A. Dayeh, et al. (2011). "Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets." Diabetologia **54**(2): 360-367.
Yang, B. T., T. A. Dayeh, et al. (2012). "Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes." Mol Endocrinol **26**(7): 1203-1212.
Yang, Y., L. Hu, et al. (2010). "Structural insights into a dual-specificity histone demethylase ceKDM7A from Caenorhabditis elegans." Cell Res **20**(8): 886-898.
Zegerman, P., B. Canas, et al. (2002). "Histone H3 lysine 4 methylation disrupts binding of nucleosome remodeling and deacetylase (NuRD) repressor complex." J Biol Chem **277**(14): 11621-11624.
Zeggini, E., M. N. Weedon, et al. (2007). "Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes." Science **316**(5829): 1336-1341.
Zhang, C. Y., G. Baffy, et al. (2001). "Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes." Cell **105**(6): 745-755.
Zhang, Y. and D. Reinberg (2001). "Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails." Genes Dev **15**(18): 2343-2360.
Zhou, Y. P. and V. Grill (1995). "Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans." J Clin Endocrinol Metab **80**(5): 1584-1590.

Zhou, Y. P. and V. E. Grill (1994). "Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle." J Clin Invest **93**(2): 870-876.
Zimmet, P. (2003). "The burden of type 2 diabetes: are we doing enough?" Diabetes Metab **29**(4 Pt 2): 6S9-18.