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Decreased TCF7L2 protein levels in type 2 diabetes mellitus correlate with downregulation of GIP- and GLP-1 receptors and impaired beta-cell function

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Recent human genetics studies have revealed that common variants of the *TCF7L2* (T-cell factor 7-like 2, formerly known as TCF4) gene are strongly associated with type 2 diabetes mellitus (T2DM). We have shown that TCF7L2 expression in the β-cells is correlated with function and survival of the insulin-producing pancreatic β-cell. In order to understand how variations in *TCF7L2* influence diabetes progression, we investigated its mechanism of action in the β-cell. We show robust differences in *TCF7L2* expression between healthy controls and models of T2DM. While mRNA levels were approximately 2-fold increased in isolated islets from the diabetic *db/db* mouse, the Vancouver Diabetic Fatty (VDF) Zucker rat and the high fat/high sucrose diet-treated mouse compared with the non-diabetic controls, protein levels were decreased. A similar decrease was observed in pancreatic sections from patients with T2DM. In parallel, expression of the receptors for glucagon-like peptide 1 (GLP-1R) and glucose-dependent insulinotropic polypeptide (GIP-R) was decreased in islets from humans with T2DM as well as in isolated human islets treated with siRNA to *TCF7L2* (siTCF7L2). Also, insulin secretion stimulated by glucose, GLP-1 and GIP, but not KCl or cyclic adenosine monophosphate (cAMP) was impaired in siTCF7L2-treated isolated human islets. Loss of *TCF7L2* resulted in decreased GLP-1 and GIP-stimulated AKT phosphorylation, and AKT-mediated Foxo-1 phosphorylation and nuclear exclusion. Our findings suggest that β-cell function and survival are regulated through an interplay between TCF7L2 and GLP-1R/GIP-R expression and signaling in T2DM.

INTRODUCTION

The strong linkage of single nucleotide polymorphisms in the *TCF7L2* gene with type 2 diabetes mellitus (T2DM) has been reported in worldwide studies (1,2). The at-risk alleles of *TCF7L2* are associated with severe diabetic phenotypes characterized by impaired β-cell function and a reduction in glucagon-like peptide 1 (GLP-1)-induced potentiation of insulin secretion and fasting and postprandial hyperglycemia (3–5). *TCF7L2* is a Wnt signaling-associated transcription factor expressed in several tissues, including the gut and the pancreas. Wnt signaling plays an important role in β-cell proliferation and insulin secretion (6,7) and influences synthesis of GLP-1 in intestinal L-cells (8). The two incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide GIP (also known as gastric inhibitory polypeptide) are important targets for diabetes therapy because of their ability to potentiate glucose-stimulated insulin secretion as well as to promote

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β-cell proliferation and survival (9). While GLP-1 secretion in carriers of the *TCF7L2* risk allele during an oral glucose tolerance test (OGTT) is normal, there is a significant reduction in GLP-1-induced insulin secretion suggesting the presence of a direct defect of GLP-1 signaling in the insulin-secreting cell (5).

In our previous study we found that reducing *TCF7L2* gene expression by siRNA in isolated human and rodent islets resulted in increased β-cell apoptosis and impaired function. In contrast, overexpression of *TCF7L2* protected islets from high glucose- and cytokine-induced apoptosis and impaired function (10), a finding that has been confirmed recently (11).

Very little is known about how *TCF7L2* expression is regulated during the development of diabetes. *TCF7L2* expression in adipose tissue is decreased in obese subjects with T2DM (12). In contrast, *TCF7L2* mRNA is increased in islets isolated from the Zucker diabetic fatty (ZDF) rat (13) and from patients with T2DM or individuals carrying an increased number of *TCF7L2* risk T-alleles (4). This apparent increase in *TCF7L2* mRNA in diabetes appears to be in contradiction to the impairment of β-cell function and survival in pancreatic islets following siRNA-induced loss of *TCF7L2* expression (10). However, it is important to emphasize that none of the studies to date have examined TCF7L2 protein levels in health and during progression to diabetes. Therefore, we posed the following question: can the loss of β-cells in T2DM be explained by altered regulation of TC7L2 at a post-transcriptional level.

RESULTS

Reciprocal changes in islet TCF7L2 mRNA and protein levels in human and rodent type 2 diabetes mellitus

Increased *TCF7L2* mRNA levels in isolated islets are correlated with T2DM (4). Here, we examined TCF7L2 protein expression in human pancreatic sections of seven healthy controls and seven patients with T2DM (see Supplementary Material, Table S1). Triple staining for TCF7L2, insulin and DAPI (4′,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to DNA) revealed localization of TCF7L2 in healthy control β-cells that was mostly, but not exclusively localized in the nucleus (Fig. 1A). In contrast, TCF7L2 was almost undetectable in all sections analyzed from patients with T2DM. Subsequently, we analyzed TCF7L2 mRNA and protein expression in three rodent models for T2DM, a diet-induced obesity model (HFD), the leptin receptor deficient *dbdb* mouse and the Vancouver Diabetic Fatty (VDF) Zucker rat. All diabetic animals showed impairments in fasting glucose and glucose tolerance during intraperitoneal glucose tolerance tests (IPGTT) or OGTT (Fig. 1E,I,M) confirming previous reports (14–17).

With the diet-induced obesity mouse model, C57Bl/6J mice were fed a high fat/high-sucrose diet 'Surwit', resulting in hyperinsulinemia and impaired glucose tolerance after 4 weeks, impaired fasting glucose after 8 weeks, hyperglycemia and loss of glucose-stimulated insulin secretion after 12 weeks of the HFD (14). Levels of TCF7L2 protein from isolated islets after 12 weeks of diet were decreased by 47% ($P < 0.01$) in HFD mice compared with normal diet (Fig. 1B and C), but there was a 2-fold increase in mRNA ($P < 0.05$, Fig. 1D). IPGTT in the mice showed higher glucose levels at all time points after glucose injection as well as a 2.1-fold increase in fasting glucose ($P < 0.01$; Fig. 1E).

Similar results were achieved in the *db/db* mouse (Fig. 1F–I). While islet TCF7L2 protein levels were almost undetectable (decreased by 56% compared with heterozygous controls, $P < 0.01$; Fig. 1F and G), mRNA was 2.5-fold increased ($P < 0.01$; Fig. 1H). Glucose tolerance was impaired and fasting glucose was 1.4-fold increased in the 8-week old *db/db* mice compared with their heterozygous db/+ control mice ($P < 0.01$; Fig. 1I).

In the VDF rat, we found a dramatic reduction of TCF7L2 protein in isolated islets from diabetic fa/fa mice, compared with those from lean Fa/? littermates (88% reduction; Fig. 1J and K), together with a tendency to increased mRNA expression (Fig. 1L). The latter is in agreement with a previous study, in which mRNA analyzed by oligonucleotide microarray demonstrated a significant 3-fold increase in the obese ZDF rat (13). Glucose tolerance was impaired, and fasting glucose was 1.7-fold increased in the 8-week-old VDF rats when compared with their lean controls ($P < 0.01$; Fig. 1M).

Depletion of *TCF7L2* results in decreased GLP-1R and GIP-R expression in human islets

GLP-1 and GIP act through their cognate G-protein-coupled receptors (GLP-1R and GIP-R), which are expressed in several tissues; including the stomach, lung, duodenum, hypothalamus and pancreatic islets (18). We investigated the localization of GLP-1R and GIP-R in human pancreatic tissue of healthy and T2DM individuals. Triple-staining for GLP-1R/GIPR, insulin and glucagon revealed that GLP-1R was exclusively expressed in β-cells, in agreement with previous reports (19), whereas GIP-R was expressed in α and β-cells (Fig. 2A, upper panel). In pancreatic tissue of T2DM individuals, both GLP-1R and GIP-R expression were downregulated when compared with healthy controls (Fig. 2A, lower panel).

Since we previously showed that loss of *TCF7L2* expression results in significant impairment of β-cell function and survival, we examined whether this process is mediated through GLP-1R/GIP-R signaling in the β-cell. First, we exposed isolated human pancreatic islets to siRNA directed to *TCF7L2* (siTCF7L2) or scrambled control siRNA (siScr) or transfected with either pCMV-TCF7L2 or an empty plasmid under control of the same promoter as described (10) and cultured for 4 days in the presence of increasing glucose concentrations (5.5–33.3 mM) or to a mixture of cytokines (2 ng/ml IL-1β+1000 U/ml IFN-γ).

SiTCF7L2 resulted in the downregulation of both GLP-1R (62%, $P < 0.01$; Fig. 2B and C) and GIP-R (51%, $P < 0.01$; Fig. 2D and E). Increasing the glucose concentrations (to 11.1 mM-data not shown) to 33.3 mM as well as the exposure to the cytokine mixture also decreased GLP-1R and GIP-R expression, a similar response to that reported for TCF7L2 protein levels (10). Overexpression of *TCF7L2* restored GLP-1R and GIP-R protein expression in islets, suggesting

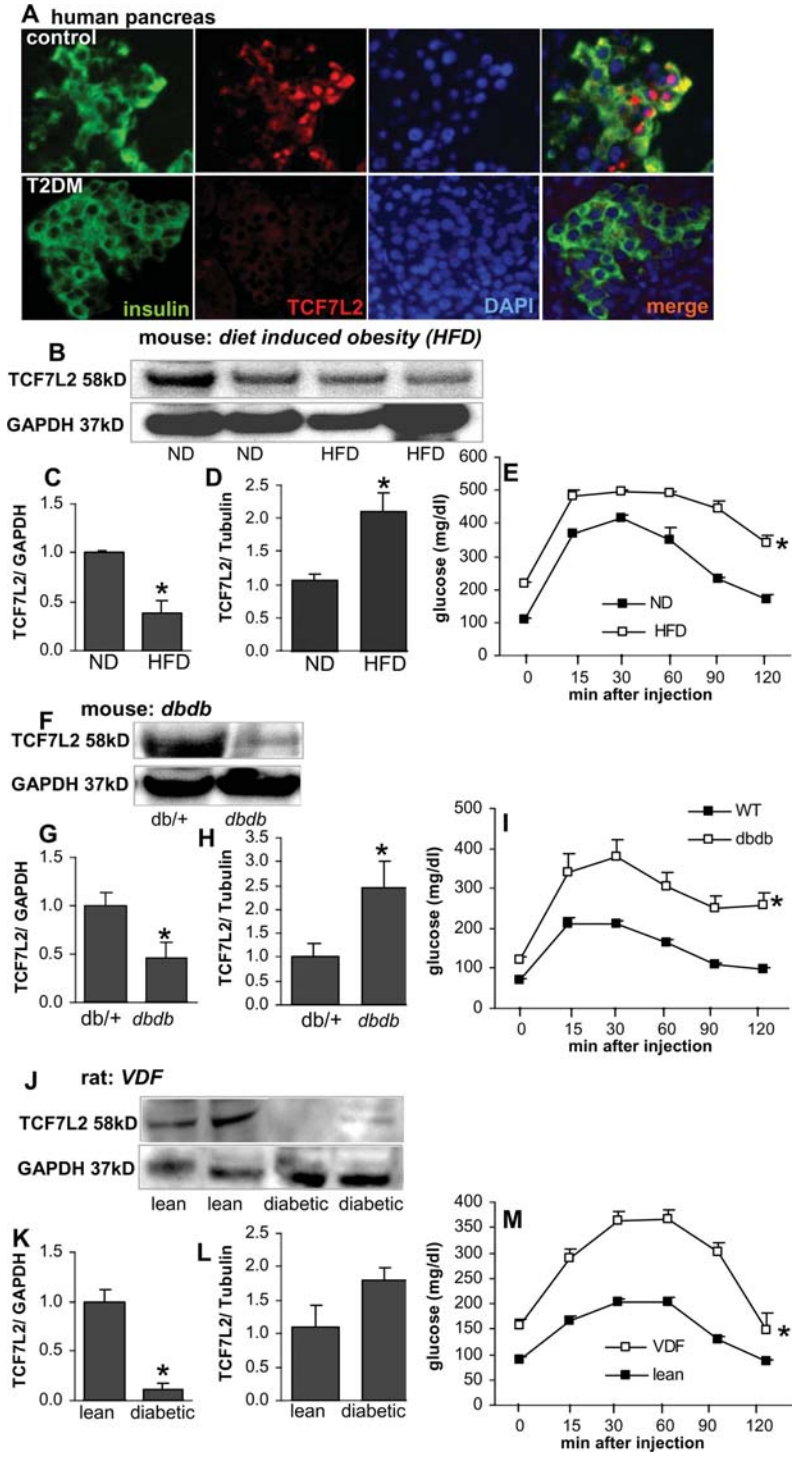

Figure 1. Reciprocal changes in *TCF7L2* mRNA and protein in human and rodent type 2 diabetes mellitus. (**A**) TCF7L2 protein expression assay in human pancreatic sections. Triple staining for TCF7L2 in red, insulin in green and DAPI in blue in human pancreatic sections from seven poorly controlled T2DM patients and seven healthy controls. (**B**–**E**) C57Bl/6J mice were fed a ND (normal diet) or HFD (high-fat/high sucrose diet) and intraperitoneal glucose tolerance tests (IPGTTs) were performed after 12 weeks of diet, their islets isolated and protein and mRNA extracted. (**F**–**I**) Eight-week-old db/db and their heterozygous db/+ littermates and (**J**–**M**) 8–10-week-old Vancouver diabetic fatty (VDF) Zucker rats (fa/fa) and their lean littermates (Fa/?) were used for the experiments. (E, I) Intraperitoneal or oral (M) glucose tolerance tests (IPGTT/OGTT) were performed after 12 h fast with 1 g/kg body weight glucose. Tests were performed in four independent experiments with a total of 16 mice in each group. $^{*}P < 0.01$ to control animals at all time points during the IPGTT/OGTT. (B–D,F–H,J–L) Islets were isolated from 16 mice each group and western blot (B,C,F,G,J,K) and quantitative RT-PCR (D,H,L) analyses of TCF7L2 protein and mRNA were performed in four independent experiments. (C,G,K) show densitometric analyses of western blot results, normalized to GAPDH and expressed as change of control. The levels of *TCF7L2* mRNA expression were normalized against β-actin and tubulin with similar results and shown as change from control. Results are means ± SE. $^{*}P < 0.05$ to non-diabetic control animals.

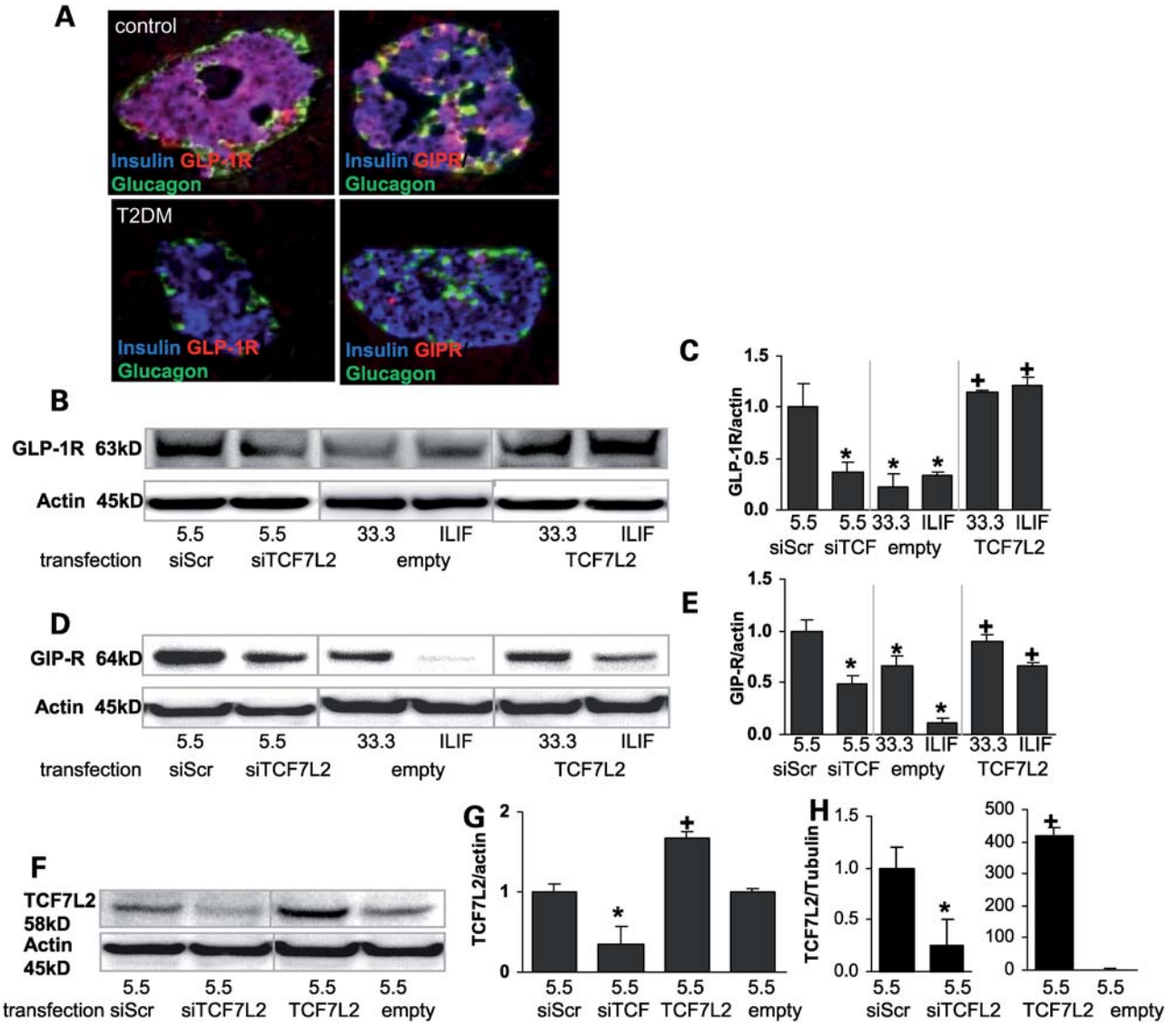

Figure 2. Depletion of *TCF7L2* results in decreased GLP-1R and GIP-R expression in human islets. (**A**) Triple staining for GLP-1R (left)/GIPR (right; red), insulin in blue and glucagon in green in human pancreatic sections from seven poorly controlled type 2 diabetic patients (T2DM; lower panel) and seven healthy controls (upper panel). (**B**–**H**) Human islets transfected with scrambled control (siScr) or TCF7L2 siRNA (siTCF7L2) or with pCMV-TCF7L2 or an empty control plasmid under the same promoter, were cultured on extracellular matrix-coated dishes for 4 days and exposed to increasing glucose concentrations (5.5, 33.3 mM) or the mixture of 2 ng/ml IL-1β+1000 U/ml IFN-γ (ILIF). Islet lysates were collected for western blot analysis of GLP-1R (**B,C**) and GIPR (**D,E**) and TCF7L2 (**F,G**). (C,E,G) show densitometric analyses of western blot results, normalized to actin and expressed as change of control ± SE. Western blots are representative of three (B–E) or seven (F–H) independent experiments from three/seven different organ donors. (**H**) Quantitative mRNA analysis of the downregulation of *TCF7L2* achieved by the siRNA (left panel) and upregulation achieved by pCMV-TCF7L2 (right panel). $^*P < 0.05$ to siScr-treated islets, $+P < 0.05$ to islets transfected with empty control vector at the same treatment conditions.

a direct interplay between TCF7L2 and GLP-1R/GIP-R (Fig. 2B–E). In all experiments, level of TCF7L2 downregulation achieved by the siRNA transfection and level of overexpression achieved by the pCMV-TCF7L2 transfection was analyzed by western blot analysis (Fig. 2F and G) and RT–PCR (Fig. 2H). SiTCF7L2 decreased *TCF7L2* mRNA levels by 75% and protein levels by 65% compared with siScr-treated islets ($P < 0.01$). *TCF7L2* overexpression resulted in a 416- and 1.7-fold induction of TCF7L2 mRNA and protein levels, respectively, compared with empty vector-transfected controls.

TCF7L2 is important for regulation of AKT activation and FOXO1 trafficking

Depletion of *TCF7L2* resulted in reduced, but not depleted, islet levels of GLP-1R/GIP-R. To determine whether this reduction was associated with functional changes, responses of the downstream kinase AKT were examined. Both GLP-1 and GIP exert their protective functions through pathways involving phosphorylation of serine 473 and activation of AKT (20–22). As we reported before, loss of *TCF7L2* leads

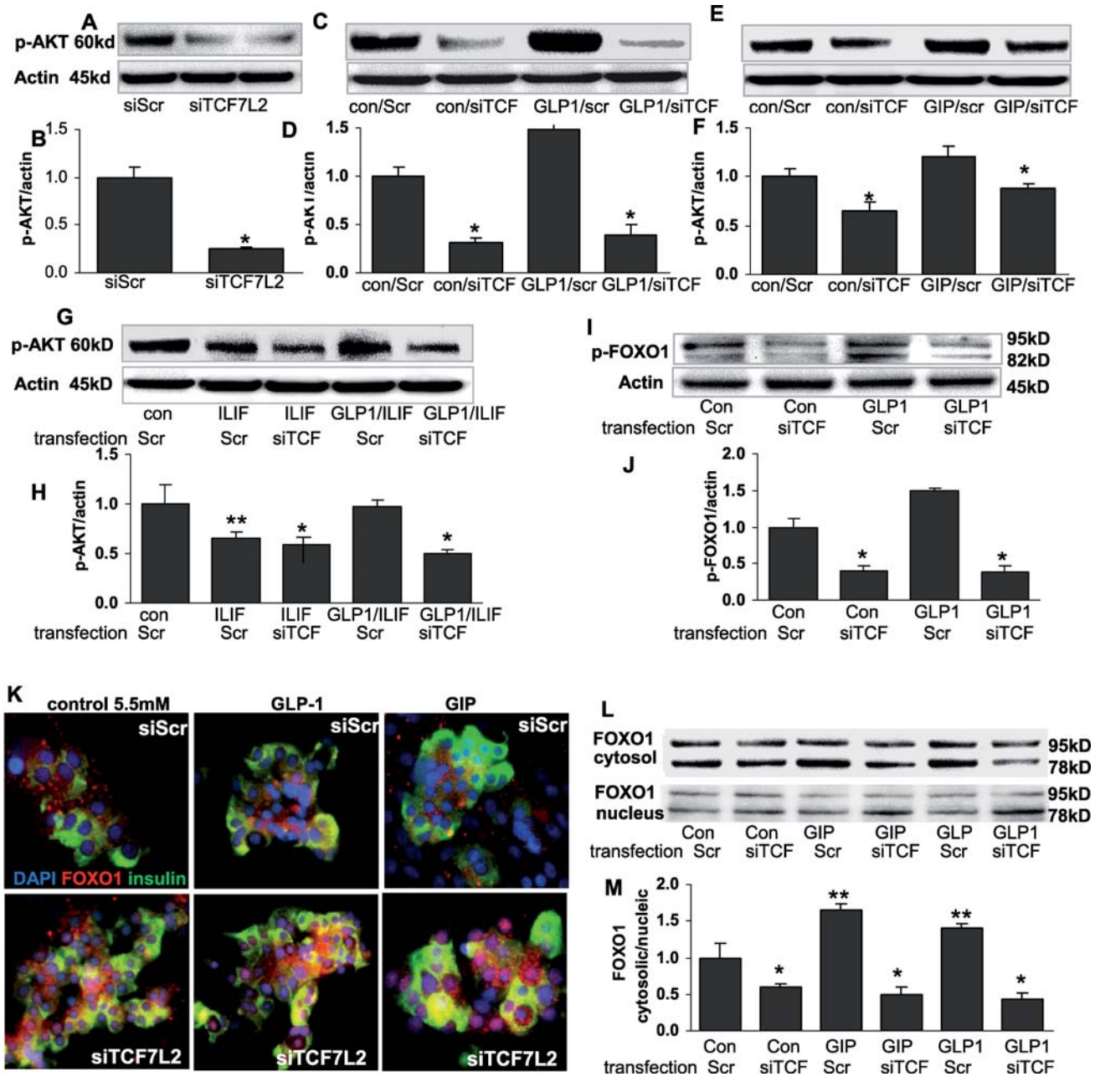

Figure 3. *TCF7L2* is important for regulation of AKT activation and FOXO1 trafficking. Isolated human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to siTCF7L2 or scrambled control siRNA (siScr) for 4 days with or without exposure to 100 nM GLP-1 or 10 nM GIP for the whole 4-day culture period (**B**–**F**) or for the last 4 h of prior to addition of 2 ng/ml IL-1β+1000 U/ml IFN-γ (ILIF) for the last 2 h of the experiment (**G,H**). Western blot analysis for p-AKT, Ser473 (**A**–**H**) or p-FOXO1 (**I,J**) was performed, actin was used as loading control on the same membrane after stripping. (**B,D,F,H,J**) show densitometric analyses of western blot results, normalized to actin and expressed as change of control ± SE. (**K**) Fixed islets were triple-stained for Foxo1 in red, DAPI in blue and insulin in green. (**L,M**) FOXO1 localization is shown in treated islets by western blot analysis after extraction of nuclei and cytoplasm. (**M**) shows densitometric analyses of the western blots of cytosolic FOXO1 normalized to nuclear FOXO1 and expressed as change of control ± SE. Western blots are representative of three independent experiments from three different organ donors. $^{*}P < 0.05$ to siScr-transfected islets at the same treatment; $^{**}P < 0.05$ to siScr-transfected control islets.

to impaired AKT activation (10). In parallel to significant reductions in basal p-AKT levels (detected by an antibody to phosphorylated AKT, Ser473; Fig. 3A and B) by *TCF7L2* depletion, also the response to GLP-1/GIP stimulation was disturbed (Fig. 3C–F). In previous studies it was shown that the cytokine mixture IL-1β/IFN-γ decreases p-AKT, a response that is prevented by GLP-1 treatment (20). Using the same experimental condition of this previous study, GLP-1 was unable to reverse the effect of treatment with the cytokine mixture IL-1β (2 ng/ml) and IFN-γ (1000 IU/ml) in TCF7L2-depleted islets (Fig. 3G and H). In view of the fact that basal and GLP-1/GIP-stimulated levels of p-AKT were

reduced in TCF7L2-depleted islets, we examined a downstream target of AKT, FOXO1, a member of the forkhead transcription factor family. Nuclear FOXO1 acts as a transcription factor when localized in the nucleus but it translocates to the cytoplasm when phosphorylated by AKT (23). As expected, GLP-1-induced Foxo1 phosphorylation in control islets but not in TCF7L2-depleted islets (Fig. 3I and J). As reported before (24), the specific FOXO1 antibody recognized the p-FOXO1 at 82 kDa, but also a hyperphosphorylated FOXO1 was detected at 95 kDa. Both forms were similarly regulated by GLP-1. Densitometric analysis of both bands show 41% decrease in p-FOXO1 by siTCF7L2 (Fig. 3J). Triple-staining for Foxo1, insulin and DAPI in control islets revealed that both GLP-1- and GIP-stimulated FOXO1 nuclear exclusion (Fig. 3K, upper panel). In contrast, in TCF7L2-depleted islets FOXO1 was mainly localized in the nucleus and neither GIP nor GLP-1 had any effect on this distribution (Fig. 3K, lower panel). These results were confirmed by western blot analysis using nuclear and cytosolic extracts from human islets transfected with scrambled RNA or siTCF7L2 treated with GLP-1 or GIP (Fig. 3L and M). The FOXO1 antibody preferentially detects endogenous levels of total FOXO1 (78 kDa), but is also sensitive to phosphorylation (95 kDa). For densitometric analysis, we only included the 78 kDa band and calculated cytosolic over nuclear FOXO1 distribution. The amount of the cytosolic FOXO1 portion decreased by 40% in the siTCF7L2-treated islets. GIP or GLP-1 treatment resulted in a 1.6- and 1.4-fold induction of the cytosolic FOXO1 portion, respectively, in the control cells but not in the TCF7L-depleted cells (Fig. 3L and M).

Depletion in *TCF7L2* results in impaired β-cell function through suppression of GLP-1 and GIP-stimulated insulin secretion

GLP-1 and GIP potentiate glucose-stimulated insulin secretion through their cognate β-cell receptors (25). Since depletion of *TCF7L2* resulted in a loss of GLP-1R and GIP-R, we tested whether TCF7L2 also affects insulin secretion stimulated by GLP-1/GIP. We first analyzed the effect of *TCF7L2* depletion on insulin secretion stimulated by GLP-1 or GIP in human islets in static incubations. In confirmation of our previous study, siTCF7L2 led to impaired glucose-stimulated insulin secretion (GSIS) compared with siScr-treated islets. *TCF7L2* did not result in changes in basal insulin secretion at 2.8 mM (Fig. 4A and C) but induced a 1.6-fold reduction in insulin secretion at 16.7 mM glucose and a 2.2-fold reduction at 16.7 mM glucose+100 nM GLP-1 compared with siScr ($P < 0.05$, Fig. 4A). The associated glucose-stimulatory index shows a 1.6-fold reduction induced by *TCF7L2* depletion. GLP-1 induced a 1.5-fold induction of stimulated insulin secretion compared with 16.7 mM glucose stimulation alone ($P < 0.05$, Fig. 4B). In contrast, GLP-1-induced potentiation of GSIS was completely absent in the siTCF7L2-treated islets (Fig. 4B).

Similar results were obtained for GIP. While GIP induced a 1.3-fold induction of GSIS ($P < 0.05$, Fig. 4C), the GIP response was absent in the siTCF7L2-treated islets. The associated stimulatory index shows a 1.6-fold reduction at 16.7 mM glucose and a 2.0-fold reduction at GIP+16.7 mM glucose in the TCF7L2-depleted islets compared with siScr-treated islets ($P < 0.05$, Fig. 4D).

To analyze the dynamics of β-cell insulin secretion in greater detail, we performed islet perifusion studies in human isolated islets that had been pre-cultured for 4 days with siScr or siTCF7L2 as previously described (10). In our previous study we observed that insulin secretion induced by glucose and/or GLP-1 is impaired during the perifusion. Here, we show results on GIP-stimulated insulin secretion (Fig. 4E). In agreement with results in the static incubation experiments (Fig. 4C), depletion of *TCF7L2* resulted in an impaired response to glucose ($P < 0.05$ at all time points, Fig. 4E). As expected, siScr human islets demonstrated a typical glucose-stimulated first phase (approximately 2-fold increase from baseline, $P < 0.05$) and second phase (1.5-fold increase from baseline, $P < 0.05$) insulin secretory response as well as a robust (approximately 5-fold increase from baseline, $P < 0.05$) KCl maximally stimulated insulin response. In addition, GIP induced a rapid and robust potentiation of GSIS in human control islets where insulin levels were increased approximately 3-fold following the start of GIP infusion (Fig. 4E). In contrast, siTCF7L2-treated islets demonstrated significantly diminished first phase (68% decrease from controls, $P < 0.05$) and second phase (100% decrease from controls, $P < 0.05$) insulin secretion. Most importantly, siTCF7L2-treated islets failed to show a significant GIP-induced potentiation of GSIS, which is consistent with our previous findings with GLP-1 and with studies showing impaired GLP-1 and GIP-potentiated insulin secretion in humans carrying at-risk alleles of *TCF7L2*. Comparison of KCl-induced insulin secretion to basal secretion resulted in an average 4.20-fold stimulation in the siScr-treated islets and an average 4.24-fold stimulation in the siTCF7L2-treated islets. These and our previous data suggest an impaired glucose, GLP-1 and GIP-stimulated insulin secretion but an effective KCl-stimulated insulin secretion in *TCF7L2*-depleted islets.

To address this question in further detail, we performed static incubations in *TCF7L2*-depleted islets without GLP-1/GIP stimulation. One hour stimulation of the islets with 20 mM KCl induced a 3.9-fold and 3.6-fold increase in insulin secretion in the siScr and siTCF7L2-treated islets, respectively ($P < 0.05$, Fig. 4F). The stimulatory index was similar in siTCF7L2 and siScr, confirming an intact exocytotic machinery in the *TCF7L2*-deleted islets (Fig. 4F and G).

Both GLP-1 and GIP enhance glucose-stimulated insulin secretion via activation of adenyl cyclase and consequent rise in intracellular cAMP levels, which results in the increase in Ca^{2+} and subsequent stimulation of insulin secretion (26). To determine if the cAMP-induced sensitization of Ca^{2+} release and subsequent insulin secretion is active in *TCF7L2*-depleted islets, we incubated islets with agents that raise intracellular levels of cAMP (forskolin and 3-isobutyl-Methylxanthine/IBMX) independent of GLP-1R/GIP-R activation. One hour exposure of the islets to IBMX (100 μM)/Forskolin (10 μM) induced a 3.2-fold and 3.4-fold rise in insulin secretion compared with 2.8 mM glucose alone in the siScr and siTCF7L2 islets, respectively ($P < 0.05$, Fig. 4F). The stimulatory index was similar in

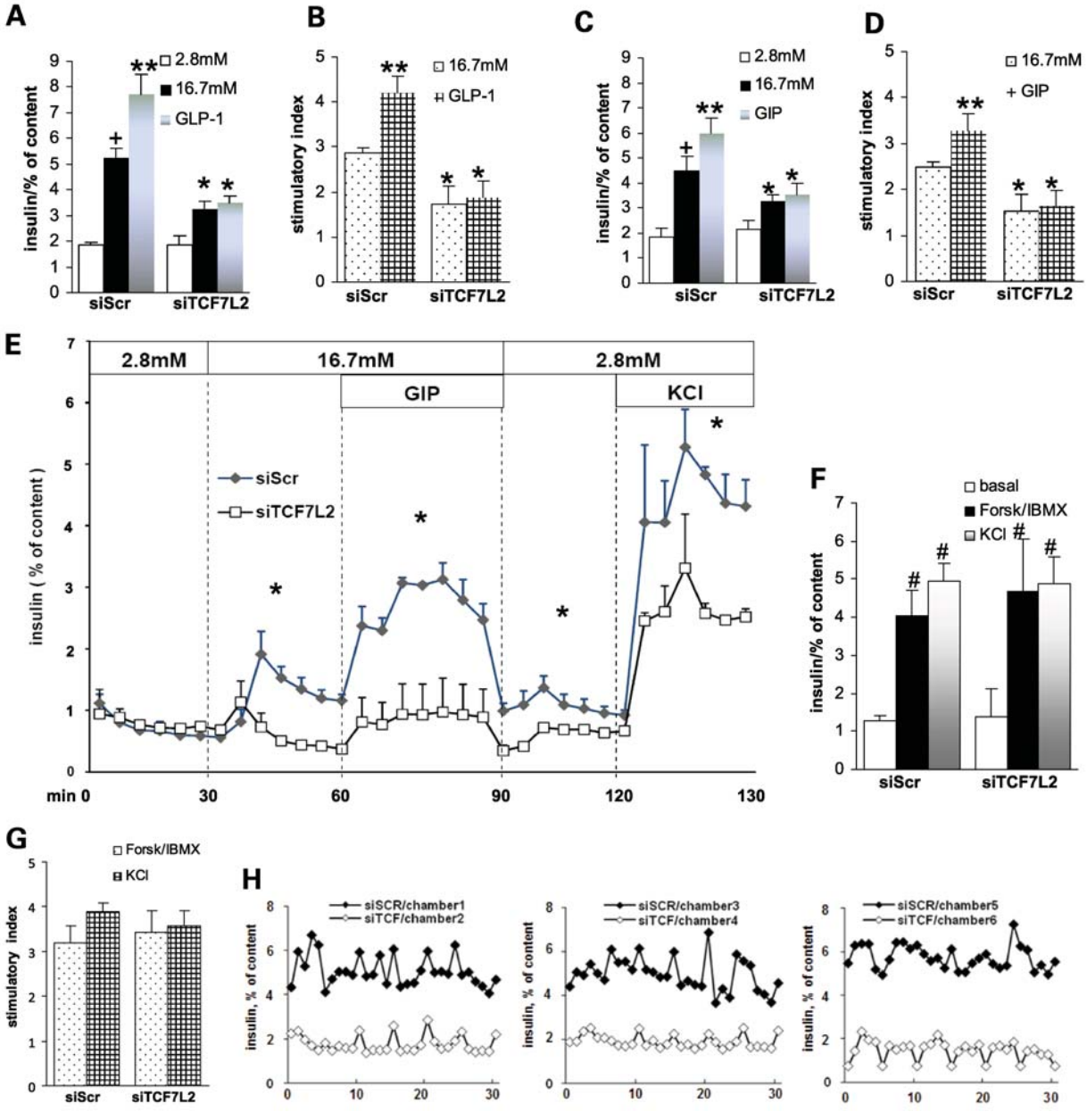

Figure 4. Depletion of *TCF7L2* results in impaired β-cell function through suppression of GLP-1 and GIP-stimulated insulin secretion. Human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to siRNA sequences to TCF7L2 (siTCF7L2) or scrambled control siRNA (siScr) for 4 days. Basal and stimulated insulin secretion indicate the amount secreted during 1-h incubations at 2.8, 16.7 mM glucose, 16.7 mM plus 100 nM GLP-1 (**A**) or 16.7 mM plus 10 nM GIP (**C**), following the 4-day culture period, normalized to whole islet insulin content. (**B,D**) Stimulatory index which denotes the amount stimulated divided by the amount of basal insulin secretion. (**E**) Human islets transfected with scrambled control (siScr) or *TCF7L2* siRNA (siTCF7L2) were placed in perifusion chambers in aliquots of 20 islets of equal size, respectively. The perifusate contained 2.8 mM glucose for 1 h (measured for the last 30 min), 16.7 mM glucose for 30 min, 16.7 mM glucose plus 10 nM GIP for 30 min, 2.8 mM glucose for 30 min (the effluent was collected in 5-min intervals) and 10 min 20 mM KCl (the effluent was collected in 2-min intervals). Insulin secretion is expressed as percentage of content. Experiments were performed in triplicate. (**F,G**) For KCl and IBMX/Forskolin-stimulated insulin secretion analysis, basal and stimulated insulin secretion indicate the amount of secreted insulin during 1-h incubations at 2.8 mM glucose (basal), and 20 mM KCl or IBMX (100 μM)/Forskolin (10 μM) following the 4-day culture period, normalized to whole islet insulin content. (**G**) Stimulatory index which denotes the amount of KCl or IBMX/Forskolin-induced insulin secretion divided by the amount of basal insulin secretion. Data represent results of three different experiments from three different organ donors in triplicate. Results are means ± SE. $^{*}P < 0.05$ siTCF7L2 versus siScr; $+P < 0.05$ 16.7 versus 2.8 mM glucose; $^{**}P < 0.05$ GLP-1/GIP+16.7 versus 16.7 mM glucose; $\#P < 0.05$ KCl or IBMX/Forskolin versus 2.8 mM glucose alone. (**H**) Transfected human islets were placed in perifusion chambers in aliquots of 20 islets of equal size. The perifusate contained 2.8 mM glucose for 1 h, 16.7 mM glucose for 30 min (not shown) and 16.7 mM glucose plus 100 nM GLP for 30 min (shown here). The effluent was collected in 1-min intervals. Data represent results from one representative experiment in six different perifusion chambers out of three independent experiments from three organ donors and show that *TCF7L2* depletion leads to a decreased mass, but not frequency of insulin secretory bursts.

siTCF7L2- and siScr-treated islets, confirming an intact cAMP response in the *TCF7L2*-depleted islets (Fig. 4F and G).

TCF7L2 depletion leads to a decreased mass, but not frequency of insulin secretory bursts

The regulation of insulin secretion is accomplished through adaptation of the insulin secretory pulse mass in response to insulin secretagogues, e.g. glucose, sulfonylureas or GLP-1 (27). A decreased insulin pulse mass is an early defect in patients with T2DM (28). The impaired GLP-1 response in carriers of the *TCF7L2* mutation and in T2DM may also be owing to a decreased insulin pulse mass. To investigate this possibility, we studied pulsatility in *siTCF7L2*-treated islets. Pulses were detected by measuring glucose+GLP-1-induced insulin secretion every minute from islets in a perifusion chamber (Fig. 4H). In each chamber, pulse mass was decreased in the siTCF7L2- versus siScr-treated control islets, whereas pulse interval remains stable at approximately 4 min. The experiment was repeated with GIP stimulation, pulse mass responses were again decreased in the siTCF7L2- versus siScr-treated control islets, whereas the insulin pulse interval was stable at approximately 4 min (data not shown). This suggests the decrease in insulin secretion induced by depletion of *TCF7L2* is because of a decrease in pulse mass, rather than pulse frequency.

DISCUSSION

In T2DM, impaired β-cell secretory function and decrease in β-cell mass can be attributed to a complex interplay between environmental factors and genetic predisposition. The underlying mechanisms of β-cell failure are still largely unknown. Recent results from genome-wide association studies revealed new gene mutations in T2DM (29). This suggests that genes may have more significant contributions to common forms of T2DM than previously recognized. First reported in 2006 (29), *TCF7L2* polymorphisms are highly associated with T2DM in different ethnic groups worldwide (1). A number of important questions such as: (i) the fact that the mutations are located in the non-coding region of *TCF7L2*; (ii) the contribution of *TCF7L2* towards a failure in β-cell survival and insulin secretion; and (iii) how *TCF7L2* expression regulated in diabetes needs further clarification. Previous studies indicated that *TCF7L2* mRNA is increased in islets isolated from the ZDF rat (13) as well as from either patients with T2DM or individuals carrying an increased number of *TCF7L2* risk T-alleles (4), which is in contradiction to findings showing that *TCF7L2* depletion directly leads to decreased insulin secretion (10). Our data show for the first time that *TCF7L2* mRNA and protein levels are oppositely regulated in diabetes and thus provide a potential answer to this apparent contradiction. In pancreatic sections of patients with T2DM we observed a drastic downregulation of TCF7L2 protein expression, compared with healthy controls. This observation was confirmed in three diabetic animal models, the *db/db* mouse, the HFD-fed mouse and the VDF Zucker rat. All three models were characterized by impaired fasting glucose and glucose intolerance compared with their wild-type littermates. This diabetic phenotype coincided with increased *TCF7L2* mRNA expression and reduced protein expression in isolated islets.

In our previous study we observed that treatment of isolated islets with siRNA to *TCF7L2* resulted in increased β-cell apoptosis and impaired β-cell function. On the other hand, increased TCF7L2 protein expression resulted in increased β-cell protection. Consequently, the increased *TCF7L2* mRNA expression in diabetes may be a consequence, not a cause, of impaired β-cell function owing to deficiency of TCF7L2 protein, and the post-transcriptional regulation of *TCF7L2* rather than changes in mRNA levels may be more important for *TCF7L2*-regulated β-cell function and survival. The mechanism underlying this opposite regulation of transcription and translation is unclear. There is evidence that translational control is disturbed in diabetes (30,31) through an inhibition of translation by unfolded protein accumulation. The β-cell is a cell with the capacity to fold and process large amounts of secreted protein; more than 5% of the whole protein content is insulin. This specific requirement together with the comparably low amount of anti-oxidative molecules, e.g. catalase, glutathione peroxidase and superoxide dismutase (32) may make the β-cell more vulnerable to stress conditions than any other cell type and could lead to disturbances in the post-transcriptional regulation. Although rarely, opposite regulation of mRNA and protein has been reported before. For example, in the Fragile X-associated tremor/ataxia syndrome, a progressive neurodegenerative disease, FMR1 mRNA is elevated, while its protein levels are downregulated and it has been suggested that reduced translational efficiency may be compensated through increased transcriptional activity (33).

TCF7L2 has been implicated in glucose homeostasis through the regulation of pro-glucagon expression, the precursor encoding glucagon-like peptide-1 (GLP-1) in L-intestinal cells (8). Carriers of the *TCF7L2* risk allele show a significant reduction in GLP-1-induced insulin secretion (5), suggestive of a direct defect in islets. Actions of GLP-1/GIP may also be altered through changes in *TCF7L2* expression. We reported earlier on a significant deficit in the potentiation of GLP-1-induced GSIS in *TCF7L2*-depleted isolated human islets (10). In the present study, we also investigated expression and localization of GLP-1R and GIP-R in human pancreas tissue. Both, GLP-1R and GIP-R expression were down-regulated in pancreatic islets from patients with T2DM. Importantly, for its application in diabetes therapy, GLP-1 stimulates insulin secretion in patients with T2DM, although responses are reduced when compared with healthy individuals (34,35). We saw a robust downregulation of GLP-1R in our samples, but responses to GLP-1 in T2DM are generally shown to be relatively mildly reduced (approximately 30%). This supports the possibility of a major autonomic component to the effect of GLP-1 on insulin secretion in T2DM.

In contrast, almost complete resistance to GIP occurs in patients with T2DM (36). Among the possible explanations proposed for this defect, desensitization or downregulation of the GIP receptor (37,38), receptor gene mutations (39) or altered signal-transduction pathways have been suggested (38). The current studies provide further evidence of GIP

downregulation in the β-cell in T2DM. In line with our data, a decrease in GLP-1R and GIP-R has been observed in response to hyperglycemia, significant reductions occurring in islets from 90% pancreatectomized hyperglycemic rats as well as in isolated rat islets cultured in high glucose for 48 h (40). Loss of GIP-R expression levels also occurs in the VDF Zucker rat (16,38), a model of mild T2DM. Reversal of hyperglycemia in both strains of rats by phlorizin treatment recovered GLP-1R/GIP-R expression (38,40), indicating that glucose levels are strongly associated with GIP-R expression in the β-cell. We now show that reduced expression of the GIP-R in the VDF rat model is associated with decreased TCF7L2 protein levels. As a result of the decreases in receptor expression, neither GLP-1 nor GIP were capable of potentiating glucose-stimulated insulin secretion in *TCF7L2*-depleted islets.

Anti-apoptotic mechanisms of GLP-1 are mediated through activation of AKT (20). In turn, impaired AKT activation has been associated with reduced GSK-3 inactivation (41). GSK-3 increases β-cell apoptosis through the WNT signaling pathway, which degrades β-catenin, and thus restricts activation of *TCF7L2* (42). In *TCF7L2*-depleted islets, p-AKT was almost undetectable. GLP-1 and GIP induced AKT phosphorylation in control but not in *TCF7L2*-depleted islets. Such loss of AKT-phosphorylation would result in inhibition of Foxo1 phosphorylation and thus nuclear accumulation and Foxo1-mediated transcription (22).

Indeed, depletion of endogenous *TCF7L2* resulted in suppressed Foxo1 phosphorylation following nuclear exclusion. In contrast, Foxo1 nuclear exclusion was activated by GLP-1 or GIP in the control islets but not in the *TCF7L2*-depleted islets, confirming absence of GLP-1R/GIP-R signals and providing a mechanism for the impaired glucose/GLP-1/GIP-stimulated insulin secretion.

Upon GLP-1R activation, adenylyl cyclase (AC) is activated and cAMP is generated in the β-cell, leading to cAMP-dependent activation of second messenger pathways and insulin secretion (26,43). In contrast to glucose, KCl-induced stimulation of insulin secretion as well as cAMP accumulation is not affected in the *TCF7L2*-depleted islets. In line with this, mouse islets treated with *TCF7L2* siRNA show abnormal glucose and intact KCl-stimulated insulin secretion and increase in syntaxin 1A mRNA levels, which provides a mechanism for the disturbed insulin secretion. This leads to an alteration in the recruitment of insulin-containing granules (44). Insulin is secreted in a pulsatile manner (45), abnormalities of which are associated with early defects in both Type 1 and Type 2 diabetes. In islet perifusions, *TCF7L2* depletion resulted in impaired insulin secretion. Although we have not performed deconvolution analyses, our perifusion data from six islet perifusates including 20 islets show that this defect can be attributed to the decreased pulse mass without altered pulsatile periodicity, similar to what has been observed in diabetes *in vivo* (28). It is possible that this is a result of the increased secretory granule movement and defective vesicle fusion in the *TCF7L2*-depleted islets (44,46), which consequently leads to diminished insulin release.

Our findings therefore suggest that *TCF7L2* directly regulates β-cell function through alterations in the GLP-1R and GIP-R pathway and restoration of *TCF7L2* signaling may be a new strategy to restore β-cell sensitivity in diabetes.

MATERIALS AND METHODS

Islet isolation and culture

Human islets were isolated from pancreata of seven healthy organ donors at the University of Illinois at Chicago and from INSERM/Université de Lille, Thérapie Cellulaire du Diabète as described previously (47). HbA1c from all donors was confirmed to be $<6\%$. Mouse and rat islets were isolated by common bile duct perfusion using Collagenase type 4 (Worthington, Lakewood, NJ, USA). Islets were cultured on matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel) (48) in CMRL-1066 medium containing 5.5 mM (human islets) or RPMI-1640 medium containing 11.1 mM glucose (mouse islets), 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (Invitrogen, Carlsbad, CA, USA) hereafter referred to as culture medium. For treatment of islets, medium was changed to culture medium containing 5.5, 11.1 or 33.3 mM glucose or 5.5 mM plus 2 ng/ml recombinant human IL-1β plus 1000 U/ml recombinant IFN-γ (R&D Systems Inc., Minneapolis, MN, USA), 100 nM GLP-1 or 10 nM GIP (Sigma).

Animals

C57BL/6J and BKS.Cg-*m*+/+*Lepr*^{db}/J (db/+) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and bred in our colony to homozygous Lepr/Lepr (db/db) and heterozygous Lepr/+ (db/+) mice. For the HFD experiments, C57BL/6J mice were fed a normal diet (ND; Harlan Teklad Rodent Diet 8604, containing 12.2%, 57.6% and 30.2% calories from fat, carbohydrate and protein, respectively) or a HFD/high sucrose diet ('Surwit' Research Diets, New Brunswick, NJ, USA) containing 58%, 26% and 16% calories from fat, carbohydrate and protein, respectively. Throughout the study period of 12 weeks, food consumption and body weight were measured weekly. Four independent experiments with a total of 16 mice (four mice/cage) in each group were performed.

Male ZDF, and Zucker lean rats were from Charles River (Ont., Canada). Male Vancouver diabetic fatty (VDF) Zucker rats (fa/fa), a sub-strain of the Zucker fatty rat, and their lean litter mates (Fa/?) are maintained at the University of British Columbia. Male VDF rats develop T2DM between 7 and 10 weeks of age; animals aged 8–10 weeks were used. Homozygous VDF *fa/fa* rats exhibit a less severe diabetic phenotype than ZDF rats, with mild fasting hyperglycemia, marked postprandial hyperglycemia, hyperlipidemia and hyperinsulinemia. All animals were housed in a temperature-controlled room with a 12-h light/dark cycle and were allowed free access to food and water conformed to guidelines of the UCLA Chancellor's Animal Research Committee in agreement to NIH animal care guidelines, the UBC Committee and Canadian Council on Animal Care and the Bremen Senate for Work, Health, Women, Youth and Social Affairs and from the Bremen Senate in agreement

with the National Institutes of Health animal care guidelines and Section 8 of the German Animal Protection Law.

Intraperitoneal or oral glucose tolerance tests

For IPGTT, mice were fasted 12 h overnight and injected intraperitoneally with glucose (40%, Phoenix Pharmaceuticals Inc., St. Josephs, MO, USA) at a dose of 1 g/kg body weight. Rats received oral glucose (1 g/kg). Blood samples were obtained at time-points 0, 15, 30, 60, 90 and 120 min. for glucose measurements using a Glucometer (Freestyle, TheraSense Inc, Alameda, CA, USA).

RNA interference and plasmid transfection

SiRNA-Lipofectamine2000 complexes and DNA-Lipofectamine2000 complexes were prepared according to manufacturer's instructions (Lipofectamine2000, Invitrogen) using 50 nM siRNA to TCF7L2 (RNAs of 21 nucleotides, designed to target human *TCF7L2*; Stealth Select^{TM} RNAi, Invitrogen) and scramble siRNA (Ambion, Austin, TX, USA) or 3 μg/ml DNA of pCMV-TCF7L2 (full length *TCF7L2*, from the Full-Length Mammalian Gene Collection, Invitrogen) or a pCMV-empty control plasmid. After overnight incubation, the transfection medium was aspirated and replaced by fresh culture medium.

Nuclear fractionation

Nuclear and cytoplasmic extractions of human islets were performed according to the instructions of NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA). The purity of fractions was analyzed by incubation of the membranes with anti-GAPDH for cytosolic and anti-PARP for nuclear extracts.

Western blot analysis

At the end of the incubation, islets were washed in phosphate-buffered saline and lysed. Polyvinyl difluoride filters were incubated with rabbit antisera against GLP-1R (no. NLS1206), GIPR (no. NLS1253) (both Novus Biologicals LLC, Littleton, CO, USA), rabbit anti-TCF7L2 (no. 2565), rabbit anti-p-FOXO1 (Ser256 no. 9461), rabbit anti-FOXO1 (no. 9462), rabbit anti-actin (no. 4967), rabbit anti-GAPDH (no. 2118), rabbit anti p-AKT (Serin473 no. 9271), rabbit anti-PARP (no. 9542), all Cell signaling, Danvers, MA, USA), followed by incubation with horseradish-peroxidase-linked IgG peroxidase. The emitted light was captured on X-ray film after adding Immun-Star HRP Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Density of the bands was analyzed using DocIT®LS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA, USA).

For detection of p-AKT, islets were pre-incubated in the absence of serum at 5.5 mM glucose for 14 h, followed by no serum and no glucose for 4 h and then exposed to the treatment conditions.

Immunofluorescence staining

Pancreatic sections from seven healthy controls and from seven patients with T2DM were obtained from the National Disease Research Interchange (NDRI) and from Kangnam St. Mary's Hospital, Seoul, Korea as described (49), and approval for the studies were granted by the Kangnam St. Mary's Hospital and by the Ethical Commission of Bremen University. Sections were deparaffinized, rehydrated and incubated overnight at 4°C with rabbit antisera against GLP-1R, GIPR, TCF7L2 or mouse anti-glucagon glucagon (Abcam, Cambridge, MA, USA). For Foxo1 localization studies, siRNA-transfected islets on ECM dishes were fixed in 4% PFA, and stained with rabbit anti-Foxo1-Ab. Staining was detected using cy3-conjugated donkey anti-rabbit or donkey anti-mouse antibodies (Jackson). Islets were double/triple-stained with guinea pig anti-insulin antibody (Dako, Carpinteria, CA, USA), followed by detection using fluorescein-conjugated donkey anti-guinea pig antibody (Jackson) and embedded in glycerol gelatin (Sigma) or Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), which visualized all cells using 4,6-diamidino-2-phenylindole (DAPI) staining. Fluorescence was analyzed using a Leica DM6000 (Leica Microsystems Inc., Bannockburn, IL, USA) or Nikon MEA53200 (Nikon GmbH Dusseldorf, Germany) microscope and images were acquired using Openlab (Improvision Inc, Waltham, MA, USA) or NIS-Elements software (Nikon).

RNA extraction and RT-PCR

Total RNA was isolated from cultured islets as described previously (50). For quantitative analysis, we used the Light Cycler quantitative PCR system (Roche Diagnostics, Indianapolis, IN, USA) with a commercial kit (Light Cycler-DNA Master SYBR Green I; Roche). Primers used were 5′-GAAGGAGCGACAGCTTCATA-3′ and 5′-GGGGGAGG CGAATCTAGTAA-3′ (hTCF7L2), 5′-CAGGGAAGAACAG GCAAAAT-3′ and 5′-GGGGGAGGCGAGTCTAGTAA-3′ (mTCF7L2) and compared with the house-keeping gene 5′-AGAGTCGCGCTGTAAGAAGC-3′ and 5′-TGGTCTTGT CACTTGGCATC-3′ (α-Tubulin) and 5′-TCACCCACACTGT GCCCATCTACGA-3′ and 5′-CAGCGGAACCGCTCATTG CCAATGG-3′ (β-actin).

Glucose-stimulated insulin secretion

For acute insulin release, islets were washed and pre-incubated (30 min) in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose. The KRB was then replaced by KRB containing 2.8 mM glucose for 1 h (basal), followed by additional 1 h incubation in KRB containing 16.7 mM glucose, 16.7 mM glucose plus 100 nM GLP-1 or 10 nM GIP, 20 mM KCl or IBMX (100 μM)/Forskolin (10 μM; Sigma). For insulin content, islets were extracted with 0.18 N HCl in 70% ethanol. Insulin was determined using a human insulin ELISA kit (Alpco, Windham, NH).

Islet perifusion

Human islets were suspended in Bio-Gel P-2 beads (Bio-Rad) and placed in perifusion chambers in aliquots of 20 islets as described before (51). The perifusion system (ACUSYST-S, Cellex Biosciences, Inc., Minneapolis, MN, USA) consisted of a multichannel peristaltic pump that delivered perifusate through six parallel tubing sets via a heat exchanger and six perfusion chambers at a constant rate of 0.3 ml/min. The perifusion buffers (KRB) were preheated to 37°C, and oxygenized with 95% O_2 and 5% CO_2. The perifusate was delivered to the perifusion chambers containing the human islets, and the effluent was collected every minute for determination of insulin concentrations. At the end of the perifusions, islets were collected by handpicking and extracted with 0.18 N HCI in 70% ethanol for determination of insulin content. Insulin was determined using a human insulin ELISA kit (Alpco, Salem, NH).

Statistical analysis

Samples were evaluated in a randomized manner by a single investigator (L.S.) who was blinded to the treatment conditions. Data are presented as mean $\pm$ SE and were analyzed by paired Student's *t*-test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of interest statement. The authors declare no conflicts of interest.

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