Characterization of the human *SLC30A8* promoter and intronic enhancer

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Abstract

Genome-wide association studies have shown that a polymorphic variant in *SLC30A8*, which encodes zinc transporter-8, is associated with altered susceptibility to type 2 diabetes (T2D). This association is consistent with the observation that glucose-stimulated insulin secretion is decreased in islets isolated from *Slc30a8* knockout mice. In this study, immunohistochemical staining was first used to show that *SLC30A8* is expressed specifically in pancreatic islets. Fusion gene studies were then used to examine the molecular basis for the islet-specific expression of *SLC30A8*. The analysis of *SLC30A8*-luciferase expression in β TC-3 cells revealed that the proximal promoter region, located between -6154 and -1 , relative to the translation start site, was only active in stable but not transient transfections. VISTA analyses identified three regions in the *SLC30A8* promoter and a region in *SLC30A8* intron 2 that are conserved in the mouse *Slc30a8* gene. Additional fusion gene experiments demonstrated that none of these *Slc30a8* promoter regions exhibited enhancer activity when ligated to a heterologous promoter whereas the conserved region in *SLC30A8* intron 2 conferred elevated reporter gene expression selectively in β TC-3 but not in α TC-6 cells. Finally, the functional effects of a single nucleotide polymorphism (SNP), rs62510556, in this conserved intron 2 enhancer were investigated. Gel retardation studies showed that rs62510556 affects the binding of an unknown transcription factor and fusion gene analyses showed that it modulates enhancer activity. However, genetic analyses suggest that this SNP is not a causal variant that contributes to the association between *SLC30A8* and T2D, at least in Europeans.

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Introduction

Zinc transporter-8 (ZnT-8) belongs to a family of zinc transporters that, along with metallothioneins, are involved in intracellular zinc homeostasis and signaling through compartmentation of the ion and regulation of its concentration (Wijesekara *et al.* 2009). The mouse *Slc30a8* gene, which encodes ZnT-8, is mainly expressed in pancreatic α and β cells (Gyulkhandanyan *et al.* 2008, Pound *et al.* 2009), with much lower levels of expression in testis and submaxillary glands (Murgia *et al.* 2009). ZnT-8 is localized to insulin secretory granules within β cells (Chimienti *et al.* 2006) where it is thought to be required for providing zinc to allow for proper maturation, storage, and secretion of insulin (Wijesekara *et al.* 2009).

In keeping with an important role for ZnT-8 in the β cell, recent genome-wide association (GWA) studies linked a non-synonymous single nucleotide polymorphism (SNP), rs13266634, which changes amino

acid 325 of human ZnT-8 from tryptophan to arginine, to increased susceptibility to type 2 diabetes (T2D; Saxena *et al.* 2007, Scott *et al.* 2007, Sladek *et al.* 2007, Zeggini *et al.* 2007), gestational diabetes (Lauenborg *et al.* 2009), impaired proinsulin to insulin conversion (Stancakova *et al.* 2009), and reduced first-phase insulin secretion (Stancakova *et al.* 2009). This same variant is also associated with autoantibody epitope specificity changes in human type 1 diabetes (Wenzlau *et al.* 2008).

The rs13266634 variant has the potential to be causative, rather than just being in strong linkage disequilibrium with the actual causative variant, since it has been reported to affect zinc transport by ZnT-8 in cultured cell studies (Nicolson *et al.* 2009). However, a key question that remains to be addressed is whether this SNP is the only variant in the *SLC30A8* gene altering susceptibility to T2D. This seems unlikely because the variants identified to date can only explain a small fraction of the calculated genetic contribution to the risk of developing T2D (Manolio *et al.* 2009).

While various explanations have been proposed for this missing heritability (Manolio *et al.* 2009), one possibility is that rare SNPs, which are not analyzed in GWA studies, exist in genes such as *SLC30A8* that markedly affect their expression or the function of the encoded protein. The studies described here were initiated with the goal of characterizing key regions of the *SLC30A8* gene so as to establish a framework for the future analysis of rare SNPs that have the potential to modulate *SLC30A8* gene transcription. We show that the proximal human *SLC30A8* promoter, located between −6154 and −1, relative to the translation start site, is only active in stable and not transient transfections. In addition, we identify a conserved islet β cell-specific enhancer in intron 2 of the *SLC30A8* gene. Finally, a SNP, rs62510556, in this conserved intron 2 enhancer is shown to affect the binding of an unknown transcription factor and thereby modulate enhancer activity. This SNP therefore had the potential to be a second causal variant that contributed to the association between *SLC30A8* and T2D. However, genetic analyses suggest that, in Europeans, this SNP is not associated with the risk for T2D.

Materials and methods

Materials

The BAC plasmids from which mouse *Slc30a8* and human *SLC30A8* genomic regions were isolated (see below) were purchased from BACPAC Resources (Children's Hospital Oakland Research Institute, Oakland, CA, USA).

Immunohistochemistry

Human pancreata were fixed in 4% paraformaldehyde in PBS (Invitrogen Corporation) for 12 h and were paraffin embedded. Immunostainings were performed on 6 μ m sections that were de-paraffinized three times with HistoClear (National Diagnostics, Atlanta, GA, USA) for 5 min, then hydrated serially in 100 (twice), 95, and 70% ethanol and water for 2 min. Antigen retrieval was performed on sections by incubating in 0.01 M citrate buffer, pH 6.0, for 5 min in a microwave using medium power settings. Sections were incubated for 1 h at room temperature with blocking buffer (TSA system; Invitrogen Corporation) and then at room temperature overnight in a humid chamber with three primary antibodies: mouse anti-human ZnT-8 (1:20; John Hutton, UC Denver; please see [Supplementary Data](#), see section on [supplementary data](#) given at the end of this article, for details on antibody generation), Guinea pig anti-insulin (1:50; Sigma), and rabbit anti-glucagon (1:50; Dako North America, Inc., Carpinteria,

CA, USA) in TNB antibody dilution buffer (Invitrogen Corporation). The slides were then drained and washed three times for 5 min in PBS before secondary antibodies (1:250) conjugated to aminomethylcoumarin acetate, Cyanine 2, or Cyanine 5 fluorophores (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were applied and incubated at room temperature for 60 min. The slides were then washed in PBS and mounted with a glycerol-based media. Images were acquired with Intelligent Imaging System software using an Olympus 1X81-inverted-motorized microscope equipped with Olympus DSU spinning disk confocal optics and a Hamamatsu ORCA IIER monochromatic CCD camera. The images were later pseudo-colored for illustration: red (ZnT-8), blue (glucagon), and green (insulin).

Fusion gene plasmid construction

Please see [Supplementary Data](#), see section on [supplementary data](#) given at the end of this article.

Cell culture, transfection, and luciferase assays

Mouse islet β cell-derived β TC-3 cells and α cell-derived α TC-6 cells were grown in DMEM containing 10% (vol/vol) fetal bovine serum. For transient transfections, cells were transfected with 0.5 μ g of an expression vector encoding SV40-*Renilla* luciferase (Promega) and 2 μ g of a firefly luciferase pGL3 or pGL4 fusion gene plasmid using the lipofectamine reagent (GibcoBRL) as described previously (Bischof *et al.* 2001). Following overnight incubation in serum-containing medium, cells were harvested by trypsin digestion and then solubilized in passive lysis buffer (Promega). After two cycles of freeze/thawing, firefly and *Renilla* luciferase activity were assayed using the Promega Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. To correct for variations in transfection efficiency, the results are expressed as the ratio of firefly:*Renilla* luciferase activity. In addition, three independent preparations of each fusion gene plasmid construct were analyzed in triplicate.

For stable transfections, cells were transfected with 2 μ g of a firefly luciferase pGL4 fusion gene plasmid using the lipofectamine reagent as described previously (Bischof *et al.* 2001). Three independent preparations of each fusion gene plasmid construct were analyzed in triplicate. Following overnight culture at 37 °C, cells were incubated with serum-containing medium supplemented with hygromycin (Sigma–Aldrich) at a final concentration of 500 μ g/ml. Every 2–3 days, hygromycin-containing medium was replaced. After 3–4 weeks, or until individual colonies could be visualized, colonies were pooled using trypsinization, transferred

to 10 cm dishes, and hygromycin treatment was discontinued. Once the cells had reached confluence, luciferase activity in the pooled cultures was then assessed and the Pierce BCA protein assay used to correct for variations in protein content.

Statistical analyses

The transfection data were analyzed for differences from the control values, as specified in the figure legends. Statistical comparisons were calculated using an unpaired Student's *t*-test. The level of significance was $P < 0.05$ (two-sided test).

Results

The human *SLC30A8* gene is expressed in both pancreatic islet α and β cells

Chimienti *et al.* (2004) had suggested that in pancreatic islets, expression of the mouse *Slc30a8* gene, which encodes the zinc transporter ZnT-8, is restricted to insulin-secreting β cells, but recent data suggest that it is also expressed in glucagon-secreting α cells (Gyulkhanyan *et al.* 2008, Pound *et al.* 2009). To assess the expression pattern of human *SLC30A8*, immunohistochemical staining was performed on human pancreas sections. Figure 1 shows that ZnT-8 was absent from exocrine tissue but was detected in both α and β cells in human pancreatic islets, consistent with observations made in mice (Gyulkhanyan *et al.* 2008, Pound *et al.* 2009).

Highly conserved putative human *SLC30A8* and mouse *Slc30a8* promoter regions are unable to drive reporter gene expression in transient transfection experiments

To identify candidate regions of the human *SLC30A8* and mouse *Slc30a8* genes that may serve as promoters, a sequence alignment using the VISTA program (Frazer *et al.* 2004) was performed with human and mouse gene sequence 10 kbp 5' of the translation start site (Chimienti *et al.* 2004). This analysis identified several highly conserved regions in the mouse and human genes in the area located ~ 8000 and 6000 bp, respectively, 5' of the translation start site (Fig. 2). Figure 2 also shows that, while the conserved regions are the same, the spacing between conserved regions is reduced in the human promoter. An analysis of *SLC30A8*-expressed sequence tags in the NCBI nucleotide database suggests that *SLC30A8* gene transcription initiates at multiple start sites in a ~ 500 bp region located immediately 5' of the translation initiation codon, consistent with the absence of a consensus TATA box sequence in this location (Smale 1997). To investigate whether this region represents the *SLC30A8* promoter, a *SLC30A8*-luciferase fusion gene was constructed, containing putative promoter sequence between -6154 and -1 , relative to the translation start site, and transiently transfected into β TC-3 cells, an islet β cell-derived cell line (Efrat *et al.* 1988). Basal luciferase expression directed by this fusion gene was below the level obtained with the promoterless luciferase expression vector (Fig. 3A). Identical results were obtained in transient transfection experiments in HIT

Figure 1 Immunohistochemical detection of ZnT-8 in human pancreas. Paraffin-embedded sections of adult human pancreata were immunostained for the presence of insulin (green) and glucagon (blue) and ZnT-8 (red) as described in the Materials and methods section. A representative section is shown. Scale bar: 10 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0055>.

Figure 2 The putative human *SLC30A8* and mouse *Slc30a8* promoters are highly conserved. The figure shows a VISTA browser plot (Frazer *et al.* 2004) comparing 10 kbp of human *SLC30A8* versus mouse *Slc30a8* (A) and mouse *Slc30a8* versus human *SLC30A8* (B) gene sequence. The translation start site is at bp 10 000 and the level of conservation is indicated on the vertical axis. Conserved regions above 70%/100 bp are highlighted in gray and three putative promoter enhancer regions in the mouse *Slc30a8* promoter are designated 1, 2, and 3. It is shown that, while the conserved regions are the same, the spacing between conserved regions is reduced in the human promoter.

cells, an alternate islet β cell-derived cell line, and with *SLC30A8*-luciferase fusion genes in both the pGL3 and the pGL4 luciferase vectors (data not shown). A *Slc30a8*-luciferase fusion gene containing putative mouse *Slc30a8* promoter sequence between -1803 and -1 was also constructed and analyzed by transient transfection in both cell lines and in both luciferase vectors. As with the putative human promoter, the putative mouse promoter was also incapable of driving reporter gene expression (Fig. 3B and data not shown). The results of stable transfection experiments (see below) suggest that these human and mouse DNA fragments are not acting as repressors. Rather the reductions in reporter gene expression below the level obtained with the promoterless luciferase expression vector (Fig. 3A and B) are more likely due to the presence of *SLC30A8* and *Slc30a8* genomic DNA further reducing low non-specific read-through transcription.

A possible explanation for the lack of promoter activity was that a cryptic RNA secondary structure, forming between the luciferase coding sequence and the 5'-untranslated region located between the transcription start site and translation start site, was impairing mRNA translation (Kozak 1989). Fusion genes were therefore generated containing genomic sequence that terminated at known human ($-6154/-235$; NM_173851) and chimpanzee ($-6154/-522$; XM_519918) transcription start sites (Fig. 3A) or at a known mouse ($-1803/-335$; NM_172816) transcription start site (Fig. 3B). However, none of these fusion genes conferred reporter gene expression in transient transfection assays (Fig. 3A and B).

Identification of human *SLC30A8* and mouse *Slc30a8* promoter regions that drive reporter gene expression in stable transfection experiments

Another possible explanation for the lack of fusion gene expression in transient transfection experiments was the known differences in chromatin structure between that on endogenous promoters and that on transiently transfected plasmid templates (Smith & Hager 1997). Thus, previous studies have demonstrated the potential for major differences in expression levels when fusion genes are compared using transient and stable transfection (Smith & Hager 1997). Some promoters/enhancers are only active, or are much more active, when analyzed in stable transfections (Smith & Hager 1997). To assess whether chromatin structure was essential for human *SLC30A8* and mouse *Slc30a8* promoter function, luciferase fusion genes containing either the human *SLC30A8* sequence from -6154 to -1 or the mouse *Slc30a8* sequence from -1803 to -1 were analyzed in the context of the pGL4 vector. This vector contains a hygromycin resistance gene, which allows for negative selection. β TC-3 cells were stably transfected with these human or mouse promoter-luciferase fusion genes and treated with hygromycin until individual colonies were visualized. The individual colonies were then pooled and luciferase activity assayed. Under these conditions, the human *SLC30A8* (Fig. 3C) and mouse *Slc30a8* (Fig. 3D) genomic regions analyzed both drove fusion gene expression at much higher levels than the promoterless pGL4 vector, indicating the presence of functional promoters. A difference in activity was observed between the human and mouse promoters, but presumably this is mainly because the human promoter fragment used (Fig. 3C) was much longer than the mouse (Fig. 3D) and is likely to contain additional enhancer regions (Fig. 2). Although zinc has been reported to regulate *Slc30a8* gene expression in INS-1E cells (Smidt *et al.* 2009), no effect of zinc on human *SLC30A8* promoter activity was observed in the stable cell lines (Supplementary Figure 1, see section on supplementary data given at the end of this article), suggesting that the effect of zinc is mediated through other genomic regions or post-transcriptionally.

The VISTA analysis identified three conserved regions in the mouse *Slc30a8* promoter, located between approximately (-7751 and -7250), (-5498 and -4730) and (-3163 and -2698) (Fig. 2B). These regions are found in similar locations in the human *SLC30A8* gene (Fig. 2A). We hypothesized that these regions might represent transcriptional enhancers important for islet-specific expression of the *Slc30a8* gene. To address this hypothesis, these regions, designated as promoter enhancers 1, 2, and 3 (Fig. 2B), were isolated and ligated 5' of a heterologous

thymidine kinase (*TK*)-luciferase fusion gene containing *TK* genomic sequence between -105 and $+51$, relative to the transcription start site (Vander Kooi *et al.* 2005). Luciferase expression directed by these fusion genes was then analyzed by transient transfection of β TC-3 cells. None of these *Slc30a8* promoter regions enhanced reporter gene expression beyond that driven by the *TK*-luciferase fusion gene alone, regardless of orientation (Supplementary

Figure 2, see section on supplementary data given at the end of this article). Similarly, when ligated 5' of a heterologous *G6PC2*-luciferase fusion gene containing the proximal human *G6PC2* promoter sequence between -150 and $+3$, again relative to the transcription start site, none of these *Slc30a8* promoter regions enhanced reporter gene expression beyond that driven by the $-150/+3$ *G6PC2*-luciferase fusion gene alone, regardless of orientation (Supplementary Figure 3, see section on supplementary data given at the end of this article). These data suggest that none of these promoter regions are enhancers.

A conserved islet β cell-specific enhancer is located in the second intron of the *SLC30A8* gene

Intronic enhancers have previously been identified in several genes whose expressions are enriched in islets including those encoding glucagon (Zhou *et al.* 2006), islet amyloid polypeptide (Ekawa *et al.* 1997), and *G6pc2* (Wang *et al.* 2008). We also recently identified an intronic enhancer located between $+20\,125$ and $+20\,745$ in intron 2 in the mouse *Slc30a8* gene (Pound *et al.* 2010). A sequence alignment using the VISTA program shows that this region is conserved and found at a location between $+16\,333$ and $+16\,954$ in intron 2 in the human *SLC30A8* gene (Fig. 4).

Figure 3 The putative human *SLC30A8* and mouse *Slc30a8* promoter regions are able to drive reporter gene expression in stable but not transient transfection experiments. (A and B) β TC-3 cells were transiently co-transfected, as described in the Materials and Methods section, with the empty pGL3 vector or the indicated human *SLC30A8* (A) or mouse *Slc30a8* (B) promoter-firefly luciferase fusion genes ($2\,\mu\text{g}$) and an expression vector encoding *Renilla* luciferase ($0.5\,\mu\text{g}$). Following transfection, cells were incubated for 18–20 h in serum containing medium and then harvested and luciferase activity assayed. Results are presented as the ratio of firefly:*Renilla* luciferase activity, expressed relative to the ratio obtained with the empty pGL3 vector, and represent the mean $\pm$ s.e.m. of three experiments each using an independent preparation of all fusion gene plasmids with each plasmid assayed in triplicate. * $P < 0.05$. (C and D) β TC-3 cells were transfected with the empty pGL4 vector or the indicated human *SLC30A8* (C) or mouse *Slc30a8* (D) promoter-firefly luciferase fusion genes ($2\,\mu\text{g}$). Following transfection, cells were incubated for 18–20 h in serum containing medium after which cells were incubated with media containing $500\,\mu\text{g/ml}$ hygromycin. After 3–4 weeks, or until individual colonies could be visualized, colonies were pooled using trypsinization, transferred to 10 cm dishes, and hygromycin treatment was discontinued. Once the cells had reached confluence, luciferase activity in the pooled cultures was then assessed and the Pierce BCA protein assay used to correct for variations in protein content. Results are presented as the ratio of firefly activity to protein content in the cell lysate, expressed relative to the ratio obtained with the empty pGL4 vector, and represent the mean $\pm$ s.e.m. of three experiments each using an independent preparation of all fusion gene plasmids with each plasmid assayed in triplicate. * $P < 0.05$.

Figure 4 Identification of a highly conserved region within intron 2 of the human *SLC30A8* and mouse *Slc30a8* genes. A VISTA browser plot (Frazer *et al.* 2004) comparing the human *SLC30A8* and mouse *Slc30a8* gene sequences between exons 2 and 3 is shown. The level of conservation is indicated on the vertical axis. Conserved regions above 70%/100 bp are highlighted in gray. Exons 2 and 3 and the putative intron 2 enhancer (Enh) are boxed.

Fusion gene analyses showed that the mouse *Slc30a8* enhancer in intron 2 was selectively active in β TC-3 but not in α TC-6 cells, indicating that this region is an islet β cell-specific enhancer (Pound *et al.* 2010). To determine whether the +16 579 and +16 954 region of intron 2 in the human *SLC30A8* gene also represents a transcriptional enhancer, this region was isolated using PCR and ligated 5' of the -150/+3 *G6PC2*-luciferase fusion gene described above. Luciferase expression directed by this fusion gene was then analyzed by transient transfection of β TC-3 and α TC-6 cells. Figure 5A shows that, in β TC-3 cells, this region elevated reporter gene expression beyond that driven by the -150/+3 *G6PC2*-luciferase fusion gene alone. This effect was dependent on orientation (Fig. 5A), which is not consistent with the strict definition of an enhancer (Blackwood & Kadonaga 1998). However, it is likely that, because of its close proximity to the *G6PC2* promoter, this *SLC30A8* enhancer is affecting transcription factor binding to the heterologous *G6PC2* promoter in a manner that is different to the mechanism by which it affects *SLC30A8* promoter activity from its native location in *SLC30A8* intron 2. Strikingly, Fig. 5B shows that, in α TC-6 cells, this region did not elevate reporter gene expression beyond that driven by the -150/+3 *G6PC2*-luciferase fusion gene alone. These data demonstrate that this region is an islet β cell-specific enhancer and suggest that the mechanisms controlling *SLC30A8* expression in α and β cells (Fig. 1) are overlapping but distinct.

Discussion

In this study, we show that the human *SLC30A8* gene, like the mouse *Slc30a8* gene, is expressed in both pancreatic islet α and β cells (Fig. 1) and we identify a conserved *SLC30A8* promoter region that drives fusion gene expression in stable, though not transient, transfections (Figs 2 and 3). Though it is uncommon

for promoters/enhancers to require a precise chromatin context to function, several examples do exist (Smith & Hager 1997). In addition, we have identified a conserved islet β cell-specific enhancer in *SLC30A8* intron 2 (Figs 4 and 5). A SNP, rs62510556, within this enhancer affects the binding of an unknown transcription factor and modulates enhancer activity (Supplementary Figure 4, see section on supplementary data given at the end of this article); however, genetic analyses suggest that, in Europeans, rs62510556 is not a causal variant that contributes to the association between *SLC30A8* and T2D (Supplementary Table 1, see section on supplementary data given at the end of this article). Future studies will examine whether an association exists in other populations. Thus, the

Figure 5 Analysis of the transcriptional enhancer activity of the highly conserved region within intron 2 of the human *SLC30A8* gene in the context of the *G6PC2* promoter. β TC-3 (A) and α TC-6 (B) cells were transiently co-transfected, as described in the Materials and methods section, with the indicated firefly luciferase fusion genes (2 μ g) and an expression vector encoding *Renilla* luciferase (0.5 μ g). The fusion genes represent the enhancerless human *G6PC2* promoter-luciferase vector (V) or the *SLC30A8* intron 2 sequence between +16 579 and +16 954 ligated to the human *G6PC2* promoter-luciferase vector in the correct (C) or inverted (I) orientation relative to the endogenous *SLC30A8* gene. Following transfection, cells were incubated for 18–20 h in serum-containing medium and then harvested and luciferase activity assayed. Results are presented as the ratio of firefly:*Renilla* luciferase activity, expressed relative to the ratio obtained with the enhancerless *G6PC2* heterologous vector, and represent the mean $\pm$ S.E.M. of three experiments each using an independent preparation of all fusion gene plasmids with each plasmid assayed in triplicate. * $P < 0.05$.

rs13266634 *SLC30A8* variant, which changes amino acid 325 of human ZnT-8, is associated with T2D risk in Asians and Europeans, but not in Africans (Xu *et al.* 2011). Similarly, a variant, rs560887, in the *G6PC2* gene has been associated with increased susceptibility to the development of T2D in some populations (Hu *et al.* 2009, 2010, Reiling *et al.* 2009) but not others (Dupuis *et al.* 2010). These observations emphasize the importance of assessing the potential causative involvement of SNPs and genes in multiple populations.

While genetic data have strongly linked the *SLC30A8* gene to increased susceptibility to the development of T2D in several, though not all, human populations (Xu *et al.* 2011), the manner in which defects in ZnT-8 expression or function lead to islet dysfunction and hence diabetes is unclear. While the *SLC30A8* rs13266634 variant has been reported to affect zinc transport by ZnT-8 in cultured cells (Nicolson *et al.* 2009), it does not affect glucose-stimulated insulin secretion from isolated human islets (Cauchi *et al.* 2010). However, this may simply relate to the small number of human islets examined (Cauchi *et al.* 2010) and the relatively small contribution of the *SLC30A8* rs13266634 variant to diabetes risk (Saxena *et al.* 2007, Scott *et al.* 2007, Sladek *et al.* 2007, Zeggini *et al.* 2007). Surprisingly, though, studies in genetically modified mice, in which more significant changes in *Slc30a8* expression can be achieved, have also failed to demonstrate a critical role for ZnT-8 in the control of glucose metabolism. Thus, mice in which the *Slc30a8* gene was globally deleted have normal glucose tolerance (Lemaire *et al.* 2009, Nicolson *et al.* 2009, Pound *et al.* 2009) and mice with a β cell-specific *Slc30a8* gene deletion have only mildly impaired glucose tolerance (Wijesekara *et al.* 2010). These observations suggest that either ZnT-8 is more important for the control of glucose metabolism in humans than mice or defects in glucose metabolism will only become apparent in older mice or mice harboring disease-associated variants in additional susceptibility genes. The latter possibilities appear more likely since the absence of ZnT-8 clearly affects β cell function, as apparent from the observed reduction in fasting plasma insulin in mice lacking ZnT-8 as well as glucose-stimulated insulin secretion and zinc content in islets isolated from those mice (Pound *et al.* 2009). Among these changes, the reduction in islet zinc content is quantitatively the most dramatic and also surprising, in relation to the mild metabolic phenotype observed, given the abundant literature supporting a key role of zinc in the proper maturation, storage, and secretion of insulin (Wijesekara *et al.* 2009). If future studies reveal that no association exists between rs62510556 and T2D, regardless of the population studied, this may indicate that the rs62510556 SNP does not change *SLC30A8* expression sufficiently *in vivo* to have a biological effect

or that the presence of a variant form of ZnT-8, as manifest by the rs13266634 SNP, increases T2D risk in some populations whereas changes in *SLC30A8* expression do not. This last possibility is particularly intriguing given the very mild phenotype of *Slc30a8* knockout mice since it would raise the interesting concept that perhaps it is the presence of a mutant form of ZnT-8 that causes problems with β cell function whereas the absence of ZnT-8, as manifest in *Slc30a8* knockout mice, is less deleterious.

The mild phenotype observed in *Slc30a8* knockout mice may also be partly explained by the existence of unknown compensatory changes that have occurred during development to offset the absence of ZnT-8. Indeed, using an shRNA-mediated approach to down-regulate *Slc30a8* mRNA expression by >90% in the INS-1 pancreatic β cell line, Fu *et al.* (2009) demonstrated diminished uptake of exogenous zinc, reduced insulin content, and decreased glucose-stimulated insulin secretion. They also predicted that SNPs that affect *SLC30A8* expression, such as the one reported here, would have the potential to affect insulin secretion and the glycemic response *in vivo* (Fu *et al.* 2009).

In summary, these experiments have shown that the human *SLC30A8* genomic region located between -6154 and -1, relative to the translation start site, contains a promoter that is only active in stable and not transient transfections. In addition, the human *SLC30A8* genomic region located between +16 579 and +16 954 in intron 2 contains a conserved islet β cell-specific enhancer. Given the critical role of ZnT-8 in islet zinc metabolism and the association between *SLC30A8* and T2D risk, it is possible that SNPs that affect *SLC30A8* expression, rather than ZnT-8 function, may also influence T2D risk. While we find that a SNP, rs62510556, in the conserved intron 2 enhancer, which modulates enhancer activity, is not associated with T2D risk, at least in Europeans, it is possible that future studies may uncover rare SNPs with a more pronounced effect on *SLC30A8* expression that do influence T2D risk.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-11-0055>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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