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Mechanisms of defective insulin secretion in type 2 diabetes

Mahdi, Taman

2013

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by

Taman Mahdi Hamed, M.D.

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With due permission from the Faculty of Medicine, Lund University, the public defense of this thesis for the degree of Doctor of Philosophy in medical science will take place

In lecture hall Medelhavet, wallenberglaboratoriet, skåne university hospital, Malmö, Sweden

On February 8, 2013 at 1:00 PM

Faculty opponent

Professor Marc Donath

Endocrinology University of Basel

Mechanisms of defective insulin secretion in type 2 diabetes

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ISSN 1652-8220
ISBN 978-91-87189-78-4
Lund University, Faculty of Medicine Doctoral Dissertation series 2013:10

Printed in Sweden by Media-Tryck, Lund University, Lund 2013

To my father

For letting me choose my own path in life and for your belief in my success

"Science knows no country, because knowledge belongs to humanity and it is the torch which illuminates the world."

Louis Pasteur (1822-1895)

Contents

Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
ADRA2A	Adrenoceptor alpha 2A
BMI	Body mass index
CIR	Corrected insulin response
FFA	Free fatty acid
GSIS	Glucose-stimulated insulin secretion
GWAS	Genome-wide association study
GLUT2	Glucose transporter-2
HbA1C	Glycated haemoglobin A1C
HI	Hyperinsulinemia of infants
IL-1β	Interleukin- 1 beta
IFG	Impaired fasting glucose
IVGTT	Intravenous glucose tolerance tests
INS 832 13	Insulin-secreting rat insulinoma cell
Kin	Connectivity within module
Kout	Connectivity outside the module
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1
LADA	Latent autoimmune diabetes in adults
Ldha	Lactate dehydrogenase A
MODY	Maturity onset diabetes of young
NADPH	Nicotinamide-adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ND	Non diabetic donor

OGTT	Oral glucose tolerance test
PDX1	Duodenal homeobox factor 1
PP cell	Pancreatic polypeptide
RRP	Readily releasable insulin
RP	Reserve pool
SAB	Secretion assay buffer
SNP	Single nucleotide polymorphisms
SFRP4	Secreted frizzled-related protein 4
SOX 5	SRY (sex-determining region Y) box 5
TCF7L2	Transcription factor 7-like 2
T1D	Type 1 diabetes
T2D	Type 2 diabetes
WGCNA	Weighted gene co-expression network analysis

List of publications

Scientific papers included in this thesis

I. Rosengren AH, Braun M, **Mahdi T**, Andersson SA, Travers ME, Shigeto M, Zhang E, Almgren P, Ladenvall C, Axelsson AS, Edlund A, Pedersen MG, Jonsson A, Ramracheya R, Tang Y, Walker JN, Barrett A, Johnson PR, Lyssenko V, McCarthy MI, Groop L, Salehi A, Gloyn AL, Renström E, Rorsman P, Eliasson L. *Reduced insulin exocytosis in human pancreatic β-cells with gene variants linked to type 2 diabetes*. Diabetes. 2012 Jul;61(7):1726-33

II. **Mahdi T**, Hänzelmann S, Salehi A, Muhammed SJ, Reinbothe TM, Tang Y, Axelsson AS, Zhou Y, Jing X, Almgren P, Krus U, Taneera J, Blom AM, Lyssenko V, Esguerra JL, Hansson O, Eliasson L, Derry J, Zhang E, Wollheim CB, Groop L, Renström E, Rosengren AH. *Secreted frizzled-related protein 4 reduces insulin secretion and is overexpressed in type 2 diabetes. Cell Metab. 2012 Nov 7;16(5):625-33.*

III. **Mahdi T**, Mecham B, Groop L, Renström E and Rosengren AH. *A module of co-expressed genes in human islets associated with type 2 diabetes and islet dedifferentiation. Manuscript.*

The published papers were reprinted with permission by the publisher.

Paper not included in this thesis

Pawel Buda, Thomas Reinbothe, Vini Nagaraj, **Taman Mahdi**, Cheng Luan, Yunzhao Tang, Annika S. Axelsson, Daiqing Li, Anders H. Rosengren, Erik Renström and Enming Zhang. *Eukaryotic translation initiation factor 3 subunit E controls intracellular calcium homeostasis by regulation of CaV1.2 surface expression. Revised.*

Introduction

There is an explosive increase in the number of people diagnosed with diabetes, which makes this disease one of the major health threats for the 21st century. The disease currently affects 285 million individuals in ages 20-79 years, which corresponds to 6.4% of the world's population. Between 2010 and 2030, diabetes is expected to increase by 70% in developing countries and by 20% in developed countries (Shaw, Sicree et al. 2010).

Diabetes leads to severe complications in the eyes, kidneys and cardiovascular system. The disease and its complications pose a heavy burden on health care systems in both developed and developing countries (Zimmet, Alberti et al. 2001). The treatment available today is not able to cure the disease or prevent the complications. A major hurdle to develop new and effective therapies is the incomplete understanding of the disease pathophysiology. Understanding the disease mechanisms and finding a way to prevent diabetes is an urgent challenge for the health care community and our society.

Diabetes is a metabolic disorder of multiple aetiology and occurs when insulin levels in the body are insufficient, or when the body cannot effectively use the insulin it produces or both. This leads to increased blood glucose levels (hyperglycaemia) (World Health Organization 2009).

According to the World Health Organization (WHO) diabetes mellitus is defined as fasting plasma glucose (FPG) ≥7.0 mmol/l and /or plasma glucose ≥11.1mmol/l at 2 hours after injection of 75 g of glucose during an oral glucose tolerance test (OGTT). An individual is considered to have prediabetes when blood glucose is higher than normal but not sufficiently high for the diagnosis of diabetes. It is characterized by impaired fasting glucose (IFG), (fasting glucose between 6.1 and 6.9 mmol/l and 2hr glucose below 7.8mmol/l) or impaired glucose tolerance (IGT) (fasting glucose < 7 mmol/l or 2h glucose between 7.8 mmol/l and 11.2 mmol/l. An individual with prediabetes is at increased risk for developing diabetes. Another way to diagnose diabetes is to analyse glycated haemoglobin A1C (HbA1c), which is a blood test that indicates average blood glucose levels for the past two to three months. HbA1c levels higher than 6.5% at two separate occasions is a sufficient criterion to diagnose diabetes (World Health Organization 2011).

Diabetes mellitus is typically classified into two main subtypes. Type 1 diabetes is a disease due to autoimmune destruction of the insulin-producing β-cells in the islets of Langerhans in the pancreas (Atkinson and Maclaren 1994; Rother 2007).

It is also known as juvenile-onset diabetes but can develop in adult as well. It is associated with islet cell antibodies. These patients are dependent on exogenous insulin. Type 2 diabetes is the most prevalent form of diabetes, and it comprises about 90% of all diabetes cases. It is characterized by two interrelated metabolic defects: impaired insulin secretion from the pancreatic β-cells and insulin resistance in target tissues (Polonsky, Sturis et al. 1996; Bell and Polonsky 2001; Kahn 2003). Type 2 diabetes increases rapidly due to the modern way of living with a sedentary life and calorie abundance (Alberti and Zimmet 1998; Zimmet, Alberti et al. 2001). The elevated insulin demands in type 2 diabetes make it difficult for the β-cells to meet the needs and ultimately result in β-cell failure.

In addition to the two classic forms of diabetes, LADA (Latent Autoimmune Diabetes in Adults) is an intermediate form with islet cell antibodies but less dramatic β-cell destruction compared with type 1 diabetes. However, most patients with LADA become dependent on insulin treatment shortly after diagnosis. There are also more rare forms of diabetes, such as MODY (Maturity-Onset Diabetes of the Young). MODY is a monogenic autosomal dominant disease and starts at young age. It can present in different forms with various degrees of β-cell dysfunction.

Pancreas and islet of Langerhans

One of the greatest medical revolutions is the discovery that insulin is a blood glucose-lowering hormone secreted by the pancreas. Insulin is secreted in response to food intake and exerts an important role in blood glucose homeostasis. In the normal physiological state, the glucose concentration is tightly regulated by the opposing actions of the insulin and glucagon. After a meal, insulin suppresses glucose output from the liver and enhances glucose uptake into skeletal muscle and adipose tissue. By contrast, glucagon increases the glucose level during fasting.

Insulin is produced in the pancreatic β-cells. The pancreas consists of two quite different types of glandular tissue. The exocrine pancreas is a lobulated, branched, acinar gland producing digestive enzymes. The endocrine pancreas, on the other hand, is composed of millions of small cell clusters, termed islets of Langerhans, scattered throughout the exocrine tissue. The endocrine cells constitute about 4% of the total pancreatic cell mass (Githens 1988).

Each islet consists of about a thousand cells. There are at least 4 types of cells in islet of Langerhans: insulin-secreting β-cells (70% of the cells), glucagon-secreting α-cells (comprising around 20% of the cells), somatostatin-secreting δ-cells, and pp-cells, which is the least abundant cell type and secret pancreatic polypeptide. There is considerable difference in the distribution of islet cells between humans and rodents. In rodents, there is a sharp segregation within the islets such that the β-cell lies in the centre and the other types at the periphery but this segregation is less clear in human islets, which also have a larger proportion of α-cells (Quesada, Tudurí et al. 2008).

Embryonically, the pancreas arises from the endoderm as a dorsal and ventral bud, which fuse together to form the single organ (Murtaugh and Melton 2003). The endocrine part of the pancreas is induced by several transcription factors, including neurogenin 3. After endocrine determination distinct sets of transcription factors restrict the expression of islet hormone genes in β-, α-, δ- and pp-cells. One of the key factors is Pancreatic and Duodenal Homeobox factor-1(PDX-1), which is expressed in endocrine pancreatic progenitor cells (Ohlsson, Karlsson et al. 1993).

During subsequent development, PDX-1 is restricted to β-cells and has an important role in maintaining β-cells in a differentiated and fully functional state. In mature β-cells, PDX-1 activates transcription of the insulin gene and other genes involved in glucose sensing and metabolism, such as GLUT2. Homozygous Pdx1 knock-out mice have pancreas agenesis, which heterozygous mice present with reduced insulin secretion and decreased Glut2 expression (Brissova, Shiota et al. 2002).

Beta cell and insulin secretion

In the pancreatic β-cell insulin is stored in small secretory granules. Each β-cell contains about 10 000 insulin granules (Dean 1973). Insulin is produced as pre-pro-insulin, and then processed to pro-insulin. Pro-insulin, in turn, is cleaved into insulin and c-peptide and stored in the secretory granules. The ratio of pro-insulin to insulin has been shown to be increased in type 2 diabetes (Pfutzner and Forst 2011).

Stimulus-secretion coupling in the β-cell

Glucose-stimulated insulin secretion (GSIS) is the principal mechanism of insulin release. Insulin is secreted in a pulsatile fashion. Pancreatic β-cells are electrically

active. At the normal physiological concentration of blood glucose (4-5 mM), the β-cell has a negative membrane potential (-70 mV) due to constant efflux of K^+ ions. When blood glucose rises, glucose enters the β-cell by facilitated diffusion through glucose transporter-2 (GLUT2) (Meglasson and Matschinsky 1986). After approximately 1 min, metabolism of glucose leads to an increased production of ATP at the expense of ADP. The elevated ATP/ADP ratio leads to closure of ATP-sensitive potassium channels (K_{ATP} channels). The closure of K_{ATP} channels results in membrane depolarization and opening of voltage-gated Ca^{2+} channels and subsequent increase in intracellular calcium ($[Ca^{2+}]_i$) (Ashcroft and Rorsman 1989). This triggers the release of insulin-containing granules by Ca^{2+}-dependent exocytosis. This process is referred to as the triggering pathway or K_{ATP}-channel-dependent pathway of insulin secretion (Fig. 1).

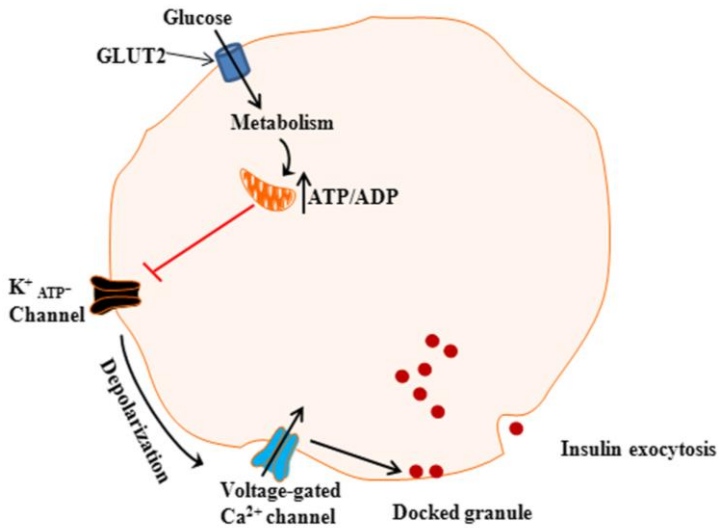

Fig. 1 Mechanisms of glucose-stimulated insulin secretion

There is also an amplifying mechanism (termed the K_{ATP}-channel-independent pathway), which further enhances insulin secretion in response to Ca^{2+} influx. The underlying mechanism is still not clear, but several factors have been suggested to contribute to this pathway, such as NADPH, ATP, GTP, malonyl-CoA and

glutamate (Orci, Amherdt et al. 1973; Orci, Malaisse-Lagae et al. 1973; Wollheim and Sharp 1981; Ammala, Ashcroft et al. 1993; Gembal, Detimary et al. 1993; Warnotte, Gilon et al. 1994; Eliasson, Renström et al. 1997; Henquin 2000; Ivarsson, Quintens et al. 2005).

Ion channels in the β-cell

Beta cells are equipped with several types of ion channels but not all of them are involved in insulin secretion. K_{ATP}-channels couple glucose metabolism to plasma membrane electrical activity by regulating membrane K^+ fluxes (Seino and Miki 2004). It consists of two parts: 4 pore-forming subunits which are sites of ATP binding (Kir6.2) and 4 regulatory subunits (Sulfonylurea receptor subunits, SUR). The channel is a key regulator of β-cell electrical activity. Mutations in the genes that encode the K_{ATP}-channel produce a wide spectrum of diseases, including hyperinsulinemia of infants (HI) and diabetes (Huopio, Reimann et al. 2000; Magge, Shyng et al. 2004). Sulfonylureas bind to and close K_{ATP}-channels, and are effective insulin secretagogues that are widely used for the treatment of type 2 diabetes (Gribble and Reimann 2003).

Another important ion channel family in the β-cell is calcium channels, which are activated upon membrane depolarization. There are 4 different kinds of calcium channels, classified according to their physiological and pharmalogical characteristics: L-, P/Q-, T- and N-type Ca^{2+} channels. There appears to be major differences in the distribution of Ca^{2+} channel between rodent and human β-cells. Blockade of L-type abolish GSIS while inhibition of T and P/Q type reduce GSIS by 60-70%. In human β-cell L-type channel is of $Ca_v2.1$ and P/Q is of Ca_v 2.1 subtype (Braun, Ramracheya et al. 2008). In mouse both $Ca_v1.2$ and Ca_v 1.3 are expressed but lack of effect of the L-type channel blocker in $Ca_v1.2$ knock mice, suggest that $Ca_v1.3$ has a minor role for β-cell function (Sinnegger-Brauns, Hetzenauer et al. 2004).

Distinct granule pools

Insulin granules need to be transported to the plasma membrane for Ca^{2+}-dependent exocytosis. There are two distinct functional pools of insulin granules that differ in release competence according to their proximity to the plasma membrane. A small fraction of the granules (1-5%) (Neher 1998) can undergo exocytosis immediately upon stimulation and is referred to as the readily releasable pool (RRP). These granules are located immediately beneath the plasma

membrane. There is also a reserve pool (RP) that accounts for the vast majority of granules (95-99%). This pool must be translocated to the plasma membrane and undergone a series of ATP- and temperature-dependent reactions (docking and priming) to become release-competent (Fig. 2).

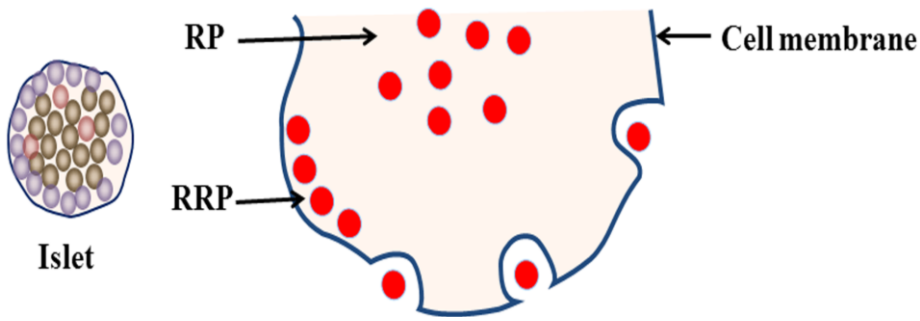

Fig. 2 RRP is located immediately under plasma membrane and is released directly upon stimulation, while granules under RP need ATP- and temperature-dependent reactions to translocate to the plasma membrane

In isolated pancreatic islets, insulin is secreted in a biphasic pattern. The biphasic release of insulin in response to glucose was first reported in 1960(Curry, Bennett et al. 1968). The prevailing hypothesis is that the release of RRP granules accounts for the rapid and transient first phase (lasting 5-10min) and that mobilization of a subsequent supply of new granules for release accounts for the second phase, which is continued at slower rate and is correlated with the ATP/ADP ratio in the β- cell (Rorsman and Renstrom 2003) (Fig. 3).

Recent studies of the dynamics of insulin release have challenged the old view and classified granules into 3 modes: mode 1, in which predocked granules are immediately fused to the plasma membrane by stimulation (old face); mode 2, in which granules are newly recruited by stimulation and immediately fused to the plasma membrane (restless newcomer); and mode 3, in which granules are newly recruited by stimulation but are first docked and then fused to the plasma membrane (resting newcomer) (Shibasaki, Takahashi et al. 2007).

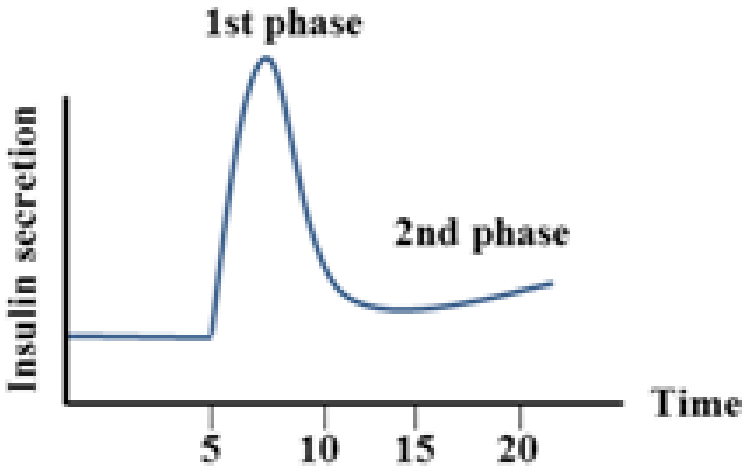

Fig. 3 Glucose-stimulated insulin secretion is characterized by a rapid first phase and a continuous slower second phase

Pathophysiology of type 2 diabetes

T2D is characterized by increased blood glucose as a result of increased glucose production by the liver and decrease glucose uptake in muscle and adipose tissue. Both defective insulin secretion and insulin action on the target tissue are main features of the pathogenesis (Fig. 4). A peripheral tissue resistance causes increased secretion of insulin by the β-cell to maintain normoglycaemia. Pancreatic β-cells can often manage this increased demand for insulin by increasing their number or capacity, resulting in hyperinsulinemia. However, if insulin resistance progresses further, the pancreatic β-cell hypersecretion of insulin may fail to compensate for insulin resistance and this may eventually leads to β-cell death in susceptible individuals (Ahren 2005). T2D usually has slow onset, and most patients remain undiagnosed for years. Transition to overt diabetes occurs when pancreatic β-cells no longer secret sufficient insulin. By the time when type 2 diabetes is diagnosed, many patients have already diabetic

complications like heart disease, stroke, and microvascular complications like blindness, renal failure and peripheral neuropathy.T2D is complex disease which is caused by both environmental and genetic factors. It has a higher incidence in monozygotic twins than in dizygotic twins (Newman, Selby et al. 1987; Kaprio, Tuomilehto et al. 1992). However environmental factors and life style like obesity account for the rapid increase of T2D. Not all obese people are diabetic, which is an account of the genetic background of T2D.This concept was tested by infusion of triglyceride emulsion that causes decrease in insulin secretion in people who have first degree relatives but not in those who did not (Kashyap, Belfort et al. 2003).

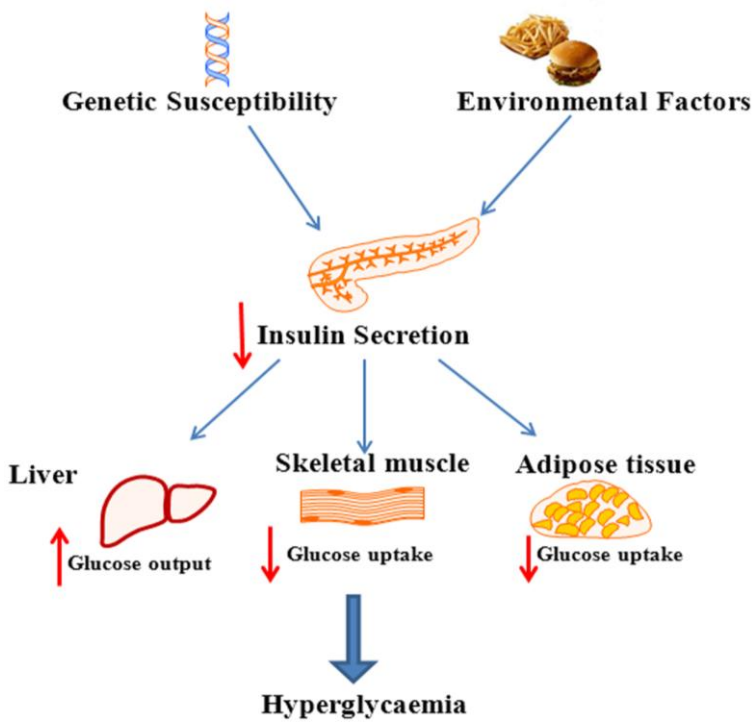

Fig. 4 The complex interplay of the various pathophysiologic defects contributing to hyperglycemia in type 2 diabetes mellitus.

Obesity is the main risk factor for type 2 diabetes due to increased peripheral resistance that leads to the upregulation of insulin secretion. It is not possible to exclude its direct effect on β cell function. Obesity causes increase circulatory level of leptin, which is a proinflammatory cytokine (Otero, Lago et al. 2005), and dyslipidemia. Leptin causes reduction in insulin secretion and promote inflammation in beta cell due to secretion of IL-1b from β- cell with subsequent reduction in beta cell mass in T2D (Donath, Schumann et al. 2008). There is some overcapacity in β-cell mass, since 40% of β-cells would be sufficient for adequate glucose control in non-diabetic individuals (Ashcroft and Rorsman 2012). On the other hand, long term exposure to FFA suppresses glucose stimulated insulin secretion, but the order of events is still not fully understood.

IL-1β has been known to induce autoimmune inflammation in T1D (Mandrup-Poulsen 1996), but it also potentiates the effect of high glucose on β-cell function and leads to the activation of NF-κB in T2D (Kwon, Corbett et al. 1995; Maedler, Sergeev et al. 2002).Taken together this indeed suggests that inflammation could participate in T2D. Finding of amyeloid in diabetic human islet by histological examination is another agreement.

Genetic background of T2D

The human genome consists of three billion nucleotides and naturally occurring variation in DNA sequences can led to associations between genetic variants and human disease. The most common genetic variation is a single nucleotide polymorphism (SNP). SNPs mean that a single nucleotide differs in a DNA sequence of paired chromosomes. GWAS is a hypothesis free approach that was successfully used to detect the genetic variants underlying many human diseases including T2D.

The emergence of GWAS capable of identifying T2D susceptible gene came after the identification of TCF7L2, which is the most important diabetes susceptible gene up today (Humphries, Gable et al. 2006). It acts in the wnt signaling pathway. However, several diabetes genes appear to be involved in the wnt signaling pathway. This pathway is activated by binding of Wnt proteins to frizzled receptors that in turn leads to stabilization and nuclear translocation of β-catenin. Over the last decade, more than 40 genetic variants have been identified by GWAS and this explains 5-10% of T2D risk. Table 1 lists some of the T2D susceptible loci identified to date.

Table 1 Genetic loci associated with T2D.

SNP	Nearest gene	Risk allele	Non-risk allele
rs7903146	TCF7L2	T	C
rs2237895	KCNQ1	C	A
rs231362	KCNQ1	G	A
rs13266634	SLC30A8	T	C
rs5219	KCNJ11	T	C
rs10830963	MTNR1B	G	C
rs553668	ADRA2A	A	G
rs1111875	HHEX/IDE	C	T
rs10946398	CDKAL1	C	G
rs10423928	GIPR	C	A
rs2191349	DGKB	G	T
rs10861975	SYT1	T	C
rs363004	SNAP25	A	G
rs11759297	RIMS1	G	A
rs560887	G6PC2	T	C
rs11920090	SLC2A2	A	T
rs4607517	GCK	A	G

Studying of a complex disease like T2D, which is a result of interaction between environmental and genetic factors, is extremely difficult in outbred populations like humans. It would therefore be interesting to investigate such interaction in animal models. This idea is supported by identification of common variant in the α2a-adrenoreceptor, which causes diabetes in GK rat and in humans is associated with decreased insulin secretion (Rosengren, Jokubka et al. 2010).

Although GWAS uncovers genetic loci with the strongest statistical association with T2D (Grant, Thorleifsson et al. 2006), there is still a lack of molecular and physiological understanding of many of these genes and how they contribute to T2D. This is not entirely unexpected because variation in DNA does not on their own directly impact on disease risk. A disease usually reflects the perturbations of the complex intracellular and intercellular network that links tissue and organ system. Therefore, an understanding of a gene network has the potential to fill in the gap in genetic understanding.

Network-based approach to human disease

For maintaining proper cellular function, there is an interaction between different cell compartments. Indeed, disease traits are rarely due to specific genetic defects but reflect various pathological processes that interact in a complex network. Only about 10% of human genes have a known disease association (Amberger, Bocchini et al. 2009). However, the emergence of network approach has provided insights to better understand the cellular interconnectedness under a variety of conditions including normal physiological condition like differentiation and to more effectively target complex disease.

In the past few years there has been a very fast increase in human molecular interaction data (Ideker and Sharan 2008). This molecular network, like other networks operating in technology or social system, is not random. A number of different procedures to build network exist. There are typically highly connected nodes which are located in the centre of the networks, with large segregation of non-essential genes which vary in expression. This argue that hub genes are essential (Jeong, Mason et al. 2001), widely expressed and have particular biological function. Taking this together, molecular networks are placed in an intermediate position between genetic loci and the resulting disease phenotypes. However, additional experimental efforts are still needed with this approach, since it is often not combined with functional studies (Fig. 5)

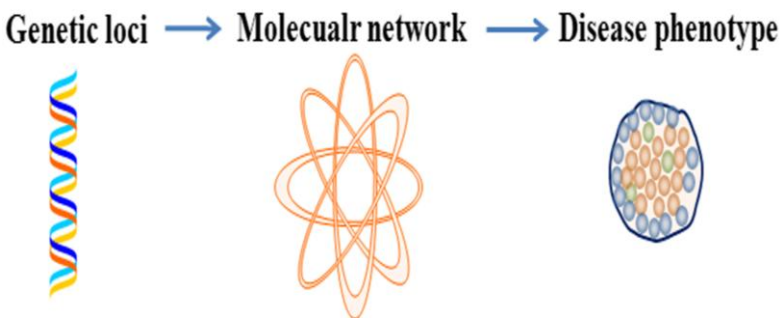

Fig. 5 Molecular networks allow a direct link between genetic variance and disease phenotype.

Network based DNA variation approach has successfully been applied to liver and adipose tissue gene expression data in mouse (Chen, Zhu et al. 2008). Although network-based approaches to human disease have multiple biological and clinical applications, little is known about the networks underlying T2D. This thesis addresses the impact of gene networks on the risk of T2D as well as the underlying mechanisms

Aims of the Thesis

The main aim of this thesis to find the genetic and cellular basis for the impaired insulin secretion in type 2 diabetes mellitus.

The specific aims were:

- To identify the functional effects of genetic risk variants for type 2 diabetes.
- To characterize the gene networks in human islets that are perturbed in type 2 diabetes.
- To investigate central genes in type 2 diabetes-associated gene networks and the mechanisms by which they contribute to the pathophysiology.

Materials and methods

Pancreatic islets and cell lines

In this thesis we used both human and rodent islets, as well as cell lines. Human islets, in particular, is a unique source for studying the mechanisms of defective insulin secretion in T2D Human islets were extracted from cadaveric multi-organ donors at Uppsala University (papers I-III) and the Oxford Centre for Islet Transplantation (paper I) . The experimental procedure was approved by the local ethics committees. The pancreas was perfused with ice-cold collagenase, cut into pieces and placed in a digestion chamber at 37°C. Separation of endocrine and exocrine tissues was achieved by a continuous density gradient. Selected fractions were then centrifuged to enrich for islets. Purity of islets was measured by dithizone staining (Goto, Holgersson et al. 2006). From this suspension, islets to be used for experiments were hand-picked under a microscope. The islets were cultured at 5.6 mM glucose in CMRL 1066 for 1 to 9 days prior to experiment. Since there is limited availability of human islets, we also used rodent islets as an alternative source. Islets from both NMRI mice and Wistar rats were prepared by collagenase digestion of the exocrine pancreas. The rodent islets were hand-picked and incubated in a humidified atmosphere in RPMI 1640 tissue culture medium with antibiotics. Insulin-secreting rat insulinoma INS832/13 cells (passages 60-70) were also used in this thesis for mechanistic studies. The INS832/13 cells are easier to transfect for siRNA experiments than primary β-cells, and are available in unlimited amounts.

Co expression network analysis

The co-expression network analysis was performed in R (version 2.11.1) using log2-transformed microarray expression data. Using the weighted gene co-expression network analysis (WGCNA) framework (Zhang and Horvath 2005) and the corresponding Bioconductor package (Langfelder and Horvath 2008), we first calculated the pair-wise co-expression for all genes and formed a similarity matrix based on the Pearson correlation coefficients $s_{i,j} = |cor(x_i,x_j)|$, where x_i denotes the expression vector for gene i across the samples.

Next, the similarity matrix was transformed into an adjacency matrix $a_{i,j} = |cor(x_i,x_j)|^{\beta}$. The adjacency matrix represents the strength of connection between two genes as a continuous weight in [0,1] and has been shown to be robust with respect to the choice of β as compared with methods using hard thresholds to dichotomize the coexpression matrix (Zhang and Horvath 2005). The connectivity of a gene in a network (the degree k) equals the sum of all connections for that gene.

Biological networks have been suggested to exhibit a scale-free property (Ravasz, Somera et al. 2002), which means that the probability that a node is connected with k other nodes (the degree distribution p(k)) decays as a power function $p(k) \sim k^{-7}$. Linear regression analysis of log-transformed k and p(k) was used to estimate how well the co-expression network satisfied the scale-free topology for different values of β. We found that for $\beta \geq 7$ R2 for the fit was >0.9 for the samples in paper II.

Based on the adjacency matrix for β=7, the topological overlap, which reflects the relative gene interconnectedness, was calculated for all gene pairs (Ravasz, Somera et al. 2002). The non-negative and symmetric topological overlap matrix $\Omega = [\omega_{i,j}]$ was converted to dissimilarity (distance) measures $d_{i,j} = 1-\omega_{i,j}$, which were used for module identification by hierarchical clustering. The clustering was unsupervised and based on the Dynamic Tree Cut method (Langfelder, Zhang et al. 2008) using 30 genes as the minimal module size (Fig. 6).

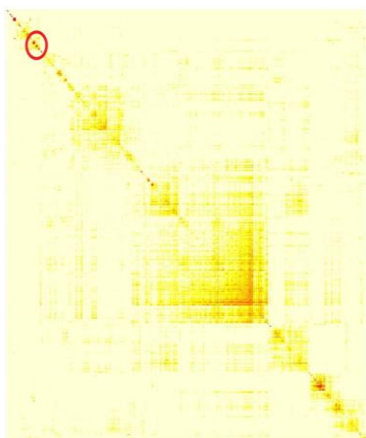

Fig. 6 Symmetrically arranged heatmap of the topological overlap matrix for which the rows and columns are sorted by the hierarchical clustering tree used to define modules. Intensity from light yellow (low) to red (high) denotes the topological overlap between genes.the red circle indicateds T2D-associated module

The eigengene, defined as the 1st principal component of the gene expression matrix, was determined for each module. The association between module eigengenes and phenotype traits was analyzed by logistic (T2D status) or linear (HbA1c and insulin secretion) regression with corrections for BMI, age and sex.

For each gene in the T2D-associated module the connectivity within the module (kin) and outside the module (kout) was determined. In addition, the association between the gene expression trait and T2D status, HbA1c or insulin secretion was calculated using logistic or linear regression and corrected for BMI, age and sex.

Insulin secretion and content measurements in vitro

Radioimmunoassay (RIA) was used in this thesis for measuring insulin secretion and insulin content from both islets and cell lines. It is based on the competitive binding of two antigens with an antibody. The insulin in the samples leads to the displacement of the radioactive antigens. The free radioactive antigens produce a signal which can be measured using a gamma counter. Krebs-Ringer bicarbonate buffer (KRBB) was used as incubation medium for the islets while SAB buffer was used for the INS832/13 cells. Prior to experiments, islets and cell lines were preincubated for 30 min and 2h, respectively, at 2.8 mM glucose, followed by 1 hour incubation at 16.7 mM glucose.

We also used enzyme-linked immunosorbent assay (ELISA) in this thesis. Here, the samples are added to wells which are coated with antibodies. Then, a detectable antibody that is linked with an enzyme is added. Binding of the detectable antibody with its substrate at the final step produces a change in the substrate colour. This change in colour reflects the amount of antigen present in the sample. Both human and mouse SFRP4 ELISA kits were used for measuring SFRP4. Absorbance was measured at 450 nm.

Cell viability

It is important to assess islet and cell line viability to exclude any toxic or apoptotic effects on cells by the various treatments. Cell viability was measured using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Reagent. The measurement is based on the spectrophotometric detection of a coloured formazan product converted from a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) compound by NADPH or NADH in metabolically active cells. For SFRP4 experiments, viability

was measure after a culture period of 24 h. The absorbance was measured at 490 nm.

Capacitance recording

Biological membranes behave as capacitors, and the cell membrane capacitance can be measured by the patch clamp technique. At exocytosis, when insulin granules fuse with the plasma membrane there will be an increase in the area of the plasma membrane. By contrast, during endocytosis, there will be a corresponding decrease in the plasma membrane area (Lindau and Neher 1988; Ammälä, Eliasson et al. 1993). The relationship between membrane capacitance (C) and surface area (A) is given by the equation

$C= \varepsilon * A/d$

Where ε is a membrane-specific constant and d is the membrane thickness. This relation means that the increased cell surface area after exocytosis can be measured as an increase in cell capacitance with the patch clamp technique. The specific capacitance for biological membrane is 9 fF/μm² (Gentet, Stuart et al. 2000).

In a patch clamp experiment, a tight seal is established between the pipette glass and the plasma membrane (Fig. 7). In this thesis we used two different configurations of the patch clamp technique. *Voltage clamp* recordings with the *standard whole cell configuration* enables measurement of all currents across the plasma membrane at the same time.

Control of the intracellular environment is achieved by removing a piece of the patch membrane by gentle suction, which allows the pipette solution to perfuse the cells. We also used *current-clamp* experiments with the *perforated patch configuration*. Here an electrical contact is established by small pores in the patch membrane due to inclusion amphotericin B in the pipette solution.

Prior to patch clamp experiments, single β-cells from human or rodent islets were prepared by incubation of islets for 10 min in a Ca^{2+}-free medium followed by mechanical disruption using a pipette.

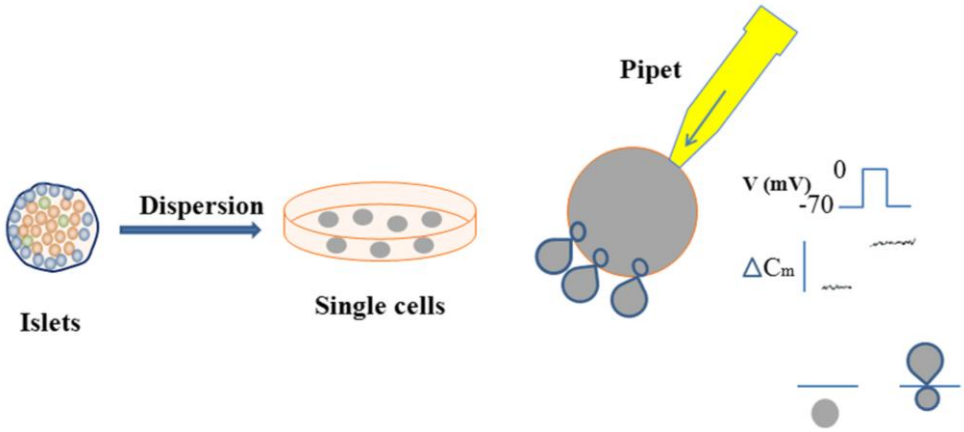

Fig.7 Principle of Capacitance recording of insulin exocytosis.

Human β-cells were identified based on their size while rodent β-cells were identified based on their size and the inactivation properties of the voltage-gated Na^+ currents (Hiriart and Matteson 1988). In the voltage-clamp experiments ,TEACL was present in the extracellular solution to block outwardly rectifying K+ currents that otherwise obscure the depolarization- evoked Ca^{2+} currents. Exocytosis was elicited by artificial depolarization. A train of ten depolarizations from -70 to 0 mV was applied to simulate glucose-induced electrical activity.

Current-voltage relationship was measured by 100 ms depolarizations from the holding potential at -70 mV. The depolarization potential was increased stepwise from -50 to +30 mV.

In current clamp experiments, the resting membrane potential was around -70 mV.

RNA interference

RNA interference is a natural biological process that is initiated by either endogenous or exogenous RNA molecules, which inhibit postranscriptional gene silencing (Nellen and Lichtenstein 1993). Cleavage of long RNA results in short double strand RNA molecules (siRNA). After entering to RNA-induced silencing comlex (RISC) in the cytoplasm, siRNA bind to mRNA and inhibits its translation. RNAi was occure in plant and invertebrates but not in mammalian cell. The latter was due to an interferon reaction that leads to unspesific gene silencing Here, we introduced 21 nucleotide long exogeneous siRNA to enable gene silencing. This short sequence of siRNA was important since it inhibited unspesific genes silencing (Elbashir, Harborth et al. 2001). RNAi by siRNA has generated a great deal of interest in biological study of protein function.

During transfection, siRNA should be enclosed into a reagent to enter the cell. In this thesis Lipofectemine RNAiMAX and Dharmafect were used as transfection reagent in primary cells and INS832/13, respectively. Gene silencing was assessed by qPCR using Taqman or Western blot.

Western blot

Western blot and confocal microscopy were used in this thesis for detection of target proteins. They are antibody-based methods with quite different procedures. Western blot is based on gel elecrophoresis for the detection of specific protein in a homogenized sample. Proteins in the sample are generally separated on a gel by size, and their molecular weight can be compare with known molecular weight marker. Then the target protein transferred to a thin membrane followed by detected with primary antibodies. A secondary antibody is then added. This antibody is typically linked to an enzyme reporter, like Horseradish peroxidase.

Confocal microscopy

In confocal microscopy a laser source is used to provide the excitation light to visualize florescently tagged secondary antibodies (fluorophores).The wavelengths of the excitation light and the colour of the emitted light are fluorophore dependant. Passing of the light through a pinhole enables the detection of only a thin slice of the sample. In this thesis Cy2, Cy3 and Cy5 flurophores were used.

Electron microscopy

Electron microscopy enables high resolution imaging of the ultrastructure of β-cells. We can thereby estimate insulin granule distribution, mitochondria and other organelles in a detailed manner that is not possible by confocal microscopy.

For these experiments, human islets were incubated in KRBB containing 16.7 mM glucose for 1h, fixated in 2.5% Glutaraldehyde, and added to freshly prepared Millonig buffer. Millonig buffer contains 2.26% NaH2PO4 and 2.52% NaOH (pH 7.2). Islets were post-fixated in osmium tetroxide (1%), and dehydrated and embedded in Durcupan. β-cells can be identified by the typical appearance of the granules with a central dense core and a surrounding halo. Cellular granule distribution was determined using in-house MATLAB-based software. A granule was considered as docked if the granule centre was within 0.15 μm, i.e. half a granule diameter, from the plasma membrane.

Ca^{2+}measurements of islets

To estimate calcium dynamics in whole islets we measured intracellular Ca^{2+} ($[Ca^{2+}]i$) by dual-wavelength fluorimetry (Grynkiewicz, Poenie et al. 1985)(7).Imaging was performed using a Polychrome V monochromator (TILL Photonics, Graefeling, Germany) on a Nikon Eclipse Ti Microscope (Nikon, Tokyo, Japan). The islets were loaded with fura-2 in the presence of pluronic acid for 35-40 minutes at 37 ºC prior to the experiments. They were then perfused (1 ml/min) with extracellular solution for 10 min prior to imaging. Fura-2 was excited alternately at 340 and 380 nm. The fluorescence intensity ratio (F340/F380) was calculated after background subtraction. For the experiments with acute SFRP4, SFRP4 was added to a final concentration of 30 nM.

TCF/LEF activity

The family of TCF/LEF transcription factor mediates major downstream events of the Wnt pathway. Therefore, to assess TCF/LEF transcriptional activity we used different reporter constructs. INS832/13-cells were transfected with an inducible transcription factor-responsive construct expressing firefly luciferase, a construct expressing Renilla luciferase constitutively, a non-inducible construct expressing firefly luciferase (negative control), and positive control constructs. For each well, the TCF/LEF-inducible or non-inducible (negative control) firefly signal was normalized for Renilla signal.

Gene expression

Both real time quantitative PCR (RT-PCR) and Affymetrix microarray were used for quantifies difference in gene expressin between different samples in this thesis.

RT-PCR

islets and cell lines were homogenized in Qiazol reagent. RNA was extracted with chloroform precipitation using the mRNeasy kit from Qiagen. RNA was then transformed into cDNA by reverse transcriptase and random primers. In this thesis gene expression was measured using TaqMan. In the TaqMan technique sequence-specific DNA probes consisting of oligonucleotides that are labeled with two fluorescent reporters (quencher) on the 5' end and (reporter) on the 3' end. These bind with complementary DNA that is amplified by a set of primers. By repeated heating and cooling reactions, the exonulease activity of taq polymerase from 5' to 3' will cleave the hybridized probe. This leads to the separation of these two probes from each other with subsequent increase in the flurescence emission by the reporter. The emitted fluorescent signal is directly proportional to the amount of target cDNA to which the probe is hybridized. The increase in florescence above threshold value is measured at each cycle (Ct-value), which permits comparison of expression of a gene of interest among different samples

Affymetrix microarray

Gene expression microarrays enable the assessment of global gene expression, and are based on hybridization between sample RNA and probes representing different genomic regions. In papers II and III we analysed global gene expression in human islets using the Affymetrix GeneChip® Human Gene 1.0 ST microarray chip. Briefly, total RNA is converted into biotin-targeted cRNA, and the biotin-labeled cRNA is fragmented into strands with 35 to 200 nucleotides. This is then hybridized onto the chip. The arrays are washed and stained, followed by scanning and image analysis. Data normalization was performed using Robust Multi-array Analysis (RMA).

In vivo experiments.

To measure the effect of SFRP4 in vivo female NMRI mice were injected intraperitoneally with SFRP4 (200 μg/kg in PBS) or PBS at 24, 16 and 8 h before

intravenous glucose tolerance tests (IVGTT). IVGTT was performed after 4 h fasting in anesthetized mice. Glucose was injected at 1.0 g/kg body weight intravenously. Serial blood sampling by the retrobulbar method at 0, 5, 10, 30, 90, and 180 min of the IVGTT was performed as previously described (Rerup and Lundquist 1966). Blood glucose was analyzed using an AccuChek blood glucose reader. Plasma insulin was analyzed by radioimmunoassay.

Genotyping.

Identification of disease-associated genetic variants is important to identify genetic factors involved in polygenic diseases like T2D. In this thesis we analysed single nucleotide polymorphisms (SNPs) that have been consistently associated with T2D in larger genetic studies. The allelic frequency for each of these SNPs differs between patients and control individuals, so that one allele is significantly more prevalent in individuals with disease. In this this thesis, TaqMan allelic discrimination assay-by-design method on an ABI 7900 analyzer(Applied Biosystems,Foster,CA) was used for genotyping. Genomic DNA was extracted from human islets from all donors for genotyping.Here two probes specific for each allele are designed to bind in the region harbouring the polymorphism. Each probe consists of an oligonuleotide with a quencher at the 3'end which preventing emission from a flurescent dye at the 5' end. During PCR running, the exonulease 5' to 3' activity of TaqDNA polymerase will separate the hybridised probes with perfectly matcheded DNA.This allows for specific discrimination between different alleles.This cleavage separate the quencher dye from the reporter dye,allowing for the the florescence to be detected. The emitted fluorescent represents the genotype of each samples(Livak 1999).

Individuals from the Botnia study

We used samples from the Botnia study both in paper I and II. In paper I, 604 non-diabetic individuals from the Botnia study, were genotyped for comparison between SNPs and data from intravenous glucose tolerance tests (IVGTT). We also used the prospective part of the Botnia study, which included 2,770 non-diabetic family members (Groop, Forsblom et al. 1996). Individuals between 18 and 70 years were invited to prospective visits. All subjects participated in a 75-g oral glucose tolerance test (OGTT) after a 12-h overnight fast. Fasting blood samples were drawn at -10, 0, 30, 60, and 120 min for the measurement of plasma

glucose and serum insulin. T2D was diagnosed on the basis of fasting plasma glucose above 7.0 mmol/l and/or 2-h glucose during the OGTT above 11.1 mmol/l. The insulin sensitivity index (ISI) was calculated as 10000/√ (fasting glucose x fasting insulin)(mean OGTTglucose x mean OGTTinsulin)). Corrected insulin response (CIR) was calculated based on insulin and glucose values at 30 min during the OGTT, and CIR = 100 x Insulin30 / (Glucose30 x (Glucose30 – 3.89)). Disposition index (DI) estimates insulin secretion adjusted for insulin sensitivity and was calculated as CIR x ISI.

In paper II serum samples were obtained from totally 226 individuals (88 in the initial analysis and 138 in the replication) participating in the Botnia Study. Fasting samples were used for analysis of serum SFRP4 concentration with ELISA.

Result and Discussion

Paper I

The aim of this paper was to investigate the β-cell phenotype associated with T2D genetic risk variants and to provide a genetic risk score for β-cell dysfunction associated with T2D.

We investigated islets from both non diabetes (ND) and T2D donors from Uppsala University and University of Oxford. We investigated glucose-stimulated insulin secretion and insulin exocytosis in ND (n=42) and T2D (n=17) donors. The individuals were divided into obese and lean according to BMI above or below 31 kg/m^2. Despite a significant reduction in insulin secretion at high glucose from T2D islets after islet size matching there was no change in insulin exocytosis and granules distribution (Fig. 8).

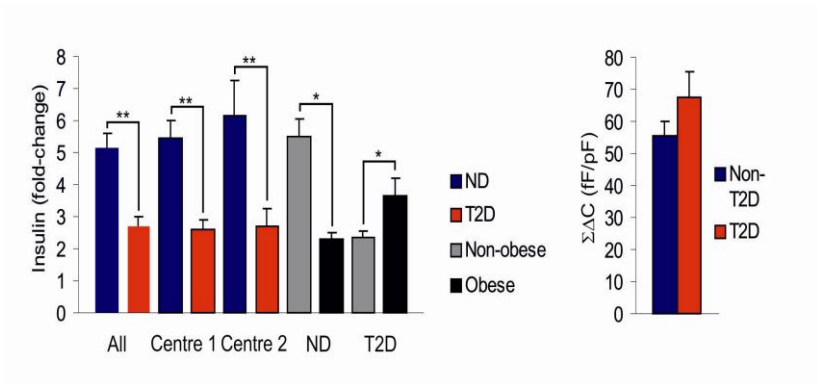

Fig. 8 Characterization of islets from diabetic and ND individuals. A: Fold stimulation of insulin secretion by glucose in batch-incubated islets from non-T2D and T2D donors, nonobese and obese ND donors as well as nonobese and obese T2D donors. Fold stimualtion of insulin secretion in non-T2D and T2D donors separated by center (1, Lund; 2, Oxford) is also displayed. B: Total exocytosis evoked by a train of 10 depolarization from -70 to 0 mV (∑ΔC).

Insulin is secreted in a characteristic biphasic manner with a rapid first phase and a sustained second phase. Secretion is triggered by fuel metabolism, resulting in increased cytosolic ATP production, electrical activity and Ca^{2+}-dependent exocytosis (Rorsman and Renstrom 2003). It has been suggested that the biphasic secretion is perturbed in T2D. This indicates that functional defects could potentially occur at multiple steps in the stimulus secretion coupling

Next we examined the effect of the genetic variants on β-cell function by the patch clamp technique and electron microscopy. Among 14 SNP that have been associated with reduced insulin secretion in vivo (Harvard, MIT et al. 2007; Sladek, Rocheleau et al. 2007; Zeggini, Scott et al. 2008; Rosengren, Jokubka et al. 2010; Voight, Scott et al. 2010), four variants were found to be associated with β exocytosis.

Ultrastructural and electrophysiological examination of rs553668 (ADRA2A), rs5219 (KCNJ11), and rs2237895 (KCNQ1) (Gloyn, Pearson et al. 2004; Grant, Thorleifsson et al. 2006; Lyssenko, Lupi et al. 2007; Unoki, Takahashi et al. 2008; Yasuda, Miyake et al. 2008; Rosengren, Jokubka et al. 2010) showed reduced number of docked granules and decreased Ca^{2+} sensitivity of exocytosis (first and second phase). For rs7903146 (TCF7L2), there was reduced Ca^{2+} sensitivity of exocytosis but no change in granule distribution. There was no effect on the integrated Ca^{2+} current. This indicates that functional secretory defects are important in these risk allele carriers.

We have also tested three other variants that in genes that are important for exocytosis including rs1111875 (HHEX/IDE), rs11920090 (SLC2A2) and rs13266634 (SLC30A8). HHEX/IDE was associated with reduced docked granules while SLC2A2 risk allele carriers had an increased number of docked granules. In this study we have also tested the effect of variants on the glucose stimulated insulin secretion in vitro. TCF7L2, which is the most common T2D associated variant to date, and ADRA2A displayed a significant reduction in insulin secretion. This corroborates previous data showing that the ADRA2A and TCF7L2 risk alleles lead to impaired insulin secretion (Table 2).

Table 2

Genotype effects on β-cell phenotypes in the complete cohort

		Insulin secretion (n=47-50 donors)		Total exocytosis* (n=249 cells from 36 donors)		Ns† (n=97 cells from 18 donors)	
SNP	Nearest gene	p value	Effect‡	p value	Effect	p value	Effect
rs7903146§	***TCF7L2***	**0.002**	**-0.530**	**0.007**	**-6.40**	**0.40**	**-0.0040**
rs553668	***ADRA2A***	**0.017**	**-0.575**	**0.059**	**-15.4**	**0.03**	**-0.14**
rs5219	***KCNJ11***	**0.776**	**0.109**	**0.021**	**-7.00**	**0.05**	**-0.12**
rs2237895	***KCNQ1***	**0.484**	**-0.171**	**0.016**	**-10.1**	**0.04**	**-0.10**
rs10946398	*CDKAL1*	0.153	-0.159	0.414	-1.73	0.187	0.081
rs2191349	*DGKB*	0.229	0.401	0.301	-14.3	0.381	0.101
rs560887	*G6PC2*	0.519	0.226	0.676	-4.97	0.06	-0.108
rs4607517	*GCK*	0.213	0.333	0.667	8.33	0.74	0.002
rs10423928	*GIPR*	0.972	-0.053	0.849	-4.56	0.8	0.004
rs1111875	*HHEX/IDE*	0.164	0.121	0.086	-10.9	0.016	-0.054
rs231362	*KCNQ1*	0.312	0.130	0.319	6.03	0.011	0.073
rs10830963	*MTNR1B*	0.851	0.001	0.655	0.129	0.074	0.095
rs11920090	*SLC2A2*	0.829	0.040	0.57	13.4	0.037	0.058
rs13266634	*SLC30A8*	0.662	0.084	0.819	-1.71	0.72	0.018

*Exocytosis measured as the total increase in single β-cell capacitance in response to ten depolarizations

†Number of docked insulin granules

‡Effect of each additional risk allele on insulin secretion (in ng/islet/h), exocytosis (fF/pF) or granule distribution (Ns: granules/μm^2) estimated from the linear model.

§SNPs in boldface were used to calculate the genetic risk score

Interestingly, these findings also show that functional defects are more pronounced in lean individuals than in obese individuals. This could be due to that insulin secretion in obese patients is compensatory enhanced (Butler, Janson et al. 2003; Rahier, Guiot et al. 2008).

Finally we constructed a genetic risk score for these four variants (coded as 0, 1, 2 depending on the number of risk alleles for each variant). Each individual was assigned a score from 0 to 8. There was an association between the risk score and the glucose stimulated insulin secretion and insulin exocytosis. Interestingly, individuals with high risk score also had reduced fasting glucose, impaired insulin secretion and elevated T2D risk (Fig. 9)

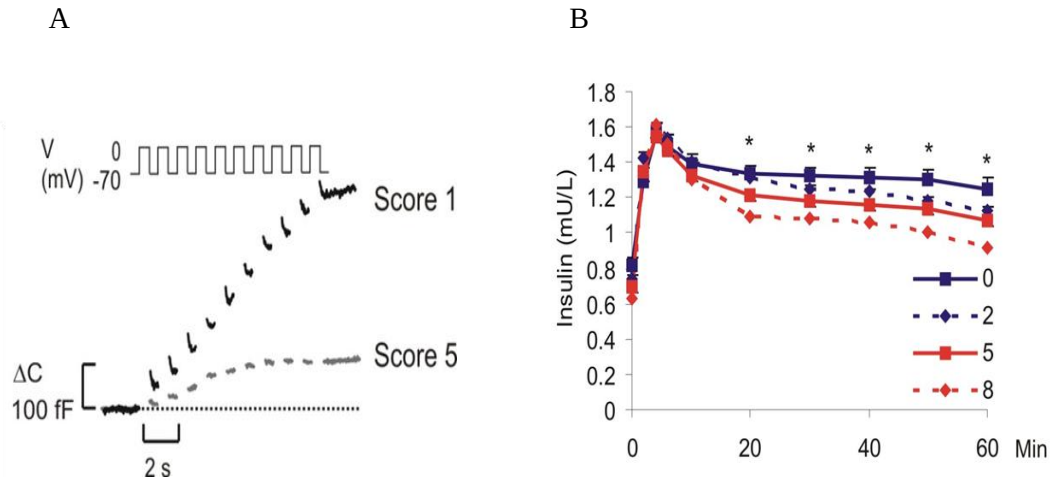

Fig. 9 Effects of the genetic risk score. A: Depolarization-evoked exocytosis in β-cells from donors with different scores. B: Effects of the risk score on insulin secretion

Paper II

Although genetic association studies have identified T2D risk genes there is a lack of understanding their underlying mechanism. As an alternative approach to better understand the disease mechanisms of T2D we investigated gene co-expressin networks in human islets (Barabási and Albert 1999; Ravasz, Somera et al. 2002; Schadt 2009; Barabási, Gulbahce et al. 2011).

In this study we have used weighted gene coexpression network analysis on global microarray data from 48 donors (Zhang and Horvath 2005). Connectivity was calculated for all pairs of gene expression traits to get a network with scale free properties (R^2 0.9) (Ravasz, Somera et al. 2002). We then identified a module with 174 genes that was associated with T2D, elevated HbA1c and impaired insulin secretion (Fig. 10).

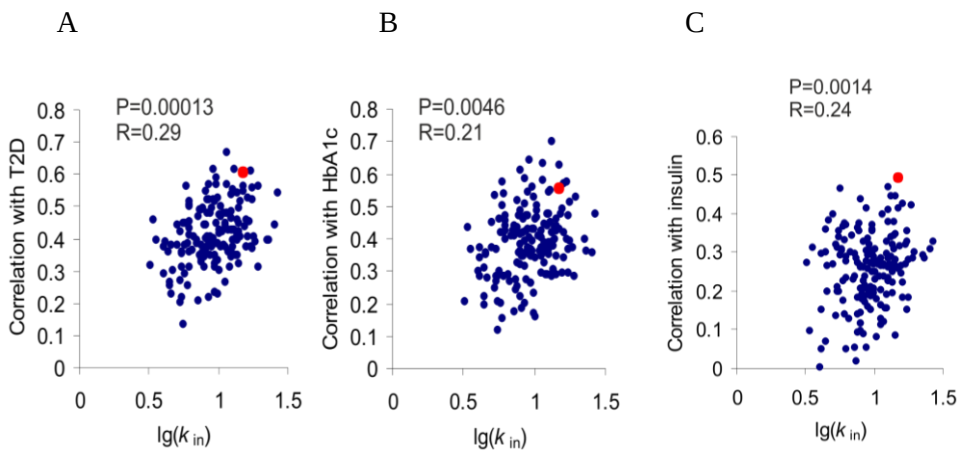

Fig.10 (A-C) For each of the 174 genes in the T2D module the absolute value of the correlation of the gene expression trait and diabetes status (A), HbA1c (B), or glucose-stimulated insulin secretion (C) is displayed against the logarithm of k_{in} for the gene. Red dots indicate SFRP4. P- and r-values for the correlation between k_{in} and gene expression association with the diabetes traits were calculated by Spearman's rank correlation.

Hub genes are genes that connect larger parts of the network and have been suggested as putative key genes in complex diseases (Casci 2006). SFRP4 is one of the hub genes in the T2D-related module. We studied the effects of SFRP4 on insulin secretion by in vitro experiment. These showed that SFRP4 causes decreased insulin secretion and insulin exocytosis in mouse and human islets when incubated for 24h in 30 nM SFRP4. The peptide had no effect on insulin content or β-cell viability.

We used mouse and INS 832 13 to knock down SFRP4 by siRNA. Interestingly, Sfrp4 silencing leads to increase in exocytosis and integrated ca current in mouse islet while in INS 832 12 there was also increase in insulin secretion. This data support that SFRP4 is secreted from β-cells.

The negative effect of SFRP4 on β-cells was due to downregulation of L-type and P/Q Ca^{2+}-channels and decreased integrated Ca^{2+} current as measured by immunostaining and the patch clamp technique. Loss of inhibitory action of Isradipine and agatoxin further supported these findings. The finding was also supported by decreased intracellular calcium concentration by ratiometric measurements with Fura-2. Furthermore electrical activity in mouse islets was measured using current-clamp technique. In SFRP4-treated islets, there was a tendency for lower action potential amplitude and frequency than in control islets. The inhibitory effect of SFRP4 in the present of Tolbutamine and high potassium in mouse islet indicate that SFRP4 act beyond K_{ATP}-channel closure and membrane depolarization.

Interestingly, SFRP4 act on the Wnt signaling pathway. Several T2D-associated variants locate near genes in the Wnt pathway (Saxena, Voight et al. 2007; Sladek, Rocheleau et al. 2007; Zeggini, Scott et al. 2008; Voight, Scott et al. 2010).

This study also showed that SFRP4 acts on Gi/o-coupled receptors as evident by the loss of inhibitory effect in the presence of pertussis toxin. Moreover, SFRP4 increased the level of unphosphorylated beta catenin and activated TCF/LEF reporter constructs. Taken together, this indicates that SFRP4 activates canonical Wnt signaling. This is in agreement with that TCF/LEF activation causes decreased Ca^{2+}-channel expression (Wisniewska, Misztal et al. 2010). More importantly, the inhibitory effect of SFRP4 on β-cell exocytosis was not influenced by cotreatment with canonical (Carmon and Loose 2008) or noncanonical Wnt proteins (Ma and Wang 2007).

In this study we also try to find the effector of SFRP4 secretion in T2D. Since the diabetes-associated module is enriched for inflammatory factors, we tested the effect of IL-1β on SFRP4 release. Incubation of human islets with IL-1β led to increased secretion of SFRP4 into the medium. This is further validated on the

mRNA level by Taqman real-time PCR. The inhibitory effect of IL-1β on insulin secretion was decreased by knock down of SFRP4 in INS 832 13.

SFRP4 was present in both α-cell and β- cells. It is not cosecreted with insulin. It is present at higher concentration in islets than in serum and is released in a constitutional way. This is due to C-terminal netrin-like domain, which binds heparin and heparan sulfate proteoglycans in the extracellular matrix and facilitates the accumulation of SFRPs at high local concentrations at the site of secretion (Salic, Kroll et al. 1997; Bafico, Gazit et al. 1999; Üren, Reichsman et al. 2000).

Importantly, we injected mice with SFRP4 to study the effect of SFRP4 on glucose stimulated insulin secretion in vivo. The peptide caused glucose intolerance and reduced insulin secretion (Fig. 11).

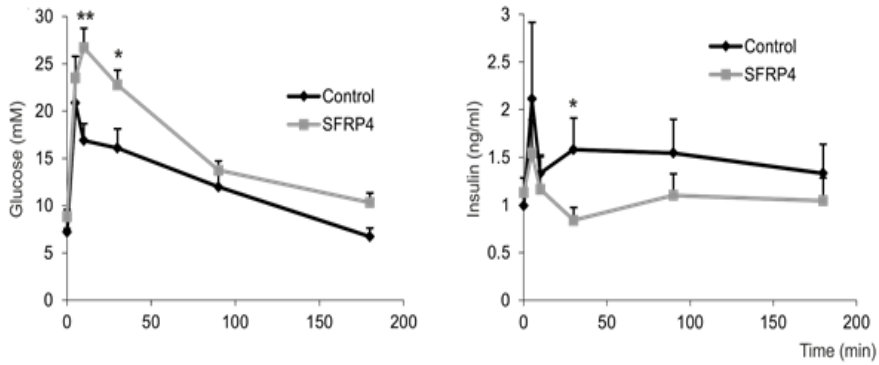

Fig. 11 Glucose and insulin levels during an IVGTT. PBS or SFRP4 were injected 24, 16 and 8 hr before the IVGTT.

Finally we investigated human serum for SFRP4 concentration. First, we investigated serum level of this peptide between diabetic and non-diabetic donors. Individuals with diabetes had higher SFRP4 concentration than non-diabetics (ND). People with serum concentration above the median are more prone to become diabetic (converters) (Fig. 12). We also investigated SFRP4 in association

with diabetes traits. SFRP4 was associated with higher fasting glucose, reduced insulin sensitivity index, and lower disposition index. Serum concentration was not affected by BMI, sex, patient age, or sample age.

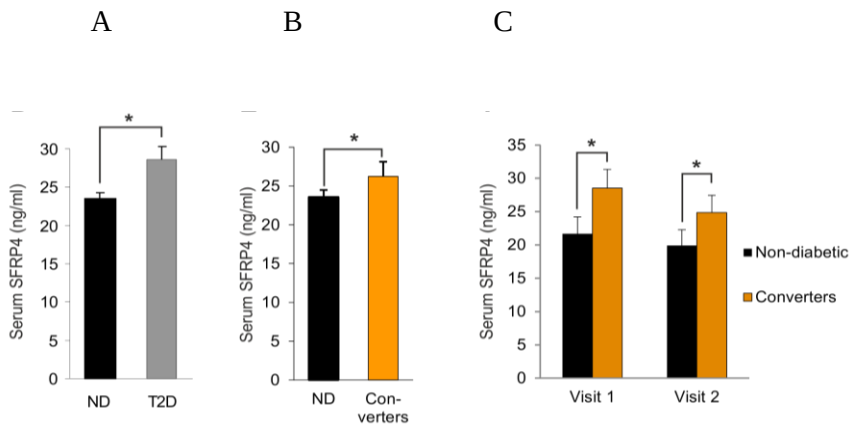

Fig. 12 (A) Serum concentration of SFRP4 in individuals who remained non-diabetic at all visits and subjects with T2D at all visits. (B) Serum SFRP4 measured at visit 1 and 2 in non-diabetic individuals who later developed T2D compared with subjects who remained non-diabetic (ND; p-value from one-sided comparisons corrected for age, sex and BMI). (C) Serum SFRP4 measured in individuals who were non-diabetic at both visit 1 and 2 and converters who were non-diabetic at visit 1 and diagnosed with T2D at visit 2.

Paper III

The aim of this paper was to use bioinformatics and cell-physiology to provide new information on human pathophysiology. Here we have combined genotype and gene expression data to study the pathophsiology of type 2 diabetes.In this study we analysed global gene expression from 64 human islets microarray data by Weighted Gene Co-expression Network analysis (Zhang and Horvath 2005). As a result of gene interconnectedness, we identified a module with 2246 genes from which the module eigengene had altered expression in islets from type 2 donors and was correlated with HbA1c and reduced insulin secretion.

Interestingly in this module 168 genes had open chromatin specifically in islets (Gaulton, Nammo et al. 2010). Moreover, most of these genes had lower expression in islets of T2D donors which indicated that these genes are important for mature islet cells. This is in line with previous data that islet cells dedifferentiation has been suggested to occur in T2D (Talchai, Xuan et al. 2012).

Next, we have also shown that SRY (sex-determining region Y) box 5 (Sox 5) is an important transcription factor for 133 of the 168 genes. Sox-5 is contained in the T2D associated module but is not among the genes with islet specific open chromatin. We also detected that expression of Sox-5 correlated with insulin secretion and associated with expression of PDX1. Later, we have used rat islets to study the effect of Sox-5 on dedifferentiated β-cells. After 48 hours incubation at high glucose and palmitate there was reduced expression of sox 5(Fig. 13).

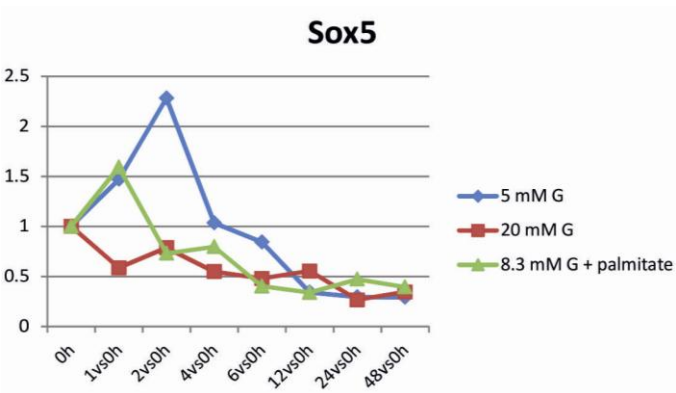

Fig. 13 Relative mRNA abundance of Sox5 in rat islets cultured under conditions as specified. Data at each time point are given relative to the level at the start of the incubation period (0h).

This reduction was also associated with reduced expression of β-cell transcription factors like Mafa and PDX1. By contrast, Ldha expression was increased. This spports the previous data that dedifferentiation of β-cell has been suggested to involve elevated expression of Ldha and reduved expression of insulin, Mafa, and PDX1(Weir and Bonner-Weir 2004). By contrast Ldha expression was increased.

We also used INS 832/13 cells for silencing of Sox 5 by siRNA and we found that Sox5-silenced cells have reduced insulin secretion compared to the control. Reduced insulin secretion in the presence of tolbutamide and high K^+ suggest that the secretory defect of Sox5-silenced cells occur at a late stage of stimulus secretion coupling, after membrane depolarization (Fig. 14).

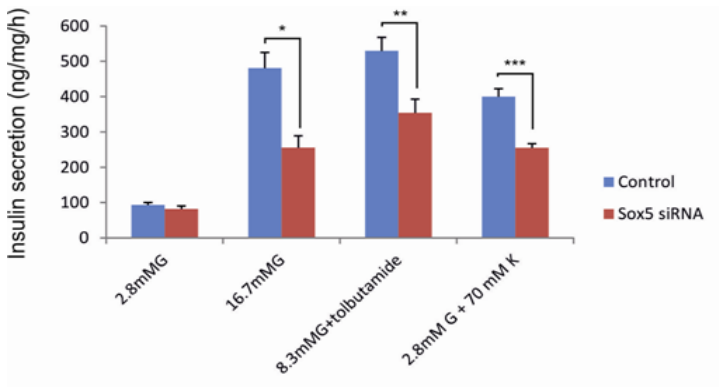

Fig. 14. Insulin secretion in INS832/13 cells incubated for 1 h under conditions as specified. Data are presented from control cells and cells treated with Sox5 siRNA.

Finally we have found that both insulin exocytosis and integrated Ca^{2+} current to the first two depolarizations (which correspond to the first phase of insulin secretion) were reduced in Sox5-silenced cells. This is of interest, since first phase of insulin secretion has been suggested to be impaired in T2D (Del Prato 2003).

Conclusions

- ✓ Genetic variants for type 2 diabetes near TCF7L2 and ADRA2A were associated with reduced glucose-induced insulin secretion, and susceptibility variants near ADRA2A, KCNJ11, KCNQ1, and TCF7L2 were associated with reduced depolarization-evoked insulin exocytosis. We combined our results to create a novel genetic risk score for β-cell dysfunction that includes aberrant granule docking, decreased Ca^{2+} sensitivity of exocytosis, and reduced insulin release. Individuals with a high risk score displayed an impaired response to intravenous glucose and deteriorating insulin secretion over time.

- ✓ We explored the pathophysiology of type 2 diabetes by analyzing global gene expression in human pancreatic islets. A group of coexpressed genes (module), enriched for interleukin-1-related genes, was associated with type 2 diabetes and reduced insulin secretion. One of the module genes that was highly overexpressed in islets from type 2 diabetes patients is SFRP4, which encodes secreted frizzled-related protein 4. SFRP4 expression correlated with inflammatory markers, and its release from islets was stimulated by interleukin-1β. Elevated systemic SFRP4 caused reduced glucose tolerance through decreased islet expression of Ca^{2+} channels and suppressed insulin exocytosis. Moreover, the protein was increased in serum from diabetic patients several years before the diagnosis, suggesting that SFRP4 could be a potential biomarker for islet dysfunction in type 2 diabetes.

- ✓ We have identified a gene co-expression module in human pancreatic islets that is enriched for genes with islet-specific open chromatin. In individuals with type 2 diabetes this module displays an expression pattern that is reminiscent of a β-cell dedifferentiation profile. The transcription factor Sox5 has putative binding sites to several of the module genes. Sox5 expression was reduced in type 2 diabetes. We found that Sox5 silencing resulted in decreased expression of key β-cell transcription factors and impaired glucose-induced insulin secretion due to a late-stage defect in β-cell exocytosis. The findings suggest that Sox5

may have a key role in the b-cell failure that is typically seen in type 2 diabetes.

Populärvetenskaplig Sammanfattning

Diabetes, i dagligt tal även kallat sockersjuka, kännetecknas av konstant förhöjt blodsocker. Sjukdomen ökar lavinartat, och har beskrivits som en av det moderna samhällets stora epidemier. Diabetes förekommer i två huvudsakliga former, typ 1 och typ 2. I typ 1-diabetes, som framför allt drabbar barn och ungdomar, har de insulinproducerande beta-cellerna förstörts av kroppens immunförsvar. Patienter med typ 1-diabetes måste därför behandlas med insulin livet ut. Beta-cellerna är förstörda även i typ 2-diabetes men till mindre grad än i typ 1. Däremot finns en samtidig störning i cellernas funktion som leder till försämrad insulinfrisättning efter födointag. Typ 2-diabetiker har ofta också så kallad insulinresistens. Insulinresistens innebär att insulinet får minskade effekter ute i vävnader där det behövs, dvs fettväv, lever och muskler. Resultatet av alla dessa komponenter är att blodsockret blir kroniskt förhöjt, vilket kan leda till svåra komplikationer i njurar, ögon och hjärt-kärlsystemet. Typ 2-diabetes drabbar framför allt individer med felaktiga kostvanor och bristande motion. Denna form av sjukdomen är den absolut vanligaste.

I denna avhandling har jag studerat sjukdomsmekanismer bakom defekt insulinsekretion vid typ 2-diabetes. Vi har använt en kombination av klassisk genetik, bioinformatik med nätverksanalyser och cellfysiologiska metoder.

I det första arbetet fann vi att riskvarianter för typ 2-diabetes i generna TCF7L2 och ADRA2A var associerade med minska insulinfrisättning. Vi fann också att varianter i generna ADRA2A, KCNJ11, KCNQ1 och TCF7L2 var kopplade till minskad insulinexocytos från beta-cellerna. Vi kombinerade våra fynd till en genetisk riskskala. Individer med hög risk hade minskad insulinfrisättning och ökad risk för typ 2-diabetes. Riskskalan skulle kunna användas för att identifiera undergrupper av diabetiker med försämrad insulinfrisättning. Dessa skulle då kunna dra nytta av behandling som specifikt riktar in sig mot att öka insulinfrisättningen.

I det andra arbete använda vi nätverksanalyser för att få en mer integrerad bild av hur genuttrycket är förändrat i beta-celler vid typ 2-diabetes. Vi identifierade en grupp av gener som hade ändrat uttryck vid typ 2-diabetes. En av de mest centrala generna i detta nätverk var SFRP4. SFRP4-uttrycket var ökat i typ 2-diabetiska öar. Dessutom fann vi att inflammation ökade frisättningen av SFRP4. Proteinet gav minskad insulinfrisättning både i cellkultur och då det injicerades i möss. Vi

såg också att SFRP4 var ökat i serum från typ 2-diabetiker flera år före diagnos. SFRP4 är den första molekylära länken mellan inflammation och typ 2-diabetes, och skulle även kunna vara en biomarkör för beta-celldysfunktion vid typ 2-diabetes.

I det tredje arbetet fann vi en annan grupp av gener vars uttryck var ändrade vid typ 2-diabetes. Dessa gener är viktiga för att bibehålla mognaden av beta-celler. Vi identifierade viktiga transkriptionsfaktorer för att hålla beta-cellerna i ett välfungerande stadium och såg att rubbning av dessa gener kan bidra till försämrad insulinfrisättning.

Sammanfattningsvis har avhandlingen visat på flera nya sjukdomsmekanismer vid typ 2-diabetes som kan ha potentiell klinisk nytta.

Acknowledgements

I raise my heart in gratitude to **Allah** for all the blessings He has showered on me throughout this study. He has been the guiding force behind all my efforts.

First of all, I would like to thank my supervisor **Anders Rosengren** for his unlimited support, constant guidance and unbelievable patience. For his great knowledge in diabetes that leading me working on diverse exciting projects and for helping me in completing this thesis.

Anders, I am very thankful for all the time you have spent discussing projects and manuscript drafts. You are the most hard working and the best energetic supervisor. Thank you for all your help and availability at all times.

My profound thanks are to my co-supervisor **Erik Renström** for involving me in the world of diabetes research, for his constructive advices and for all your intellectual and practical help.

Claes Wollheim for being always willing to share his enormous knowledge in diabetes, for presenting to me a new dimension in diabetes research and your belief in me as a scientist. You are my primary resource for getting my science questions answered.

Lena Elliasson and **Albert Salehi** for insightful discussions about the research, experimental expertise and successful collaboration. Thank you for all help.

Leif Groop, thank you for your devotion to and interest in my work and for your valuable contributions to the published work in this thesis. It has been a pleasure to collaborate with you.

I have great pleasure in expressing my hearty thanks to Anna Maria and Britt Marie for their kind help in fulfilling my lab needs and supplies and for all the help you have given me in countless experiments during the last four years. No research possible without your expert support.

I want to express my gratitude to **Dr. Saleem Saed Qader** for introducing me into science and for still showing me strong support, whose support, stimulating suggestions and good will kept me going through my study time.

I would like to give my special thanks to **Jonathan Esquerra** for sharing your stern opinions and your excellent statistical advice concerning qPCR.

I am particularly grateful to **Annika**, **Yunzhao**, **Thomas, Hannah**, **Marwa** and **Mojgan** for sincere friendship, for providing a very friendly working group. It was really nice to have you in my group.

I express my heartfelt gratefulness to **Enming Zhang**, a wonderful person with great lab skills ,for his guide and support in confocal reading that I believed I learned from the best.

Thank you **Vini** for being my roommate and for having a smile for everyone. You have been helpful in providing advice many times during my study. You are one of the best my friend and I am proud to call myself your friend. Thanks for all your help and never getting tired of listening to my complains.

I would also like to thank **Anna Edlund**, she was and remains my best role model for a scientist. I have been patching next to you for most of the time.

I thank all the lovely past and present LER group members: **Pawel Budda**, **Anna Wendt, Ulrika Krus**, **Sofia**, **Yang De Marinis**, **Arvind Soni**, **Thomas T**., **Ines Mollet**, **Helena Malm**, **Vishal**, **Cheng** and **Jones**. I want to thank you all for creating a great working- and family-like atmosphere, for all the fun we have had in the last four years. I am realy happy that I have met you all.

A major research like this is never the work of any one alone, I would like to extend my thanks all my coauthors in sweden, Seatle, China, Swisland, Barcelona and oxford.

I would like to thank my family: first and foremost my parents **Mamosta Mahdi** and **St. Ramsia** for giving birth to me and supporting me spiritually throughout my life. My mother, for always being on my side and she always telling me that work is not the only important thing in life. She taught me that even the largest task can be accomplished if it is done one step at a time. Thank you for all prayers for me. My father who has always been proud of me. My biggest regret is that he is not with me to share this joy…… He is always in my mind. I miss you so much .My brothers; **Raber** and **Rebwer** for their love and deserve my whole hearted thanks as well. My beloved sister **Tara** with her husband **Handren** for being supportive throughout my time here. I am looking forward to being able to spend more time with you soon.

I would like to thank my beloved parents-in-law **Jabar** and **Kstan** who believed in me and supported me since the beginning of my study. Also special and big thank to my brothers and sisters -in-law for their continuous emotional support and endless love.

I will forever be thankful to my dear husband **Sarheed** who supported me each step of the way. For never complaining about my hard working or my many travels without you.Thank you for giving me strength to reach for the star and get my dream. Thank you for understanding and encouragement in my many many moments of crisis. This thesis would be incomplete and might not have been written without his helping with proofreading and graphics. My lovely son **Sardam** for being patient considerate while I spent most of my time on the work. I love you.

I also wish to sincerely thank all my Kurdish friends making these years much more interesting and complete.

Finally, special thanks should be given to **Kurdistan Regional Government (KRG)** for supporting and funding during my study period.

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