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ORIGINAL ARTICLE

Identification of novel genes for glucose metabolism based upon expression pattern in human islets and effect on insulin secretion and glycemia

Jalal Taneera^{1,*}, Joao Fadista¹, Emma Ahlqvist¹, David Atac¹, Emilia Ottosson-Laakso¹, Claes B. Wollheim^{1,2}, and Leif Groop¹

¹Department of Clinical Sciences, Diabetes and Endocrinology, Lund University Diabetes Centre, Skåne University Hospital, Lund University, 205 02 Malmö, Sweden, and ²Department of Cell Physiology and Metabolism, Université de Genève, University Medical Centre, 1 rue Michel-Servet, Geneva 4 1211, Switzerland

*To whom correspondence should be addressed at: Department of Clinical Science, Diabetes and Endocrinology, Lund University Diabetes Centre, Lund University, 205 02 Malmö, Sweden. Email: jalal.taneera@med.lu.se

Abstract

Normal glucose homeostasis is characterized by appropriate insulin secretion and low HbA1c. Gene expression signatures associated with these two phenotypes could be essential for islet function and pathophysiology of type 2 diabetes (T2D). Herein, we employed a novel approach to identify candidate genes involved in T2D by correlating islet microarray gene expression data (78 donors) with insulin secretion and HbA1c level. The expression of 649 genes ($P < 0.05$) was correlated with insulin secretion and HbA1c. Of them, five genes (GLR1A, PPP1R1A, PLCXD3, FAM105A and ENO2) correlated positively with insulin secretion/negatively with HbA1c and one gene (GNG5) correlated negatively with insulin secretion/positively with HbA1c were followed up. The five positively correlated genes have lower expression levels in diabetic islets, whereas GNG5 expression is higher. Exposure of human islets to high glucose for 24 h resulted in up-regulation of GNG5 and PPP1R1A expression, whereas the expression of ENO2 and GLR1A was down-regulated. No effect was seen on the expression of FAM105A and PLCXD3. siRNA silencing in INS-1 832/13 cells showed reduction in insulin secretion for PPP1R1A, PLXCD3, ENO2, FAM105A and GNG5 but not GLR1A. Although no SNP in these gene loci passed the genome-wide significance for association with T2D in DIAGRAM+ database, four SNPs influenced gene expression in cis in human islets. In conclusion, we identified and confirmed PPP1R1A, FAM105A, ENO2, PLCXD3 and GNG5 as potential regulators of islet function. We provide a list of candidate genes as a resource for exploring their role in the pathogenesis of T2D.

Introduction

Diabetes is a globally expanding health issue, causing a great burden on healthcare in both developing and industrialized countries (1). The incidence of type 2 diabetes (T2D) is increasing globally and expected to affect >550 million people by 2030 (www.idf.org/diabetesatlas), of which T2D accounts for >90% of cases. T2D is characterized by increased blood glucose owing to defects in insulin secretion from the pancreatic beta cells and impaired insulin action.

The pathogenesis of T2D is still not fully understood. It is well established that T2D develops from an interaction between genetic susceptibility and a westernized lifestyle characterized by increasing obesity. In the past 10 years, several genome-wide association studies (GWAS) have discovered >100 genetic variants associated with T2D or blood glucose/insulin secretion (2–12). Despite this large number, these variants can only explain 10–15% of the heritability of the disease (13,14). One main reason is that the effect size of each individual variant is very modest.

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The key question is therefore whether more variants (close to 1000) would be able to explain the full genetic background of T2D or whether we need more holistic approaches combining DNA, RNA and protein information (15).

Diabetes is defined as a condition of chronic hyperglycemia, which can be monitored by measuring glycated hemoglobin A1c (HbA1c) (www.who.int/diabetes/publications/report-hba1c_2011). In untreated individuals, one would expect elevated HbA1c to be associated with impaired insulin secretion and vice versa, i.e. a low HbA1c value in normoglycemic individuals with normal or high insulin secretion.

Herein, we introduced a novel approach by correlating islet gene expression with insulin secretion and HbA1c levels in islets from 78 organ donors. Five genes correlated positively with insulin secretion/negatively with HbA1c (GLR1A, PPP1R1A, PLCDXD3, FAM105A and ENO2), whereas one gene (GNG5) correlated negatively with insulin secretion and positively with HbA1c. We then followed up these findings by siRNA silencing of the genes in an INS-1 cell line. We also analyzed whether SNPs in these genetic loci are associated with T2D in the DIAGRAM+ database or could influence gene expression in cis expression quantitative trait loci (eQTLs).

Results

Correlation between gene expression and insulin secretion and HbA1c

In this study, we utilized human pancreatic islet microarray expression data (GeneChip® Human Gene 1.0 ST) obtained from 78 donors (68 non-diabetic and 10 diabetic, see Materials and Methods). Each donor was characterized by measuring insulin response to glucose in the islet preparation and glycemic control (HbA1c) from the same individual. Spearman's test was used to perform a global correlation of gene expression (adjusted for age and sex) with insulin secretion and HbA1c in the same 78 donors (each array has ~22 000 genes) (Fig. 1). The expression of 649 genes (P -value < 0.05) showed significant correlation with insulin secretion and HbA1c levels (Fig. 1B). Of these, 518 genes were positively correlated with insulin secretion and negatively with HbA1c, 128 genes were negatively correlated with insulin secretion and positively with HbA1c, 2 genes were positively correlated both with HbA1c and insulin secretion and only 1 gene negatively correlated with HbA1c and insulin secretion (Fig. 1B, Supplementary Material, Table S1).

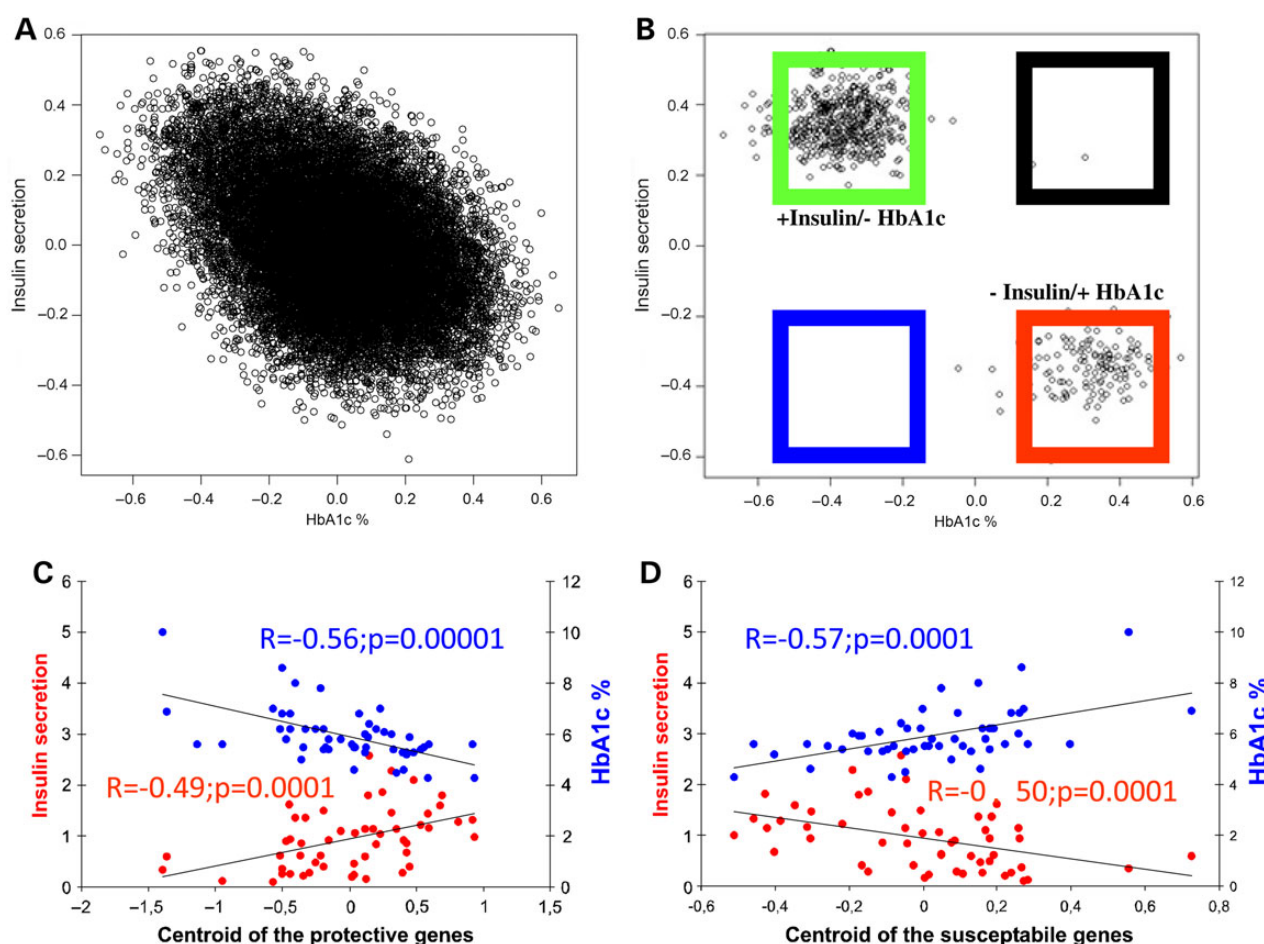


Figure 1. Spearman correlation of the expression of 22 000 genes on the chip against insulin secretion and HbA1c levels in human islets (A). (B) In total, 649 genes showed a significant correlation with insulin and HbA1c (P -value < 0.05). Of these, 518 genes correlated positively with insulin secretion and negatively with HbA1c (green square), 128 genes correlated negatively with insulin secretion and positively with HbA1c (red square), 2 genes correlated positively both with insulin secretion and HbA1c (black square), and one gene correlated negatively with insulin secretion and HbA1c (blue square). The X-axis and Y-axis scales represent the correlation coefficient. FDR and Bonferroni thresholds for each correlation are shown in Supplementary Material, Table S1. (C) Correlation of mean centroid of the 11 positively correlated genes to insulin secretion and HbA1c level. (D) Correlation of mean centroid of the 10 negatively correlated genes to insulin secretion and HbA1c levels.

Table 1. Ten top-ranked genes ordered by the lowest sum of P-value for the correlation with HbA1c and insulin secretion

Gene	Name	p-insulin	p-HbA1c
A: genes correlated positively with insulin secretion and negatively with HbA1c			
GLRA1	Glycine receptor, alpha 1	0.0006	0.00001
ATP2A3	ATPase, Ca++ transporting, ubiquitous	0.0001	0.00004
MCF2L2	MCF.2 cell line-derived transforming sequence-like2	0.0020	0.00000
PTEN	Phosphatase and tensin homolog	0.0025	0.00000
PPP1R1A	Protein phosphatase 1, regulatory (inhibitor) subunit 1A	0.0022	0.00002
SYT13	Synaptotagmin XIII	0.0012	0.00011
WSCD2	WSC domain containing 2	0.0010	0.00016
TAGLN3	Transgelin 3	0.0017	0.00009
PLCXD3	Phosphatidylinositol-specific phospholipase C, X domain	0.0025	0.00004
ENO2	Endolase 2 (gamma, neuronal)	0.0011	0.00022
FAM105A	Family with sequence similarity 105, member A	0.0043	0.000004
B: genes correlated negatively with insulin secretion and positively with HbA1c.			
B3GALNT2	Beta-1,3-N-Acetylgalactosaminyltransferase 2	0.00268	0.00206
KCNH8	Potassium Voltage-Gated Channel, Subfamily H, Member 8	0.00010	0.00543
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog	0.00671	0.00379
IL1R2	Interleukin 1 Receptor, Type II.	0.01557	0.00020
RPGR	Retinitis Pigmentosa GTPase Regulator	0.00442	0.00861
KLF6	Kruppel-Like Factor 6	0.01621	0.00173
CCL13	Chemokine (C-C Motif) Ligand 13	0.00323	0.01161
SLC16A7	Solute Carrier Family 16, Member 7	0.00738	0.00887
ATP4A	ATPase, H+/K+ Exchanging, Alpha Polypeptide	0.01333	0.00388
GNG5	Guanine Nucleotide Binding Protein (G Protein), Gamma 5	0.02021	0.00084

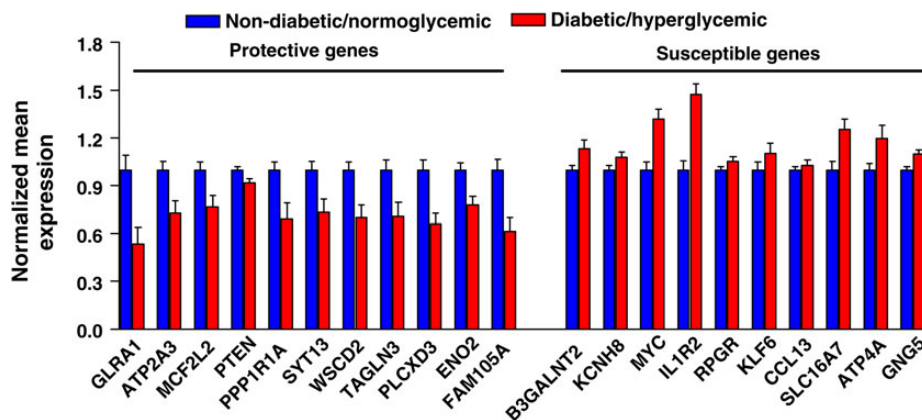


Figure 2. Fold change differences in islet expression of the top correlated genes between diabetic/hyperglycemic and nondiabetic/normoglycemic donors. The expression of the positive correlated genes GLRA1 ($P = 0.001$), ATP2A3 ($P = 0.002$), MCF2L2 ($P = 0.003$), PTEN ($P = 0.007$), PPP1R1A ($P = 0.0002$), SYT13 ($P = 0.001$), WSCD2 ($P = 0.003$), TAGLN3 ($P = 0.003$), PLCXD3 ($P = 0.0001$), ENO2 ($P = 0.003$) and FAM105A ($P = 0.0003$) was lower in diabetic/hyperglycemic ($n = 23$) donors compared with nondiabetic/normoglycemic ($n = 43$). The expression of the negative correlated genes B3GALNT2 ($P = 0.047$), MYC ($P = 0.001$), IL1R2 ($P = 0.00002$), SLC16A7 ($P = 0.01$) and GNG5 ($P = 0.007$) was lower in diabetic/hyperglycemic donors compared with nondiabetic/normoglycemic, whereas no change was seen in the expression of KCNH8, RPGR, KLF6, CCL13 and ATP4A. Bars represent mean \pm SEM.

Ranking of positively and negatively correlated genes

We ranked the 518 genes showing positive correlation with insulin and negative correlation with HbA1c (+insulin/−HbA1c) and the 128 genes showing negative correlation with insulin and positive with HbA1c (insulin/+HbA1c) based upon the sum of their P-value correlation with insulin and HbA1c levels, given a rank from 1 to 518 or 128, respectively; the lowest ranking sum represents the top-ranked genes. We selected the 10 top-ranked genes from each group for further functional analyses.

The top 10 ranked genes from the positively correlated group were GLRA1, ATP2A3, MCF2L2, PTEN, PPP1R1A, SYT13, WSCD2, TAGLN3, PLCXD3 and ENO2 (Table 1A). To ensure that the 10 selected genes are indeed expressed in β -cells, the genes were

filtered based upon recently published RNA-sequencing expression data from sorted human pancreatic endocrine cells (16). All of them showed higher expression in β -cells compared with α -cells or exocrine cells. Analysis of the expression profile of these genes in the islet expression array revealed a significant down-regulation in diabetic/hyperglycemic donors compared with non-diabetic/normoglycemic (Fig. 2). Plotting expression centroid of the top 10 positively correlated genes against insulin secretion and HbA1c level showed a strong positive correlation with insulin secretion and an inverse correlation with HbA1c (Fig. 1C). Moreover, analysis of the co-expression of these genes with glucagon (GCG) expression (probes for the insulin gene were not on the affy chip) showed a significant positive

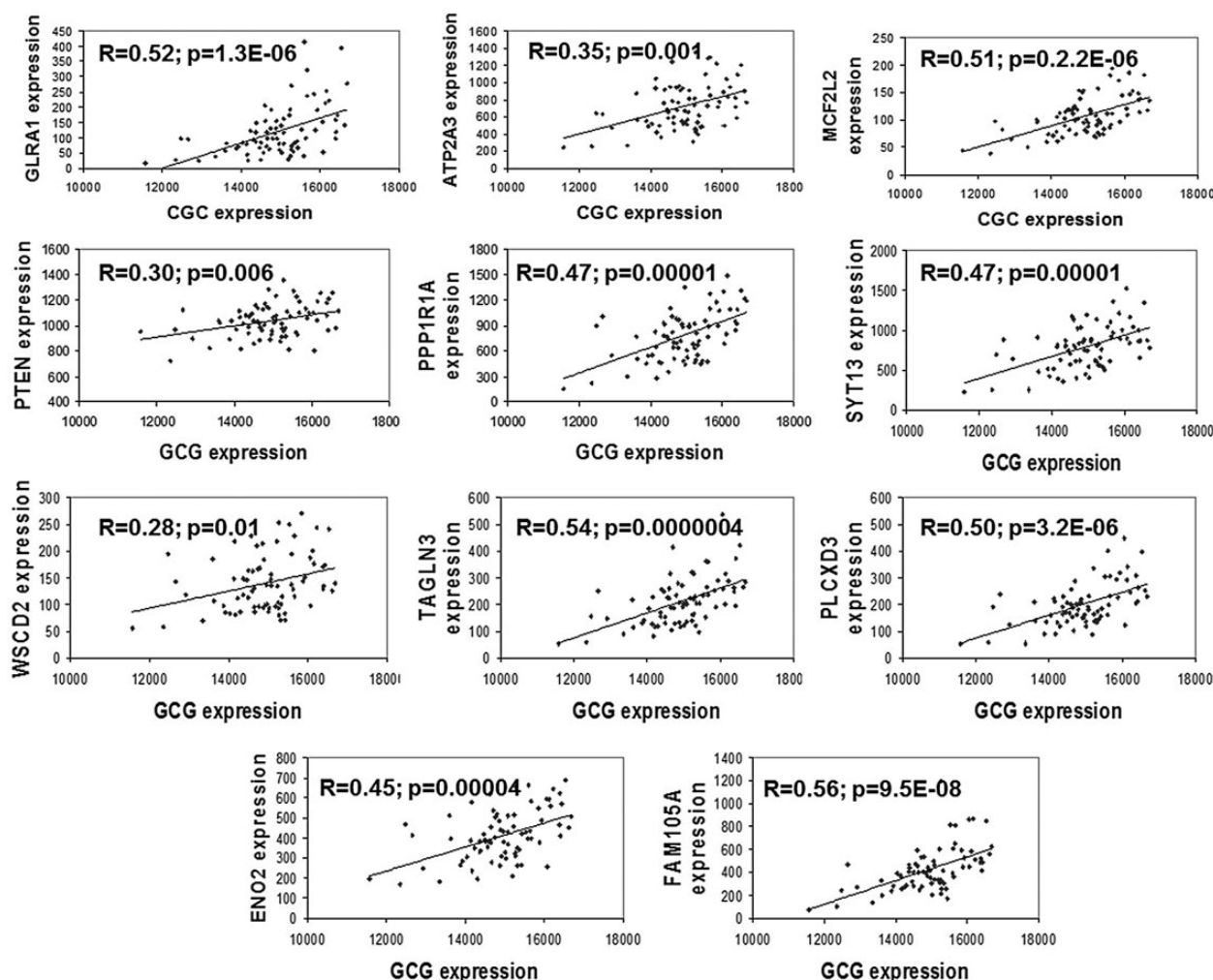


Figure 3. Co-expression analysis of the 11 positively correlated genes against glucagon (GCG) expression in human pancreatic islets. R, correlation coefficient; P, P-value.

correlation with GCG (Fig. 3). Some genes, e.g. ATP2A3 (17–19), PTEN (20,21) and SYT13 (22), have previously been implicated in insulin secretion and were not further examined. FAM105A was ranked as number 12, but because it belongs to the genes with unknown function in islets, we included it for functional follow-up. Hence, of the positively correlated genes, we selected five genes GLRA1, PPP1R1A, PLCXD3, ENO2 and FAM105A for further investigations.

Of the top-ranked negatively correlated genes B3GALNT2, KCNH8, MYC, IL1R1, RPGR, KLF6, CCL13, SLC16A7, ATP4A and GNG5 (Table 1B), the expression of B3GALNT2, MYC, IL1R1, SLC16A7 and GNG5 was higher in diabetic/hyperglycemic compared with non-diabetic/normoglycemic donors (Fig. 2). The expression centroid of the top 10 negative genes showed a strong negative correlation with insulin secretion and positive correlation with HbA1c (Fig. 1D). Moreover, these 10 genes were significantly negatively correlated with glucagon (GCG) expression (Supplementary Material, Fig. S1). KCNH8 expression showed no correlation with GCG. Out of the 10 negatively correlated genes, 8 showed higher expression in pancreatic α -cells compared with β -cells, CCL13 was equally expressed and GNG5 showed higher expression in pancreatic β -cells than that in α -cells (16). Therefore, only GNG5 was selected for functional studies.

Effect of glucose exposure on gene expression

The correlation with high HbA1c can either be the cause or consequence of elevated glucose levels. To address this question, we exposed human pancreatic islets from 51 donors to low (5.5 mM) and high (18.9 mM) glucose concentration for 24 h, assuming that genes whose expression increased after exposure to 24 h of glucose would represent secondary changes but still could mediate glucotoxic effects on islets. Given that these experiments were performed a year later than the microarray experiments, we switched to RNA sequencing for the glucose exposure experiments. Short-term exposure to high glucose showed a significant up-regulation of the expression of GNG5 ($P = 0.0001$) and PPP1R1A ($P = 0.0002$), whereas the expression of ENO2 ($P = 0.002$) and GLRA1 ($P = 0.02$) was down-regulated (Fig. 4). No effect of acute high glucose exposure was seen on the expression of FAM105A ($P = 0.9$) and PLCXD3 ($P = 0.4$) (Fig. 4).

Down-regulation of gene expression in clonal rat β -cells (INS-1)

To explore whether the down-regulation of genes seen in hyperglycemic islets would result in impaired insulin secretion and, in turn, hyperglycemia, we used RNA interference (siRNA) to silence the expression of 6 genes in INS-1 832/13 cells. We used RT-qPCR

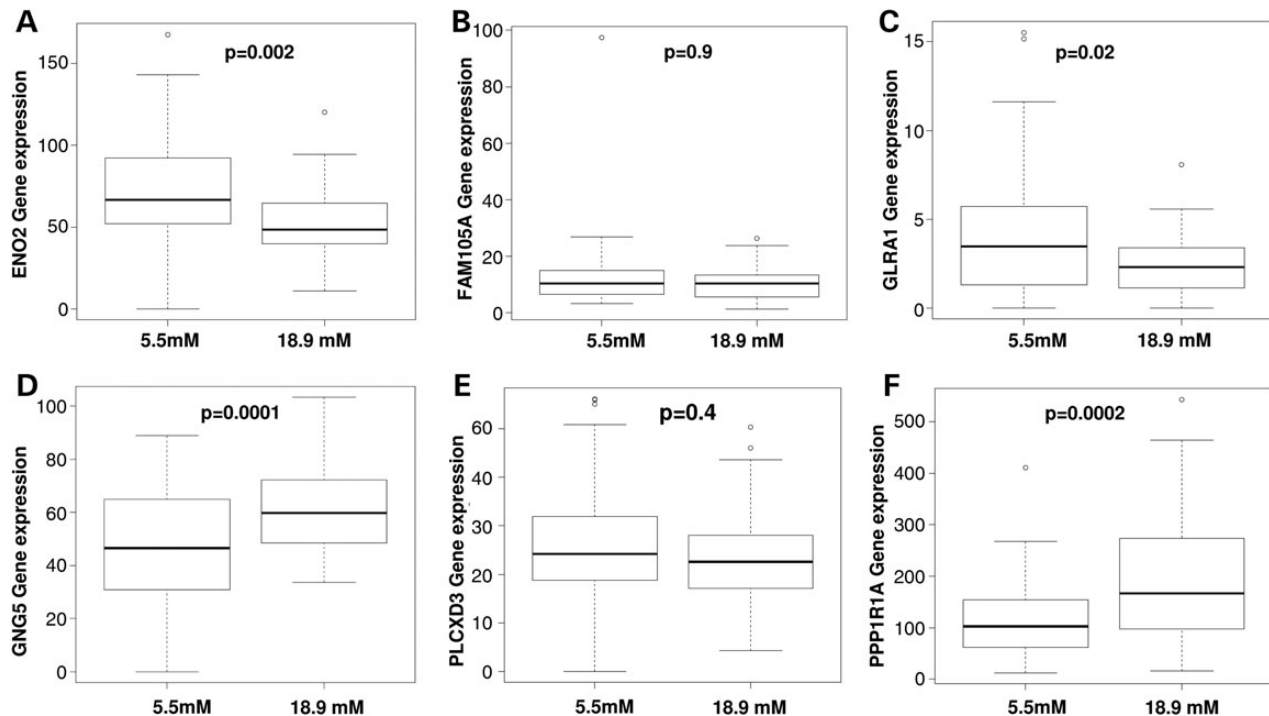


Figure 4. Effect of glucose exposure on gene expression. Human pancreatic islets ($n = 51$) treated with low (5.5 mM) and high (18.9 mM) glucose concentration for 24 h. Analysis of differential expression by RNA-seq showed a similar down-regulation of ENO2 ($P = 0.002$) and GLRA1 ($P = 0.02$) as seen during chronic hyperglycemia. In contrast, the expression of GNG5 ($P = 0.0001$) and PPP1R1A ($P = 0.0002$) was up-regulated by short-term elevated glucose. Glucose exposure had no effect on the expression of FAM105A ($P = 0.9$) and PLCXD3 ($P = 0.4$).

to demonstrate sufficient knock-down efficiency that ranged from 50 to 85% (Fig. 5A). Silencing of PPP1R1A, FAM105A, PLCXD3 ENO2 and GNG5 significantly reduced glucose-stimulated insulin secretion after 1 h of incubation in 16.7 mM glucose, whereas there was no effect of down-regulation of GLRA1 on insulin secretion (Fig. 5B–F).

Association between SNPs in the selected genes and risk of T2D in the DIAGRAM+ database

We also analyzed whether SNPs within a region spanning ± 50 kb up- and down-stream of the six selected genes were associated with T2D in the DIAGRAM+ database. This analysis included 193 SNPs in or around the GLR1A gene, 94 SNPs in PPP1R1A, 114 SNPs in FAM105A, 304 SNPs in PLCXD3, 139 in GNG5 and 28 in ENO2. While none of them as expected would pass the threshold for genome-wide significance, we cannot from the data exclude that any SNP in these genes could be pathogenically involved in T2D (Table 2).

SNP effect on gene expression (eQTLs)

We also analyzed whether SNPs influenced gene expression in cis (within 250 kb of the SNP). For analysis purposes, we identified a single best 'sentinel' SNP for each gene, defined as the SNP with the lowest P-value per eQTL gene. Applying these criteria and thresholds, we identified four cis-eQTLs in four genes. The strongest eQTLs were seen for rs35039612 (ENO2), rs1512906 (PPP1R1A), rs2347598 (GLRA1) and rs26173 (FAM105A) (Fig. 6).

Discussion

In this study, we applied a novel approach to uncover potential candidate genes that contribute to the pathophysiology of T2D.

The material used included a large number of islets from cadaver donors, in which DNA and RNA profiles could be related to glucose-stimulated insulin secretion as well as HbA1c levels. We first tried to identify a gene pattern associated with low HbA1c and normal or high insulin secretion ($n = 518$). To verify the link between insulin secretion and HbA1c, we disrupted the expression of some of the genes showing this pattern. Secondly, we assumed that genes associated with high HbA1c and low insulin secretion could contribute to disease susceptibility ($n = 128$). However, the latter scenario could also be secondary to elevated HbA1c with the so-called glucotoxicity [for review, see Weir and Bonner-Weir (23)]. To explore this possibility, we also exposed human islets to short-term high glucose to identify genes whose expression would change. These gene lists should provide a resource for further studies (see gene description in the Supplementary Material for the top correlated genes). We could only select a small number of genes for follow-up studies (siRNA) and focused on genes with little prior information on their potential involvement in the regulation of insulin secretion. These genes are primarily expressed in β -cells and, therefore, the siRNA experiments were performed in a clonal rat insulinoma cell line.

ATP2A3, PTEN and SYT13 from the positively correlated genes were excluded, as they have previously been implicated in insulin secretion and islet function. ATP2A3 plays a vital role in the sequestration of cytosolic Ca^{2+} into the endoplasmic reticulum (24). Several reports have shown that loss of ATP2A3 or down-regulation of gene expression in β -cells was associated with diabetes in db/db mice (19), Goto-Kakizaki Wistar (GK) rats (18) and humans (17). SYT13 is a member of the synaptotagmin family and involved in β -cell exocytosis (25). Recently, we showed that siRNA silencing of SYT13 expression reduced glucose-stimulated insulin secretion in INS-1 cells, which suggests that SYT13 contributes to impaired insulin secretion (22). PTEN is a phosphoinositide phosphatase

that regulates the PI3K/Akt signaling pathway (20). It was identified as a negative regulator of insulin signaling with a major role in regulating glucose metabolism via the Akt/PKB signaling pathway (21). Additionally, one report has identified three mutations in the PTEN gene in T2D patients, suggesting that the PTEN gene is associated with insulin resistance and T2D (26).

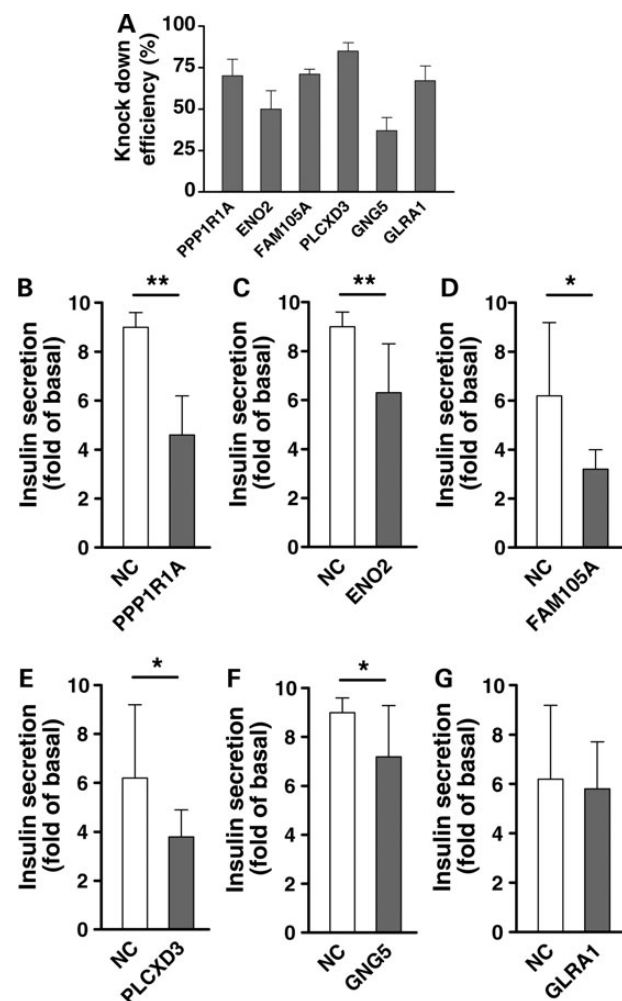


Figure 5. Glucose-stimulated insulin secretion in transfected clonal β-cells. (A) Knock-down efficiency of siRNA of PPP1R1A, ENO2, FAM105A, PLCXD3, GNG5 and GLRA1 in INS-1 cells. (B) siRNA experiments for the above-listed genes show insulin secretion in response to 2.8 mM and 16.7 mM glucose 72 h after transfection as measured during 1 h of static incubation. Secretion was expressed as fold increase (insulin secreted at 16.7 mM/insulin secreted at 2.8 mM glucose) and normalized for protein content (ng/mg/h). Data are shown from three experiments. Bars represent mean ± SD. * <0.05 , ** <0.01 .

PPP1R1A was correlated positively with insulin secretion and negatively with HbA1c. The gene has lower expression in diabetic islets compared with non-diabetic islets (Fig. 2) but was up-regulated by short-term exposure to high glucose (Fig. 4), suggesting that the decreased expression in hyperglycemic islets hardly is a consequence of hyperglycemia. RNA sequencing has also verified higher expression in β-cells than that in α-cells or exocrine cells (16). Knock-down of PPP1R1A resulted in markedly reduced insulin secretion, suggesting that a high expression could enhance insulin metabolism–secretion coupling (Fig. 5). PPP1R1A was previously found as a novel β cell marker with high-molar protein abundance and β cell-restricted expression in human pancreas (27). Moreover, using mass spectrometry of sorter-purified β-cells, PPP1R1A was suggested as a biomarker for acute β-cell destruction (28). PPP1R1A belongs to a family of 200 proteins (encoding inhibitor-1 proteins) that bind to and target the isoforms of protein phosphatase 1 (PP-1) to their substrates in the cell [for review, see Gregorio et al. (29)]. PPP1R1A is itself regulated by protein phosphorylation. Another I-1, R6 (encoded by PPP1R3D) regulates AMP kinase and its knock-down in MIN6 cells attenuated glucose-stimulated insulin secretion by impairing dephosphorylation/inactivation of AMP kinase (30). In contrast, blockade of PP-1 acutely by the nonselective inhibitors okadaic acid or Cal A decreased glucose-induced insulin secretion of pancreatic β-cells owing to inhibition of Ca^{2+} influx and impaired regulation of vesicular fusion of secretory vesicles (31,32). It remains to be demonstrated how long-term alteration of PP-1 activity influences these mechanisms in the β-cell.

ENO2 was down-regulated in hyperglycemic islets and also by exposure to high glucose in vitro (Figs. 2 and 4). Disruption of ENO2 resulted in impaired insulin secretion, suggesting that appropriate expression of ENO2 is required for normal islet function (Fig. 4). ENO2 functions as a neuron-specific enolase (NSE) in neurons, glia and also neuro-endocrine cells (33,34). NSE is a phosphopyruvate hydratase, which is a key enzyme in the conversion of glucose to pyruvate. As glucose enters the cell, it has to be converted to pyruvate for the formation of ATP in the mitochondria to initiate depolarization of the cell by closure of ATP-dependent K^{+} -channels and subsequently increased Ca^{2+} influx by voltage-gated Ca^{2+} -channels (35). This strongly suggests that NSE could regulate glycolysis within the β-cell and insulin secretion. Hence, it is conceivable to hypothesize that decreased expression of ENO2 in pancreatic β-cells may contribute to increased susceptibility to T2D by limiting pyruvate generation, leading to inadequate production of ATP, causing impaired Ca^{2+} influx and attenuated insulin secretion. Further studies are required to elucidate the potential role of ENO2 in β-cell failure and the pathogenesis of hyperglycemia.

FAM105A correlated positively with insulin secretion and negatively with HbA1c. The gene was down-regulated in hyperglycemic islets, but acute increase in glucose concentration had no influence on its expression (Figs. 2 and 4). However,

Table 2. Association between SNPs in the six selected genes and T2D in the DIAGRAM+ database

Gene	SNP	Chromosome	Position	Risk allele	P-value	P-Genome-wide significance
GLRA1	rs170020	5	151 324 751	A	1.10E – 03	2.60E – 04
PPP1R1A	rs1153146	12	53 317 187	C	3.00E – 02	5.40E – 04
FAM105A	rs153822	5	14 680 833	G	1.50E – 02	4.40E – 04
PLCXD3	rs17263168	5	41 320 500	C	3.20E – 03	1.60E – 04
ENO2	rs7955412	12	6 868 632	C	7.10E – 01	3.60E – 04
GNG5	rs6677436	1	84 687 888	T	5.30E – 01	1.80E – 03

SNPs within a region spanning ±50 kb up- and down-stream of the six selected genes were analyzed for association with T2D in the DIAGRAM+ database.

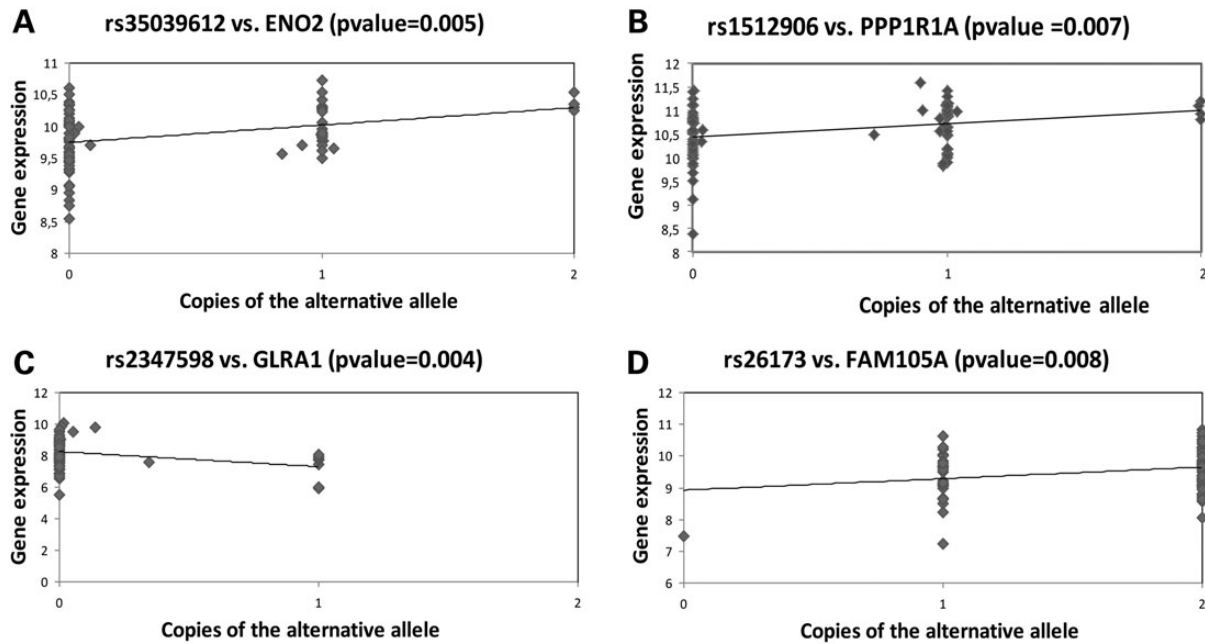


Figure 6. cis-eQTL effects on four of the selected genes. SNP genotyped in human islets were analyzed for influence on gene expression (eQTL) in cis (within 250 kb of the SNP) using array expression data. The strongest eQTLs were seen for rs35039612 (ENO2), rs1512906 (PPP1R1A), rs2347598 (GLRA1) and rs26173 (FAM105A).

knock-down of FAM105A resulted in a clear decrease in insulin secretion suggesting that it could play a pathogenic role in diabetes (Fig. 5). FAM105A has previously been ascribed a role in Amenorrhea (www.genecards.org). A paralog of this gene is FAM105B, which is required for angiogenesis, craniofacial and neuronal development by regulating the canonical Wnt signaling ([genecards.org](http://www.genecards.org)). The cellular mechanisms how FAM105A influences insulin secretion remain to be clarified.

PLCDX3 correlated positively with insulin secretion and inversely with HbA1c and its disruption reduced insulin secretion (Fig. 5). PLCDX3 was down-regulated in islets from hyperglycemic donors, but short-term exposure to high glucose did not influence its expression (Fig. 4).

PLCXDs are a subtype of the phosphatidylinositol-specific phospholipase C (PLCs) protein family. PLCs are receptor-regulated phosphodiesterases that regulate hormones, growth factors and neurotransmitters by generating the calcium-mobilizing messenger inositol-1,4,5-trisphosphate (IP3) and the membrane-bound protein kinase C activator diacylglycerol (DAG) (36–39). Several lines of evidence suggest that PLC plays an important role in the regulation of insulin secretion in the pancreatic β cells. In addition to receptor activation, the influx of calcium has been shown to activate PLC, resulting in exocytosis (40,41). Such positive feedback is important in the regulation of insulin secretion by neurotransmitters (42). Thus, the failure of PLC activation resulted in impairment of insulin secretion (43–45).

GLRA1 is an ion channel that binds glycine to increase chloride conductance. The gene was on top of the list showing positive correlation with insulin and negative with HbA1c. The gene is also down-regulated in hyperglycemic islets and in response to high glucose exposure (Figs. 2 and 4). However, siRNA of the GLRA1 gene did not influence insulin secretion (Fig. 4). In support of this, a recent report by Li *et al.* showed that glycine does not stimulate insulin secretion in human islets but rather increases glucagon release instead (46). Additionally, GLRA1 showed a positive correlation with glucagon (GCG) expression in human islets (Fig. 2). Glycine has been suggested as a biomarker for future

T2D risk, perhaps participating in the hyperglucagonemia of the disease (47,48).

Somewhat surprisingly, the expression of the positively correlated genes (associated with low HbA1c and high insulin secretion) showed a very consistent positive correlation with the glucagon gene, whereas the opposite was seen for the negatively correlated genes. The positive correlation between proglucagon mRNA and low HbA1c (Fig. 3) could point to a protective effect of the incretin hormone GLP-1, which potentiates insulin secretion, protects beta cells from apoptosis and increases cell-to-cell coupling in human islets (49). It has been demonstrated that 20% of human islet alpha cells contain and release GLP-1 in response to stimulatory glucose concentrations (50). GLP-1 production in the islet is rendered possible as this subpopulation, in contrast to the bulk of alpha cells, expresses the prohormone convertase PC1/3, necessary for the processing of the proglucagon peptide to GLP-1. In addition, islet GLP-1 production is increased by exercise, an effect that appears to be mediated by IL-6. Moreover, glucagon itself has been shown to participate in GSIS in human islets, and alpha-beta cell contacts improve insulin secretion caused by glucose (51–53). Future work should focus on the further elucidation of the role of intra-islet GLP-1 in the maintenance of beta cell function.

In contrast, GNG5 expression correlated negatively with insulin secretion and positively with HbA1c, and its expression was up-regulated in diabetic islets (Fig. 2). This could potentially represent a homeostatic adaptation to the hyperglycemic milieu, as knock-down of GNG5 in INS-1 cells resulted in decreased insulin secretion (Fig. 5). This is also supported by the finding of up-regulation of GNG5 in response to high glucose exposure (Fig. 4). GNG5 encodes an isoform of the G_γ subunit, which together with a G_β subunit forms a $G_{\beta\gamma}$ dimer, and couples signals from G protein-coupled receptors to activate adenylyl cyclases or potassium channels (54–56). In β -cells, heterotrimeric G proteins are involved in several regulatory pathways influencing glucose-stimulated insulin secretion, such as through mediation of adrenergic inhibition and effects on insulin granule exocytosis. Which specific role GNG5 would play in β -cells is not known (57,58), but disruption

of GNG5 caused embryonic lethality in mice linked to reduction of cardiac precursor cell numbers (59).

In conclusion, we introduced a novel approach to discover new candidate genes involved in the pathogenesis of T2D. Five genes PPP1R1A, FAM105A, PLCXD3, ENO2 and GNG5 were identified and experimentally validated as potential players in the pathogenesis of islet dysfunction in T2D. We also provide a list of potential genes involved in protection from and susceptibility to T2D based upon correlation between their expression in human pancreatic islets and insulin secretion and glycemia (HbA1c). This gene list should serve as a resource for future studies exploring their potential role in β -cell failure and the pathogenesis of hyperglycemia and T2D.

Materials and Methods

Human pancreatic islets

Islets from cadaver donors (78 donors) were provided by the Nordic Islet Transplantation Program (www.nordicislets.org), Uppsala University. All procedures were approved by the ethics committees at Lund University. Islets were obtained from 68 non-diabetic donors (30 females, 37 males, age 59 ± 10 , BMI 25.9 ± 3.5 , HbA1c 5.5 ± 1.1 and days of culture 3.5 ± 1.9) and 10 T2D donors (4 females, 6 males, age 60.7 ± 12 , BMI 28.1 ± 4.5 , HbA1c 7.1 ± 1.2 and days of culture 2 ± 0.9). Purity of the islet preparations was assessed by diethylenetriamine staining amounted to $60.1 \pm 20\%$ in the T2D and $70 \pm 17\%$ in the non-diabetic islets ($P = 0.10$). Insulin content was measured as a surrogate marker for β -cell mass in pancreatic islets from hyperglycemic (HbA1c $> 6.0\%$) and normoglycemic (HbA1c $< 6.0\%$) donors, which revealed a modest and insignificant decrease in insulin content in islets from hyperglycemic donors (4.8 ± 3.2 versus 5.6 ± 3.2 ng/ml in normoglycemic donors; $P = 0.4$). We also measured the contribution of exocrine and endocrine tissue as previously described (15). The estimated contribution of endocrine tissue showed no difference between non-diabetic and T2D donors (72 versus 68%, respectively, $P = 0.29$). RNA-seq showed higher insulin expression in non-diabetic donors [351 arbitrary unit (AU)] compared with diabetic donors (255 AU; $P < 0.05$). The islets were cultured in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 10 mM/l HEPES, 2 mM/l l-glutamine, 50 μ g/ml gentamicin, 0.25 μ g/ml Fungizone (GIBCO, BRL, Gaithersburg, MD, USA), 20 μ g/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany) and 10 mM/l nicotinamide at 37°C (5% CO₂) prior to RNA preparation.

Microarray gene expression in human pancreatic islets

RNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). RNA quality and concentration were measured using an Agilent 2100 bioanalyzer (Bio-Rad, Hercules, CA, USA) and Nanodrop ND-1000 equipment (NanoDrop Technologies, Wilmington, DE, USA). The microarrays (GeneChip® Human Gene 1.0 ST) were performed using the Affymetrix standard protocol as previously described (15). The array data were summarized and normalized with Robust Multi-array Analysis method using the ligo package from BioConductor. Also, batch correction was done with COMBACT function from SVA package from Bioconductor. All data are MIAME compliant, and the raw data have been deposited in a MIAME database (GEO, accession number: GSE 50398 and GSE 50397).

RNA sequencing

Human islets from 51 donors were divided in two pools of 3000 to 5000 islets and were then incubated in CMRL 1066 medium (ICN

Biomedicals) containing 5.5 mM or 18.9 mM glucose for 24 h. Following treatment, the total RNA including miRNA was extracted using miRNeasy mini kit (Qiagen) and quality was assessed with the 2200 TapeStation (Agilent Technologies). One microgram of total RNA of sufficient quality (RIN > 8) was used for sample preparation for sequencing with a TruSeq RNA sample preparation kit (Illumina). The size selection was done using Agencourt AMPure XP beads (Beckman Coulter) aiming at a fragment size of ~ 300 bp. The resulting libraries were quality controlled on a 2200 TapeStation (Agilent Technologies) before combining six samples into 1 pool for sequencing on 1 lane on a Flow cell sequenced on a HiSeq 2000 (Illumina). Illumina Casava v.1.8.2 software was used for base calling. Paired-end 101-bp-length output reads were aligned to the human reference genome (hg19) with STAR (60), and the number of reads aligned to specific transcripts was counted using HTSeq (61) (Simon Anders, Paul Theodor Pyl, Wolfgang Huber). Raw data were normalized using trimmed mean of M-values and transformed using voom (limma R-package) before linear modeling. The data extracted is part of an unpublished data set.

Genotyping

Genotyping was performed on the Illumina HumanOmniExpress 12v1 C chips, and genotype calling was done with the Illumina Genome studio software. All the samples passed standard genotyping QC (quality control) metrics: sample call rate $> 98\%$.

Glucose-stimulated insulin secretion

Islets were hand-picked under a stereomicroscope and pre-incubated for 30 min at 37°C in Krebs Ringer bicarbonate (KRB) buffer (pH 7.4) containing (in mM) 120 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 1.2 KH₂PO₄, 10 HEPES supplemented with 0.1% bovine serum albumin, N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (10 mmol/l) and 1 mM glucose. Each incubation vial contained 12 islets in 1.0 ml KRB buffer solution and was treated with 95% O₂-5% CO₂ to obtain constant pH and oxygenation. After pre-incubation, the buffer was changed to a KRB buffer containing either 1 mM (basal secretion) or 16.7 mM glucose (stimulated secretion). The islets were then incubated for 1 h at 37°C in a metabolic shaker (30 cycles per min). Immediately after incubation, an aliquot of the medium was removed for analysis of insulin using a radioimmunoassay kit (Euro-Diagnostica, Malmö, Sweden). Insulin content in homogenized human islets was assessed by ELISA (Mercodia, Uppsala, Sweden), and values were normalized to the total DNA in each sample as determined by a fluorometric assay (Quant-iT Picogreen, Invitrogen Molecular Probes, Stockholm, Sweden).

Culturing of INS-1 cell line

INS-1 832/13 is a clonal β -cell line derived from INS-1 rat insulinoma cells containing the human pro-insulin gene (18,19). Cells were cultured at 37°C in humid environment with 95% O₂/5% CO₂ and split 1:4 by using 0.05% trypsin-EDTA (Invitrogen). We used RPMI 1640 culture medium (Gibco) containing 11.1 mM D-glucose, 10% fetal bovine serum (Sigma), 5 ml penicillin/streptomycin (1000 μ /10 mg/ml; Sigma), 5.6 ml Hepes (1 M, Sigma), 2 mM l-glutamine (200 mM, Sigma), 1 mM sodium pyruvate (Sigma) and 50 μ M β -mercaptoethanol (14.3 M, Sigma).

RNA interference and insulin secretion assay

Clonal INS-1 832/13 β -cells were cultured as previously described (15,20) and transfected using a mixture of DharmaFECT® 1

(Dharmacon; Lafayette, CO, USA) and the respective siRNAs. Different sets of siRNA sequences were purchased with siRNA identification numbers ID: s130439 and 130437 (GLRA1), s133049 and s133049 (PPP1R1A), s160229 and 160230 (FAM105A), s160317 and s160319 (PLCXD3), s127701 and s127702 (ENO2), and s135096 and s135097 (GNG5) (Ambion). For control purposes, a previously described control sequence Silencer® Negative Control #2 from Ambion was employed. Cells were cultured in medium for 72 h at 37°C in the presence of 40 nM siRNA in 24-well cell culture microplates. After transfection, insulin secretion measurements were performed. Confluent plates containing transfected INS1-832/13 cells were washed twice with 1 ml pre-warmed Secretion Assay Buffer (SAB), pH 7.2 (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂, 25.5 mM NaHCO₃ and 0.2% bovine serum albumin) containing 2.8 mM glucose. The cells were then pre-incubated for 2 hours in new 2 ml SAB with 2.8 mM glucose. Subsequently, separate wells were incubated for 1 h in 1 ml SAB containing either 2.8 mM or 16.7 mM glucose. Secreted insulin was measured in the supernatant using Coat-a-Count Insulin radioimmunoassay kit (Siemens), and the values were normalized using total protein content individually for each well.

Quantitative RT-qPCR

Total RNA from transfected INS-1 cells was performed 48 h post-transfection using RNeasy Plus Mini Kit 50 (Qiagen). Assessment of quality and quantity of extracted RNA was performed using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Synthesis of cDNA from extracted RNA was performed by using Fermentas RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). Knock-down efficiency was assessed by RT-qPCR of the target genes as described earlier using the following Taqman® gene expression assays (Life Technologies): GLRA1 (Rn00565582_m1), PPP1R1A (Rn00575374_m1), FAM105A (Rn01524442_m1), PLCXD3 (Rn01762608_m1), ENO2 (Rn00595017_m1) and GNG5 (Rn00821205_g1) in triplicate on 384-well plates. qPCR was performed in ABI 7900 platform (Applied Biosystems, Foster City, CA, USA). The rat HPRT1 (Rn01527840_m1) was used to normalize gene expression by $\Delta\Delta C_t$ method, where the final normalized quantity was expressed as $2^{(C_t \text{ target} - C_t \text{ control})}$.

cis-eQTL analysis

The cis-eQTL analysis of the genes GLRA1, PPP1R1A, PLCXD3, FAM105A, ENO2 and GNG5 was performed on islets microarray from 89 individuals. A linear model adjusting for age and sex as implemented in the R Matrix eQTL package (21) was used to compute associations between these gene expression levels and SNPs within 250 kb up- or down-stream of each of these genes.

DIAGRAM database

DIAGRAM (DIAbetes Genetics Replication And Meta-analysis) is a meta-analysis of eight GWASs for T2D (WTCCC, DGI, FUSION, Decode, DGDG, KORAGEN, Rotterdam and EUROSPAN) comprising 8130 T2D cases and 38 987 controls of European descent. Details of the samples and the GWAS platforms used in each scan have been described elsewhere (8). Briefly, analyses of fixed-effects, additive-model for 2 426 886 imputed and genotyped SNPs were combined using the inverse-variance method. Imputation was performed using IMPUTE (<http://mathgen.stats.ox.ac.uk/impute/impute.html>). The replication sample included in silico data from independent three GWASs (ARIC, Nurses' Health Study,

Framingham Heart Study: 2832 cases, 15 843 controls) and *de novo* genotyping (31 580 cases and 44 082 controls) totaling 34 412 cases and 59 925 control subjects of European descent.

Statistical analysis

Differences in expression levels were analyzed by Student's t-test or nonparametric Mann-Whitney test (two tails). Expression correlation with Glucagon (GCG) was analyzed by nonparametric Spearman's test. The Global correlation of gene expression with HbA1c and insulin secretion was done using Spearman's tests in R. All Statistical tests were performed using the Statistical Package for the Social Sciences (SPSS) version 19.0 software (SPSS, Chicago, IL, USA). P-values of <0.05 were considered as significant for all tests.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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