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ARTICLE

A common variant upstream of the *PAX6* gene influences islet function in man

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Abstract
Aims/hypothesis Impaired glucose tolerance and impaired insulin secretion have been reported in families with *PAX6* mutations and it is suggested that they result from defective proinsulin processing due to lack of prohormone convertase 1/3, encoded by *PCSK1*. We investigated whether a common *PAX6* variant would mimic these findings and explored in detail its effect on islet function in man.
Methods A *PAX6* candidate single nucleotide polymorphism (rs685428) was associated with fasting insulin levels in the Diabetes Genetics Initiative genome-wide association study. We explored its potential association with glucose tolerance and insulin processing and secretion in three Scandinavian cohorts (N=8,897 individuals). In addition, insulin secretion and the expression of *PAX6* and transcriptional target genes were studied in human pancreatic islets.
Results rs685428 G allele carriers had lower islet mRNA expression of *PAX6* (p=0.01) and *PCSK1* (p=0.001) than AA homozygotes. The G allele was associated with increased fasting insulin ($p_{replication}$=0.02, p_{all}=0.0008) and HOMA-insulin resistance ($p_{replication}$=0.02, p_{all}=0.001) as well as a lower fasting proinsulin/insulin ratio

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(p_{all}=0.008) and lower fasting glucagon (p=0.04) and gastric inhibitory peptide (GIP) (p=0.05) concentrations. Arginine-stimulated (p=0.02) insulin secretion was reduced in vivo, which was further reflected by a reduction of glucose- and potassium-stimulated insulin secretion (p=0.002 and p=0.04, respectively) in human islets in vitro.

Conclusions/interpretation A common variant in *PAX6* is associated with reduced *PAX6* and *PCSK1* expression in human islets and reduced insulin response, as well as decreased glucagon and GIP concentrations and decreased insulin sensitivity. These findings emphasise the central role of *PAX6* in the regulation of islet function and glucose metabolism in man.

Keywords Genetic association · Incretin · Insulin · Insulin processing · Insulin secretion · Meta-analysis · *PAX6* · *PCSK1* · Proinsulin · Type 2 diabetes

Abbreviations

AIR	Acute insulin response
BPS	Botnia Prospective Study
DGI	Diabetes Genetics Initiative
DIAGRAM+	Diabetes Genetics Replication and Meta-analysis Consortium
EC	Endogenous control
GIP	Gastric inhibitory peptide
GLP	Glucagon-like peptide
HBCS	Helsinki Birth Cohort Study
HOMA-IR	HOMA-insulin resistance
LD	Linkage disequilibrium
MEIS2	Meis homeobox 2
MM	Malmö Men
PAX6	Paired box 6
PC	Prohormone convertase
PDX1	Pancreatic and duodenal homeobox 1
PPP	Prevalence, Prediction and Prevention of Diabetes
SNP	Single nucleotide polymorphism

Introduction

Paired box 6 (PAX6) is a key regulator of pancreatic development and a transcription factor for genes involved in glucose homeostasis, including insulin, incretins and prohormone convertases, making the gene that encodes it an interesting candidate for involvement in the pathogenesis of type 2 diabetes and insulin secretion.

Mice that have point mutations in the *Pax6* gene, resulting in a truncated PAX6 protein, have disrupted islet morphology and decreased numbers of alpha, beta, delta and pancreatic polypeptide cells and, accordingly, markedly reduced hormone production [1]. Biochemical studies have also demonstrated binding of PAX6 protein to regulatory elements in the glucagon, insulin and somatostatin genes [1, 2].

In humans *PAX6* mutations cause aniridia, a panocular disorder characterised by complete or partial absence of the iris and malformations of the eye [3]. There have been several reports of *PAX6* mutation carriers having abnormal glucose tolerance, and of type 2 diabetes co-segregating with aniridia in families with *PAX6* mutations [4–6]. Recently, Wen et al. reported that carriers of a *PAX6* R240Stop mutation developed impaired glucose tolerance and/or diabetes with age [6]. Mutation carriers had an increased proinsulin/insulin ratio and reduced insulin response to an OGTT [6]. By generating mice with a corresponding stop-mutation, the authors could demonstrate that PAX6 deficiency reduced the production of prohormone convertase (PC)1/3 and that the *PCSK1* gene encoding PC1/3 is a direct transcriptional target of PAX6 [6]. Confusingly, another study reported that mice lacking *Pax6* developed diabetes but had normal PC1/3 levels, making the role of PAX6 in *PCSK1* transcription unclear [7].

PAX6 also plays a major role in the production of incretins. In mice, PAX6 is needed for the differentiation of gastric inhibitory peptide (GIP) and glucagon-like peptide (GLP)-1- and GLP-2-producing cells in the intestine [8]. Furthermore, PAX6 regulates the production of GIP and proglucagon [9] as well as the main enzymes involved in GIP, proinsulin and proglucagon processing: PC2 and PC1/3 [2, 10–13]. PAX6 could thus affect incretin levels either by changing the expression of the propeptide genes or the levels of processing enzymes.

In addition, PAX6 has been reported to regulate the levels of pancreatic and duodenal homeobox 1 (PDX1) and GLUT2 in mice [14]. PDX1 plays a crucial role in pancreatic development, beta cell function and transcription of genes involved in metabolism (for review see Kaneto et al. [15]). GLUT2 is a glucose sensor expressed in the pancreas, and mice lacking GLUT2 have a blunted first-phase insulin secretion [16].

In the present study we assessed the effect of a common genetic variant in *PAX6* on glucose homeostasis. Using genome-wide association data from the Diabetes Genetics Initiative (DGI) [17] study we identified a single nucleotide polymorphism (SNP; rs685428) that is associated with insulin levels, reduced *PAX6* mRNA expression in human pancreatic islets and reduced expression of target genes including *PCSK1*. A detailed description of the metabolic consequences of the identified variant is provided.

Methods

Study samples and clinical characteristics Clinical characteristics of the study participants are reported in Table 1. The DGI genome-wide association study has been described in detail [17]. The non-diabetic control participants

Table 1 Characteristics of the study participants

Variable	DGI (controls)	BPS	PPP	HBCS	MM
N	1,467	2,629	4,580	1,688	166
men/women (*n*/*n*)	707/760	1,191/1,438	2,131/2,449	745/943	166/0
Age at visit (years)	58.8±10.1	52.2±14.8	47.3±15.3	61.4±2.9	65.8±2.0
BMI (kg/m^2)	26.6±3.7	26.5±4.2	26.2±4.3	27.1±4.3	26.9±3.3
Fasting glucose (mmol/l)	5.3±0.5	5.4±0. 7	5.3±0.6	5.5±0.6	6.1±2.4
2 h OGTT glucose (mmol/l)	5.6±1.3	5.4±0.7	5.2±1.5	6.9±1.7	10.7±5.1
Fasting insulin (pmol/l)	47.9±36.1	54.2±32.6	44.4±29.2	69.5±77.9	87.5±72.2
2 h OGTT insulin (pmol/l)	259.0±211.8	314.2±317.2	224.3±266.0	502.1±407.7	448.3±360.6
CIR	174.6±157.7	193.3±213.6	211±4.87	192.5±4.74	66.96±3.80
Fasting total proinsulin (pmol/l)	10.2±4.5	–	10.5±5.5	12.9±10.8	–
Fasting intact proinsulin (pmol/l)	–	–	–	4.7±4.4	–
Fasting split proinsulin (pmol/l)	–	–	–	8.2±7.3	–
2 h total proinsulin (pmol/l)	36.0±16.1	–	37.9±20.3	–	–
HOMA-IR	1.7±1.4	1.04±0.6	1.5±1.1	2.5±2.9	3.6±3.4
Fasting GIP (pmol/l)	–	–	34.3±0.5	–	–
2 h OGTT GIP (pmol/l)	–	–	179.4±1.9	–	–
Fasting glucagon (ng/l)	–	–	70.1±0.5	–	–
2 h OGTT glucagon (ng/l)	–	–	62.7±0.4	–	–
MAF (rs685438 G)	0.22	0.17	0.16	0.14	0.13

Data are means±SD

CIR, corrected insulin response; MAF, minor allele frequency

were individuals with no first-degree relatives with type 2 diabetes, fasting plasma glucose <6.1 mmol/l and 2 h glucose <7.8 mmol/l, originating from Finland (Botnia region) and Sweden (southern Sweden and Skara) [17]. Type 2 diabetes was classified according to the WHO (1999) criteria as fasting plasma glucose ≥7.0 mmol/l or 2 h glucose ≥11.1 mmol/l during an OGTT. The Prevalence, Prediction and Prevention of Diabetes (PPP) study is a population-based study including ~10% of the population aged 18–74 years in the Botnia region in Finland.

The Botnia Prospective Study (BPS) is part of the Botnia study, which includes 2,770 non-diabetic family members and spouses of diabetic individuals. Of these, 138 developed diabetes during the follow-up period (median 7.7 years follow-up).

The Helsinki Birth Cohort Study (HBCS) consists of a subset of 1,999 participants, aged 56–70 years, from the original Helsinki birth cohort (n=8,760) that have undergone an OGTT [18]. Of these, 311 individuals had type 2 diabetes.

For these studies, individuals with type 2 diabetes were excluded from the analysis of quantitative traits.

In the Malmö men (MM) study, acute insulin response (AIR) during a glucose-dependent arginine stimulation test was analysed in a subgroup of 168 men from the Malmö Preventive Project study who had impaired glucose tolerance at the screening visit 20 years earlier [19–21]. At the time of the test 70 men had developed type 2 diabetes and were either treated with diet alone (42%) or with oral hypoglycaemic agents that were discontinued the day before the test.

All participants gave written informed consent and the studies were approved by the local research ethics committees of Lund University, Malmö University Hospital or Helsinki University hospital.

Measurements In the BPS, blood samples were drawn at −10, 0, 30, 60 and 120 min of the OGTT, and in the PPP study at 0, 30 and 120 min. For the BPS, results from the first visit were used. Plasma glucose was measured by glucose oxidase methods. Plasma insulin concentrations were measured using a fluoroimmunometric assay (AutoDelfia, Perkin Elmer, Turku, Finland). In the Botnia PPP study, proinsulin was measured using a human proinsulin RIA (95% cross-reactivity with 32–33 split proinsulin, no cross-reactivity with 65–66 split proinsulin, insulin or C-peptide) (Millipore, Malmö, Sweden). In the HBCS proinsulin was measured by a two-site immunometric assay (66% cross-reactivity with 65–66 split proinsulin, no cross reactivity with 32–33 split proinsulin) [22].

GIP was measured using an ELISA kit for total human GIP [100% cross-reactivity with GIP(1–42) and GIP(3–42)] (Millipore). Glucagon was measured using a radioimmunoassay kit specific for pancreatic glucagon (cross-reactivity with oxyntomodulin <0.1%) (Millipore).

For a detailed description of the measurements in the DGI see [17].

The basal HOMA-insulin resistance (HOMA-IR) index was calculated from fasting insulin and glucose concentrations (www.dtu.ox.ac.uk).

Corrected insulin response during OGTT was calculated as in [23, 24].

Arginine-stimulated insulin secretion Insulin secretion in response to an acute bolus of arginine was measured at three glucose concentrations (fasting, 14 and 28 mmol/l) as previously described [21]. The AIR was calculated from the +2 to 5 min values using the trapezoidal rule. Potentiation of insulin secretion by glucose was calculated as the slope between AIR at fasting and 14 mmol/l glucose [21].

Genotyping Genotyping was performed using a TaqMan® assay on the ABI 7900 platform (Applied Biosystems, Foster City, CA, USA). Average genotyping success was 96.9% and the concordance rate was 99.8%. Hardy–Weinberg equilibrium was fulfilled in all populations.

Human islets Islets were provided by the Nordic Network for Islets Transplantation by the courtesy of O. Korsgren, Uppsala University. All donors had given consent to donate organs for medical research, or alternatively consent was obtained from the closest relative in accordance with the ethics approval by the ethics committees at Uppsala and Lund Universities. The study included islets from 48 donors, six of whom were diagnosed with type 2 diabetes (25 women, 23 men), with a mean (±SD) age of 55.3±11.6 years, BMI of 26.1±3.4 kg/m^2, purity 69.5±16.8% and HbA_{1c} 5.8±0.87% (39.9±9.5 mmol/mol). Islets were either directly subjected to Affymetrix analysis (see below) or collected under a stereomicroscope and preincubated for 30 min at 37°C in KRB, pH 7.4, supplemented with 10 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 0.1% BSA and 1 mmol/l glucose. Each incubation vial contained 12 islets in 1.0 ml KRB and was treated with 95% O_2/5% CO_2 to obtain constant pH and oxygenation.

Insulin secretion in islets Preincubation was followed by 1 h incubation at 37°C in a metabolic shaker (30 cycles per min) in a medium containing either 1 or 20 mmol/l glucose. Insulin was then measured using a radioimmunoassay (Linco Research, Saint Charles, MO, USA).

RNA isolation Islets were cultured in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 10 mmol/l HEPES, 2 mmol/l L-glutamine, 50 μg/ml gentamicin, 0.25 μg/ml fungizone (GIBCO, BRL, Gaithersburg, MD, USA), 20 μg/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany) and 10 mmol/l nicotinamide at 37°C (5% CO_2) for 1–9 days prior to RNA preparation. RNA was isolated from islets using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). Concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (A_{260}/A_{280} >1.8 and A_{260}/A_{230} >1.0) (NanoDrop Technologies, Wilmington, NC, USA). No signs of degradation were observed using agarose gel electrophoresis and Experion RNA gel chips (Bio-Rad, Hercules, CA, USA).

Microarray gene expression in human pancreatic islets The microarrays were performed following the Affymetrix standard protocol. Briefly, 100–200 ng total RNA was processed following the GeneChip Expression 3′-Amplification Reagents One-cycle cDNA synthesis kit instructions (Affymetrix, Santa Clara, CA, USA) to produce double-stranded cDNA, which was used as a template to generate biotin-targeted cRNA following the manufacturer's specifications. This was fragmented and hybridised onto the GeneChip Human Gene 1.0 ST whole transcript based assay overnight in the GeneChip Hybridisation oven 6400 using standard procedures. The arrays were washed and stained in a GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 3000. Image analysis was performed using GeneChip operating software. The array data were summarised and normalised with the robust multi-array analysis method using the Expression Console software (Affymetrix).

Quantitative PCR *PAX6* and *PCSK1* mRNA expression was confirmed using RT quantitative PCR. Reactions were performed in triplicate with three endogenous controls (ECs). Reverse transcription was performed on RNA samples using an Omniscript RT kit (Qiagen) and Oligo(dt) primers according to the manufacturers' instructions. Real-time quantitative PCR was carried out using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The following assays were used: *PAX6* (assay id: Hs01088112_m1), *PCSK1* (assay id: Hs00175619_m1), *HPRT1* (EC) (assay id: 4326321E, VIC-MGB), *PPIA* (EC) (assay id: 4326316E, VIC-MGB) and *POLR2A* (EC) (assay id: Hs00172187_m1). The PCR reaction was monitored using an ABI PRISM 7700 Sequence Detector (Applied Biosystems).

Relative expression was calculated by normalising the expression ($2^{-\Delta C_t}$) using the geometric mean of the EC as described in [25]. Correlation between microarray and quantitative PCR expression was 0.85 for *PAX6* and 0.97 for *PCSK1*.

Pathway analysis Pathway analysis was performed using GENECODIS [26, 27]. GO biological processes were searched at the lowest GO level. Hypergeometric tests and

false discovery rate corrections were used to test for significance.

Statistical methods We estimated the effect of genetic variants on quantitative traits using linear regression correcting for sex, age and BMI in the PPP, HBCS and MM studies. The BPS (corrected residuals) was analysed in PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) [28] using the QFAM-total method with adaptive permutation to correct for family structure. Variables that were not normally distributed were log-transformed (natural) before analysis. Islet expression was analysed by Spearman correlation and multiple linear regression adjusted for the donor's sex, age, BMI and diabetes status.

All statistical analysis was performed with the Statistical Package for the Social Sciences version 17.0 (SPSS), PLINK version 1.06 [28] or R version 2.11.0. A two-tailed p value of 0.05 was considered significant. When the number of homozygous individuals allowed, both additive and recessive models were used and the p values corrected for two tests by Bonferroni correction.

Results

Identification of candidate SNP rs685428 in the DGI genome-wide association study We analysed ten genotyped and 54 imputed SNPs in the *PAX6* gene ±50 kbp in the DGI study, in which 1,361 non-diabetic individuals had insulin and 307 had proinsulin data available [17]. We found a linkage disequilibrium (LD) block 35 kbp upstream of the *PAX6* gene, tagged by the directly genotyped marker rs685428, where the minor G allele was associated with increased fasting insulin (p=0.001, $p_{\mathrm{corrected}}$=0.01) and increased HOMA-IR (p=0.0008, $p_{\mathrm{corrected}}$=0.008). The same marker was associated with a low fasting proinsulin/insulin ratio ($p_{\mathrm{additive\ model}}$ [p_{add}]=0.007). The effect was even stronger when applying a recessive rather than additive model (Table 2).

Gene expression in human pancreatic islets The associated LD block did not include the coding region of *PAX6*, making it likely that the rs685428 variant or another SNP in the LD block could affect gene expression. We measured mRNA levels of *PAX6* by quantitative PCR in islets from six diabetic and 42 non-diabetic human organ donors and related it to the *PAX6* rs685428 genotype. We also measured *PCSK1* expression, since PCSK1 is thought to be the mediator of the *PAX6* effect on proinsulin processing. *PAX6* and *PCSK1* expression correlated strongly (R=0.72, p=5.43×10^{-8}). *PAX6* was slightly, although not significantly, lower in diabetic than non-diabetic individuals (Fig. 1a). The expression of *PCSK1* was lower in diabetic (p=0.04) than non-diabetic islets (Fig. 1b). Both *PAX6* and *PCSK1* expression was lower in rs685428 AG than in AA individuals (p=0.008 and p=0.001). The fraction of variance explained by the SNP was 0.12 for *PAX6* and 0.14 for *PCSK1*. We also analysed the correlation between *PAX6* and putative *PAX6* target genes involved in metabolic control: *INS*, *SLC2A2*, *PCSK2*, *PDX1*, *SST* and *GCG* using microarray expression data from the same islet donors (Fig. 1c–h). All genes correlated with *PAX6* expression and expression of *INS*, *SLC2A2* and *PDX1* was decreased in diabetic individuals.

We next analysed cis-expression of ten other genes (*DCDC5*, *DCDC1*, *DNAJC24*, *IMMP1L*, *ELP4*, *RCN1*, *WT1*, *EIF3M*, *CCDC73* and *PRRG4*) within a 2 Mb region of the rs685428 SNP. None of these was differentially expressed between rs685428 AA and AG individuals (p>0.05).

Genome-wide correlation with PAX6 expression We performed a co-expression analysis of genes in human islets showing correlation with *PAX6* at a genome-wide level to identify new potential *PAX6* targets. Out of 22,201 genes represented on the microarray, 403 correlated with *PAX6* with a p value <2.25×10^{-6}, corresponding to a p value of 0.05 after Bonferroni correction. An R>0.75 cut-off resulted in a list of 60 genes, all with p values <5×10^{-9}, including several key players in pancreatic development and function such as *ISL1* and *NEUROD1* (electronic supplementary material [ESM] Table 1). Interestingly, one of the strongest correlating genes was *MEIS2* (R=0.84), which regulates *PAX6* expression during lens development (Fig. 1i) [29]. *MEIS2* expression was lower in AG than AA genotype carriers of the *PAX6* variant (p=0.04; Fig. 1j). Pathway analysis of the top 60 genes showed an over-representation of genes involved in signal transduction (ten genes), regulation of transcription (seven genes), ion transport (seven genes) and synaptic transmission (six genes).

The PAX6 rs685428 genotype influences fasting insulin levels and fasting proinsulin/insulin ratio The effect of the rs685428 G allele on fasting insulin could be replicated in the additional cohorts ($p_{\mathrm{recessive\ model}}$ [p_{rec}]=0.02 in meta-analysis) and HOMA-IR (p_{rec}=0.02, meta-analysis). In a meta-analysis of all studies, including the DGI, GG homozygous individuals had 10% higher fasting insulin levels (p_{rec}=0.0008) and HOMA-IR indices (p_{rec}=0.0016) than AA/AG individuals (Table 2, Fig. 2). The 2 h insulin response to an OGTT was slightly increased (p_{add}=0.04, p_{rec}= 0.2) whereas glucose levels did not differ by genotype (Table 2).

We also searched the SNP results in the large meta-analysis performed by the Meta-analyses of Glucose and Insulin-related Traits Consortium (MAGIC) but found no

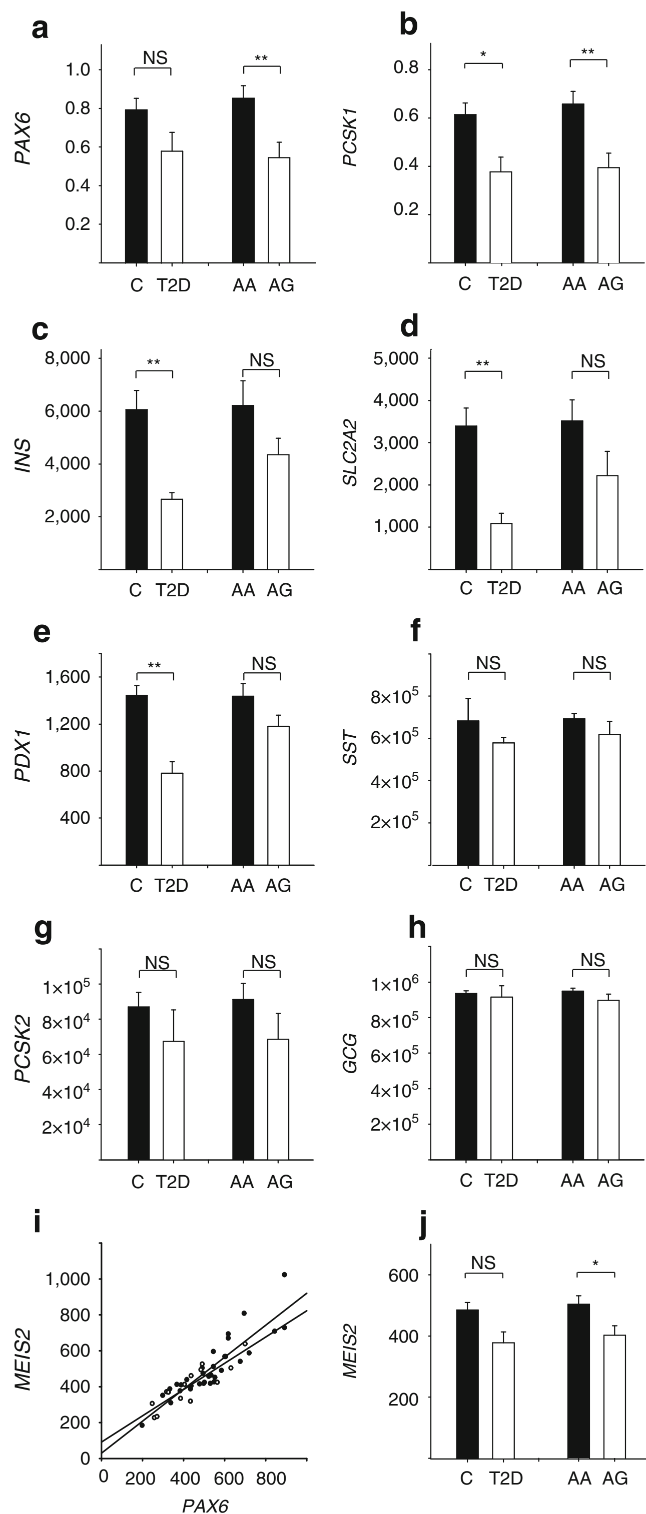
Table 2 Association between rs685428 genotype and phenotypes

Phenotype	n	Additive model			Recessive model (GG vs AA+AG)		
		β (SEM)	p value	$p_{corrected}$	β (SEM)	p value	$p_{corrected}$
Fasting proinsulin[a]							
DGI	307	−0.11 (0.053)	0.04	0.08	–	–	–
PPP	2,221	0.014 (0.017)	0.4	0.8	0.098 (0.054)	0.07	0.14
HBCS	1,585	0.01 (0.03)	0.7	1	−0.015 (0.046)	0.7	1
Meta-analysis	4,113	0.004 (0.014)	0.77	1	0.033 (0.035)	0.35	1
Fasting insulin[a]							
DGI	1,361	0.17 (0.054)	0.001	0.002	0.78 (0.21)	1.6×10^{-4}	3.2×10^{-4}
PPP	3,914	−0.001 (0.016)	1	1	0.12 (0.052)	0.02	0.04
HBCS	1,586	0.056 (0.027)	0.04	0.08	0.11 (0.046)	0.02	0.04
BPS	1,848	0.006 (0.02)	0.8	1	−0.056 (0.068)	0.4	0.8
Meta-analysis	8,709	0.023 (0.013)	0.08	0.16	0.10 (0.029)	0.0004	0.0008
Proinsulin/insulin ratio[a]							
DGI	307	−0.31 (0.12)	0.007	0.014	–	–	–
PPP	2,191	0.006 (0.020)	0.8	1	−0.051 (0.065)	0.4	0.8
HBCS	1,585	−0.05 (0.025)	0.07	0.14	−0.12 (0.042)	0.004	0.008
Meta-analysis	4,083	−0.021 (0.016)	0.18	0.36	−0.10 (0.035)	0.004	0.008
Fasting glucose							
DGI	1,428	0.02 (0.053)	0.7	1	0.016 (0.19)	0.9	1
PPP	3,982	−0.012 (0.017)	0.5	1	0.0030 (0.056)	1	1
HBCS	1,586	0.038 (0.026)	0.2	0.4	0.066 (0.044)	0.1	0.2
BPS	1,848	−0.024 (0.025)	0.5	1	−0.054 (0.08)	0.5	1
Meta-analysis	8,844	−0.002 (0.11)	0.84	1	0.037 (0.038)	0.40	0.8
HOMA-IR[a]							
DGI	1,366	0.18 (0.053)	7.8×10^{-4}	1.6×10^{-3}	0.79 (0.2)	6.7×10^{-5}	1.3×10^{-4}
PPP	3,912	−0.003 (0.016)	0.8	1	0.12 (0.055)	0.02	0.04
HBCS	1,586	0.062 (0.029)	0.03	0.06	0.12 (0.048)	0.02	0.04
BPS	1,848	0.0044 (0.02)	1	1	−0.060 (0.068)	0.4	0.8
Meta-analysis	8,712	0.017 (0.011)	0.12	0.24	0.098 (0.031)	0.0006	0.0012
2 h insulin[a]							
DGI	1,010	0.075 (0.063)	0.24	0.48	−0.016 (0.24)	0.5	1
PPP	3,802	0.033 (0.024)	0.17	0.35	0.12 (0.081)	0.15	0.31
HBCS	1,586	0.049 (0.035)	0.17	0.34	0.047 (0.059)	0.42	0.84
BPS	1,848	0.032 (0.035)	0.38	0.76	0.12 (0.12)	0.3	0.6
Meta-analysis	8,246	0.039 (0.017)	0.018	0.036	0.070 (0.043)	0.11	0.22
2 h glucose							
DGI	1,410	−0.038 (0.053)	0.47	0.94	0.087 (0.019)	0.065	0.13
PPP	3,958	0.082 (0.045)	0.07	0.14	0.18 (0.152)	0.25	0.5
HBCS	1,586	0.046 (0.084)	0.59	1	−0.036 (0.14)	0.8	1
BPS	1,848	0.036 (0.065)	1	1	0.25 (0.21)	0.23	0.46
Meta-analysis	8,802	0.034 (0.029)	0.23	0.46	0.095 (0.083)	0.25	0.5
CIR[a]							
DGI	947	0.15 (0.064)	0.019	0.038	0.18 (0.24)	0.45	0.9
PPP	4,187	0.034 (0.022)	0.14	0.27	0.15 (0.076)	0.045	0.09
HBCS	1,579	−0.031 (0.038)	0.42	0.84	0.061 (0.063)	0.33	0.66
BPS	1,848	−0.0003 (0.034)	1	1	−0.043 (0.11)	0.7	1
Meta-analysis	8,561	0.022 (0.016)	0.17	0.34	0.079 (0.043)	0.071	0.14

[a] Log_e transformed

CIR, corrected insulin response

Fig. 1 Relative expression of *PAX6* and target genes in human pancreatic islets. **a** Expression of *PAX6* measured by quantitative PCR. Whereas the mRNA expression of *PAX6* was lower in AG (n=13) than in AA genotype carriers (n=33), the mRNA of *PAX6* was not significantly reduced in diabetic (T2D) (n=6) vs non-diabetic islets (n=42). **b** *PCSK1* expression was lower both in G allele carriers and in islets from diabetic patients. **c**–**h** Expression of suggested *PAX6* target genes in human pancreatic islets measured by microarray. **i** *MEIS2* expression correlated strongly with *PAX6* (R=0.84) in human pancreatic islets. Filled circles are AA homozygous, open circles are AG heterozygous. **j** The mRNA expression of *MEIS2* was lower in AG (n=13) than in AA genotype carriers of the SNP rs685428 in the *PAX6* gene. There was no significant difference in *MEIS2* mRNA expression between islets from diabetic and non-diabetic cadaver donors. Error bars show SEM. *p<0.05, **p<0.01. C, controls



significant association with fasting insulin (p=0.3) nor HOMA-IR (p=0.2) [30] However, since this study only used an additive model it does not exclude a recessive effect of the SNP.

To replicate the effect of rs685428 on the proinsulin/insulin ratio we analysed 2,191 individuals from the PPP–Botnia study and 1,585 from the HBCS, for whom proinsulin data was available. In the HBCS the proinsulin/insulin ratio was significantly lower in GG compared with AA homozygous individuals (P_{add}=0.14, P_{rec}=0.008; Fig. 2b). A similar, non-significant trend was seen in the PPP–Botnia study. In a meta-analysis of all studies, GG genotype carriers had a 10% lower proinsulin/insulin ratio (p_{rec}=0.008) than AA/AG carriers (Table 2). There was no difference in total proinsulin levels (P>0.3, meta-analysis) [31].

Effect of PAX6 rs685428 on glucose and arginine-stimulated insulin secretion in vivo and in vitro We also studied the effect of rs685428 on arginine-stimulated insulin secretion in 167 Malmö men (Table 1) [21, 32]. The AIR to arginine was lower in AG/GG than in AA genotype carriers at all glucose concentrations (p=0.04; Fig. 3a) whereas the slope of potentiation of insulin secretion by glucose did not significantly differ between the two genotype groups [31]. A meta-analysis of corrected insulin response in all studies was not significantly different (Table 2).

To link these effects directly to impaired glucose-stimulated insulin secretion in vitro we investigated insulin secretion at low (1 mmol/l) and high (16.7–20 mmol/l) glucose in human islets. Insulin secretion was reduced in islets from AG vs AA genotype carriers at both high and low glucose (p=0.002 and p=0.009, respectively). Potassium-stimulated insulin secretion was also reduced in risk genotype carriers (p=0.04; Fig. 3b). Further, we analysed the correlation between *PAX6* and *PCSK1* expression and HbA_{1c} and insulin secretion (Fig. 3c,d). Expression of *PAX6* was positively associated with insulin secretion (r_s=0.46, p=0.004). A higher expression of *PCSK1* was associated with higher insulin secretion (r_s=0.45, p=0.005) and lower HbA_{1c} (r_s=−0.34, p=0.04).

Effect of PAX6 rs685428 on plasma glucagon and GIP levels Next we measured plasma glucagon (n=2,197) and GIP (n=1,601) levels in a subset of the PPP study.

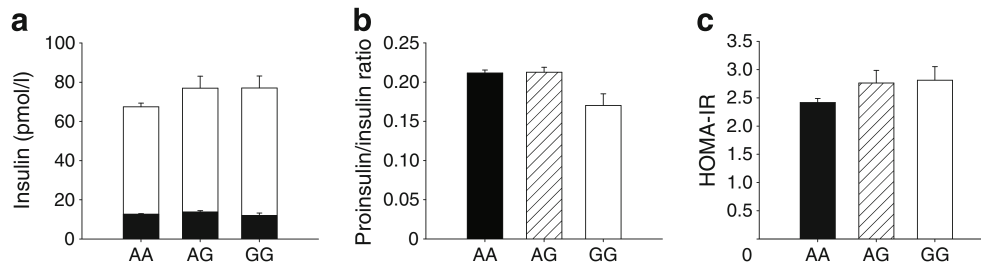


Fig. 2 **a** Fasting insulin levels in the HBCS study. The proinsulin level (black bars) was not significantly different between the genotype groups. Total insulin levels (white bars) were higher in rs685428 heterozygous and GG homozygous individuals than in AA homozygotes (p=0.04). **b** The fasting proinsulin/insulin ratio was significantly lower in GG homozygous individuals (p=0.008). **c** Insulin resistance estimated by HOMA-IR was higher in AG and GG individuals (p=0.04). Error bars show SEM

The G allele was associated with lower fasting plasma glucagon levels (p_{add}=0.04, p_{rec}=0.08; Fig. 4a). The glucagon/insulin ratio was lower in G allele carriers both in the fasting state (p_{add}=0.006, p_{rec}=0.004) and at 2 h (p_{add}=0.04, p_{rec}=0.32; Fig 4b). Furthermore, G allele carriers displayed lower fasting GIP concentrations (p_{add}= 0.05, p_{rec}=0.5; Fig. 4c). These differences were not influenced by the prevailing glucose concentration.

Risk of type 2 diabetes The putative risk of developing type 2 diabetes for carriers of the rs685428 variant was analysed in all the cohorts studied (N=2,101 cases/9,577 controls), and in the large Diabetes Genetics Replication and Meta-analysis Consortium (DIAGRAM+) meta-analysis [33]. No significant association was seen in any of the cohorts (DGI with 1,464 cases and 1,467 controls: p=0.7, OR 0.96; DIAGRAM+ with 2,832 cases and 15,843 controls: p=0.5,

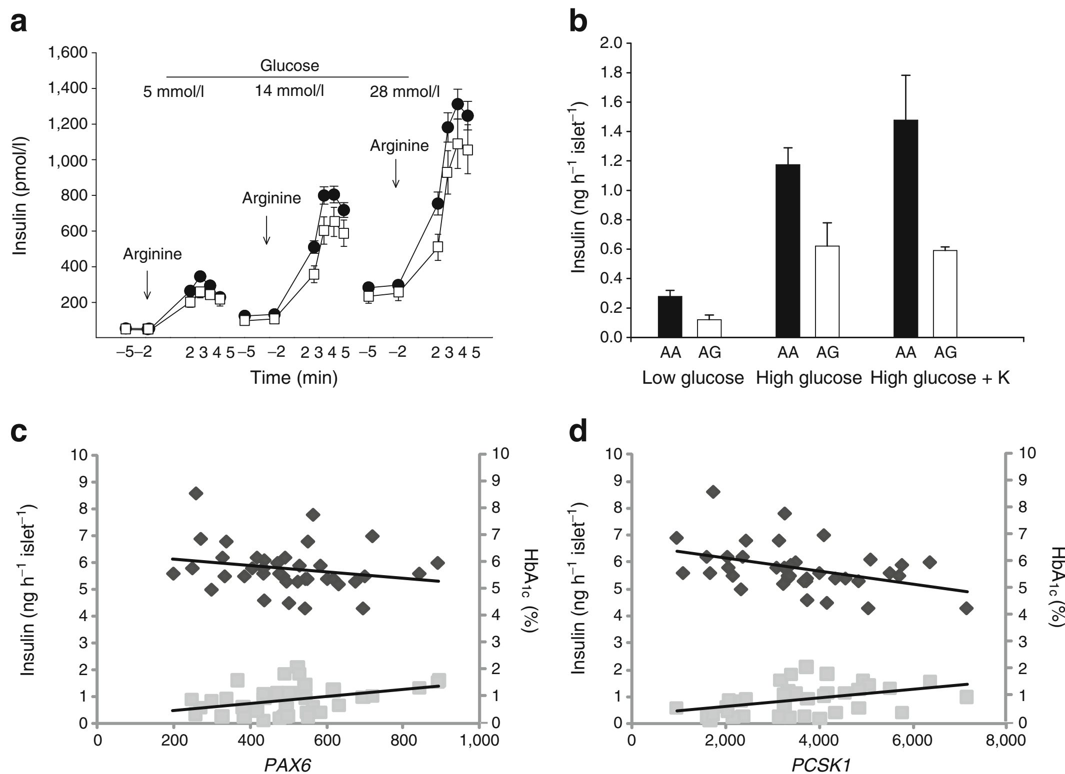


Fig. 3 **a** Insulin secretion in response to arginine stimulation at basal level (5 mmol/l) (p=0.02), 14 mmol/l (p=0.04) and 28 mmol/l (p= 0.04) glucose, in relation to SNP rs685428 in the *PAX6* gene (n_{AA}= 127, $n_{AB/BB}$=40). **b** Insulin secretion from human islets in vitro in response to low (1 mmol/l) glucose (n_{AA}=22, n_{AB}=8, p=0.002), high (16.7–20 mmol/l) glucose (n_{AA}=21, n_{AB}=8, p=0.009) and high glucose plus K^+ (16.7 mmol/l+70 mmol/l K^+) (n_{AA}=12, n_{AB}=6, p= 0.04). High glucose values were corrected for the differing glucose stimulation. **c** Correlation between *PAX6* expression and HbA_{1c} level (r_s=−0.26, p=0.1) and insulin secretion measured at 16.7 mmol/l glucose (r_s=0.46, p=0.004). **d** Correlation between *PCSK1* expression and HbA_{1c} (r_s=−0.34, p=0.04) and insulin secretion measured at 16.7 mmol/l glucose (r_s=0.45, p=0.005). Dark grey diamonds, HbA_{1c} results; light grey squares, insulin results. To convert values for HbA_{1c} in % to mmol/mol, subtract 2.15 and multiply by 10.929. Error bars show SEM

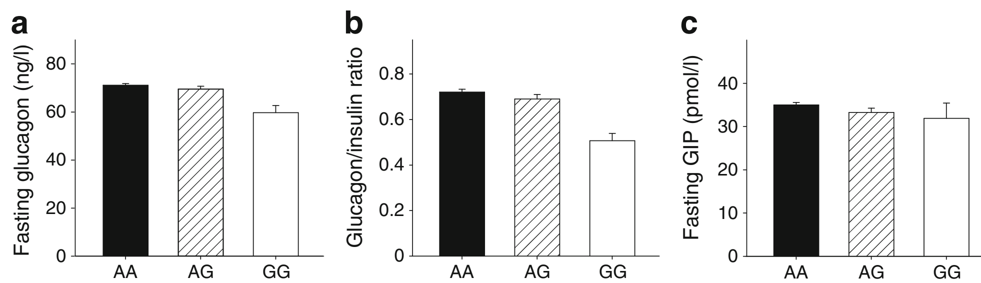


Fig. 4 **a** G allele carriers have lower levels of fasting serum glucagon compared with A allele carriers (p=0.04). **b** Fasting glucagon/insulin molar ratio according to rs685428 genotype (p=0.006). **c** Fasting serum GIP concentration was also reduced in G allele carriers (p=0.04). p values are from linear regression analysis, additive model adjusted for sex, age and BMI. Error bars show SEM

OR 0.98; PPP with 215 cases and 4,116 controls: p=0.2, OR 0.77; BPS with 27 cases and 2,408 controls: p=0.3, OR 0.81; HBCS with 295 cases and 1,586 controls: p=0.8, OR 1.03) nor in a meta-analysis of all cohorts (4,933 cases and 25,420 controls, p_{meta}=0.3, OR 0.97).

Discussion

In this study we demonstrated that a common variant in the *PAX6* gene was associated with marked changes in expression of key genes in human islets and thereby altered islet function. The similarity of effects in vivo and in vitro in human islets provides strong support for the central role of *PAX6* in development of islet function and regulation of glucose metabolism.

The finding that low *PAX6* expression causes impaired insulin secretion and affects glucose metabolism is in line with previously published findings in both animal models and human families with protein-altering mutations in the *PAX6* gene [4–7]. However, these studies were performed in individuals with more severe mutations making generalisation of the findings to potential effects of common variants in the gene difficult. Further, there is evidence from mouse models that the effect of mutations and deletions in *Pax6* on pancreatic development depends upon which functional domain of the protein is affected [34].

Three studies have previously evaluated whether common variants in the *PAX6* gene affect risk of type 2 diabetes, analysing two, four and eight SNPs, respectively, but none of them found any significant association [35–37]. Although risk genotype carriers showed impaired insulin response to several secretagogues we could not detect any association with type 2 diabetes. It should be noted that only additive models were tested and that the strongest effect of the SNP was seen using a recessive model. A recessive effect on diabetes can thus not be excluded. Another explanation for this finding is that there are mechanisms that neutralise the effect of impaired insulin secretion on risk of diabetes. One such mechanism could be the decreased glucagon concentrations in risk genotype carriers. Glucagon, particularly an increased glucagon/insulin ratio, has been ascribed a role in the pathogenesis of type 2 diabetes [38–40]. Here we observed decreased glucagon levels as well as a markedly decreased glucagon/insulin ratio in G allele carriers, suggesting that altered alpha cell function could contribute to the decreased risk of type 2 diabetes seen in G allele carriers.

A high proinsulin/insulin ratio can indicate a defect in prohormone processing or beta cell stress due to insulin resistance, and elevated proinsulin levels predict future type 2 diabetes [40–42]. Impaired proinsulin processing was expected in risk allele carriers based upon the concomitant decrease in expression of genes encoding the proinsulin processing enzymes *PCSK1* and *PCSK2*. This was also the case in the family study by Wen et al. where an elevated proinsulin/insulin ratio was associated with a decreased expression of *PCSK1* [6]. In contrast, in the present study risk allele carriers showed a decreased proinsulin/insulin ratio despite lower mRNA levels of both *PCSK1* and *PCSK2*. There might be several explanations for this unprecedented finding. One explanation is that the decrease in proinsulin/insulin ratio reflects the change in the denominator, that is increased insulin levels, rather than altered proinsulin processing. This is supported by a lack of difference in proinsulin concentrations between different genotype carriers.

While the effects of *PAX6* on islet gene expression and function were expected, the increase in fasting insulin concentration is less obvious. To the former, decreased expression of *PAX6* was associated with decreased expression of *PDX1*, *ISL1*, *NEUROD* and *MEIS2*. The strong correlation between *PAX6* and *MEIS2* expression is particularly interesting since Meis homeobox 2 (MEIS2) has been shown to act as a direct upstream regulator of *PAX6* expression during lens development [29]. MEIS binding sites have also been demonstrated in a *PAX6* transcription enhancer element involved in pancreatic development [43]. *MEIS2* could thus also be a major regulator of *PAX6* transcription in the pancreas, possibly acting directly on the *PAX6* gene. However, more detailed investigation is required to determine whether these genes are targets or regulators of

PAX6 expression or regulated by common upstream factors. If the *PAX6* polymorphism changes the islet cell ratio, as is seen in mouse models with mutated *Pax6* [34], this could also contribute to the many correlations.

The most common cause for an increase in fasting insulin concentration is insulin resistance, also evidenced by an increase in the HOMA-IR. There is, however, no obvious explanation for a direct effect of the SNP on insulin sensitivity, nor did we see an effect of the SNP on insulin sensitivity measured during a euglycaemic–hyperinsulinaemic clamp in the MM study. In addition, no effect on insulin sensitivity was reported in mice with a truncated *Pax6* gene [6]. We are thus left with the possibility that the increase in fasting insulin levels is unrelated to insulin resistance and possibly the result of an altered feedback loop in islets, for example altered clearance of insulin.

In conclusion, a common variant in *PAX6* is associated with reduced *PAX6* and *PCSK1* expression in human islets, reduced insulin response and decreased glucagon and GIP concentrations. The impairment in insulin response was not translated into an increased risk of type 2 diabetes. These findings emphasise the central role of the *PAX6* gene in the regulation of islet function and glucose metabolism in man.

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Contribution statement EA: Study coordination and design, genotyping, statistical analysis, data interpretation and draft of the manuscript. FT: Genotyping, real-time PCR and statistical analysis. STL: Microarray analysis. JT: Generation and analysis of microarray and insulin secretion data. YZ: Real-time PCR and analysis. PA: Statistical analysis. OH: Analysis of expression data. BI and TT: Sample collection, phenotyping (Botnia) and data interpretation. KE: Phenotyping in the MM study and data interpretation. JGE: Phenotyping (HBCS) and data interpretation. VL: DGI genome-wide association study and study design, data interpretation and study supervision. LG: Study conception and design, data interpretation, study supervision and draft of the manuscript. All authors critically revised the draft and approved the final manuscript.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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