Survival of pancreatic beta cells is partly controlled by a TCF7L2-p53-p53INP1-dependent pathway

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The transcription factor T-cell factor 7-like 2 (TCF7L2) confers type 2 diabetes risk mainly through impaired insulin secretion, perturbed incretin effect and reduced beta-cell survival. The aim of this study was to identify the molecular mechanism through which TCF7L2 influences beta-cell survival. TCF7L2 target genes in INS-1 cells were identified using Chromatin Immunoprecipitation. Validation of targets was obtained by: siRNA silencing, real-time quantitative polymerase chain reaction, electrophoretic mobility shift assay, luciferase reporter assays and western blot. Apoptosis rate was measured by DNA degradation and caspase-3 content. Islet viability was estimated by measuring metabolic rate. TCF7L2 binds to 3646 gene promoters in INS-1 cells in high or low glucose, including Tp53, Pten, Uggt1, Adamts9 and Fto. SiRNA-mediated reduction in TCF7L2 activity resulted in increased apoptosis and increased expression of Tp53, which resulted in elevated p53 protein activity and an increased expression of the p53 target gene Tp53inp1 (encoding p53-induced-nuclear-protein 1). Reversing the increase in p53INP1 protein expression, seen after Tcf7l2 silencing, protected INS-1 cells from Tcf7l2 depletion-induced apoptosis. This result was replicated in primary rat islets.

The risk T-allele of rs7903146 is associated with increased TCF7L2 mRNA expression and transcriptional activity. On the other hand, in vitro silencing of TCF7L2 lead to increased apoptosis. One possibility is that the risk T-allele increases expression of an inhibitory TCF7L2 isoform with lower transcriptional activity. These results identify the p53-p53INP1 pathway as a molecular mechanism through which TCF7L2 may affect beta-cell survival and established a molecular link between Tcf7l2 and two type 2 diabetes-associated genes, Tp53inp1 and Adamts9.

INTRODUCTION

Type 2 diabetes arises through an interaction between genes and environment when pancreatic islets beta-cell mass cannot sufficiently compensate for increased needs imposed by insulin resistance (1). Therefore, genetic factors that affect functional beta-cell mass are important in the pathogenesis of the disease.

TCF7L2 (T-cell factor 7-like 2, also known as TCF-4) is a transcription factor involved in Wnt/beta-catenin signaling, and genetic variations in the TCF7L2 gene are associated with type 2 diabetes (2). The risk T allele of the single nucleotide polymorphism (SNP) rs7903146 confers the strongest risk of type 2 diabetes known to date in Caucasians (3).

The risk T allele is associated with impaired insulin secretion, partially due to an impaired incretin effect, as well as hepatic but not peripheral insulin sensitivity (4). In pancreatic islets, TCF7L2 also influences beta-cell functions by affecting beta-cell survival (5). Depletion of TCF7L2 in human pancreatic islets was shown to result in impaired glucose-, glucagon-like peptide-1 (GLP-1)- and potassium chloride-stimulated insulin secretion. TCF7L2 depletion also impaired cell proliferation, and promoted apoptosis. The authors also showed that over-expression of TCF7L2 protected beta cells from glucose- and cytokine-induced apoptosis (5).

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TCF7L2 has an established role in the pathogenesis of different cancers, especially colorectal cancer, where it regulates the expressions of several oncogenes, e.g. c-Jun, c-Myc and CCND1 (6–8). Two studies have identified TCF7L2 target genes in colorectal cancer cell lines and demonstrated that TCF7L2 binds thousands of promoters (9,10). However, TCF7L2 may exert different functions in different tissues, which could in part be a consequence of its complex splicing pattern (11–14). No information is available on target genes of TCF7L2 in pancreatic islets. The aim of this study was therefore to identify TCF7L2 target genes in pancreatic beta cells, and explore their potential role in beta-cell survival.

RESULTS

Screening of TCF7L2 target genes in INS-1 cells

In an effort to identify TCF7L2 target genes, we screened for binding of TCF7L2 to gene promoters in the rat beta-cell line INS-1 (832/13) using Chromatin Immunoprecipitation on tiling arrays (ChIP-on-chip) and an antibody directed against the N-terminus of TCF7L2. The screening was performed in triplicate at both low and high glucose concentrations. Different glucose concentrations were chosen because glucose potentially could influence the binding of TCF7L2 to its target gene promoters. This hypothesis was based on a previous observation of a correlation between TCF7L2 total expression level and exon 4 incorporation with HbA1c levels (12). Using a Hidden Markov Model (HMM) method (15), 3646 gene promoters were identified as being bound by TCF7L2 in either low or high glucose conditions (Supplementary Material, Table S1: TCF7L2 binding gene symbols; Supplementary Material, Tables S2 and S3: TCF7L2 binding positions in these gene promoters in high and low glucose). Several previously known TCF7L2 target genes were found, e.g. UGCGL1 (Preproglucagon), Gipr and Myc, but not Gip1r. The 3646 potential target genes were also compared with two published data sets from colorectal cancer cells (9,10). Approximately 36% (1309) and 9% (341) of the 3646 genes are found in the LS174T colorectal cancer cell line (9) and in the HCT116 colorectal carcinoma cell line (10), respectively [or ~60% of the target genes published are covered by the 3646 genes identified in INS-1 cells, 1309/2147 (9) and 341/548 (10)]. This overlap could be regarded as relatively large given the different experimental designs, technologies and model systems used.

Potentially enriched binding motif sequences in the ChIP-on-chip experiment were identified using the Motif Discovery scan (MDscan) tool. The most highly enriched 6 bp sequence found was the previously reported TCF7L2 consensus sequence ‘TCAAG’ (9). This together with the large overlap with the two previously published data sets indicates that the TCF7L2 binding pattern is relatively comparable between INS-1 and colorectal cancer cells.

Among the 3646 potential target genes, we focused on: (i) genes previously associated with type 2 diabetes (proximal to associated SNPs) (16) or (ii) hypothesis-based selection, i.e. genes associated with mechanisms known to be involved in the pathogenesis of type 2 diabetes (5,17–19); for example, associated with apoptosis or cell proliferation. A diagram outlining the study is presented in Supplementary Material, Figure S1. In keeping with previous results in colorectal cancer cells, we found that TCF7L2 binds to the promoter region of two type 2 diabetes-associated genes, i.e. ADAMTS9 and FTO (9,10). Pten was also studied since it is involved in the insulin signaling pathway and beta-cell mass (17). Tp53 was bound by TCF7L2 at high glucose concentrations. Given that genetic variation in TP53INP1 (encoding p53-induced-nuclear-protein 1), a direct target gene of p53 (19), has been associated with type 2 diabetes (16) and p53INP1 is known to induce apoptosis in human cancer cell lines (18,19), the p53-p53INP1 pathway was further investigated.

Silencing of TCF7L2 in INS-1 cells leads to increased expression of Tp53 and Tp53inp1, and decreased expression of Adams9 and Pten

To test whether manipulation of Tcf7l2 levels influences expression of target genes, we reduced the Tcf7l2 mRNA level in INS-1 cells using siRNA in both low and high glucose conditions. Cells were transfected with siRNA targeting the variable exon 1 of Tcf7l2, thereby reducing the expression of all splice variants (12,13). A 75% reduction in low glucose and an 80% reduction in high glucose in Tcf7l2 mRNA were obtained (Fig. 1A and B). This corresponded to a 91% decrease in TCF7L2 transcriptional activity compared with scrambled control in low glucose (Fig. 1C). This marked reduction in TCF7L2 activity was in low glucose associated with a 56% reduced expression of Adams9, a 26% reduced expression of Pten, a modestly increased expression of Tp53 by 11% and a 43% increased expression of Tp53inp1 (Fig. 2A). Similar mRNA changes were observed in high glucose for Tp53 with a 14% increase, a 65% increase for Tp53inp1, an 82% decrease in Adams9 and a 52% decrease in Pten mRNA expression. No obvious change was observed for Fto mRNA expression (Fig. 2B).

To test the functional consequence of a reduced Adams9 expression, we tested if the activity of the NOTCH signaling pathway is affected, as it is known that other members of the ADAM-protein family have the ability to cleave and activate NOTCH receptors (20,21). The activity of the NOTCH signaling pathway was measured by recombining binding protein Jk-promoter activity in Tcf7l2 knocked down INS-1 cells. However, no significant difference was observed in Tcf7l2 siRNA-treated cells versus scrambled control (1.00 ± 0.23 versus 1.26 ± 0.37, n = 2) or in Adams9 siRNA-treated cells versus scrambled control (1.00 ± 0.23 versus 1.22 ± 1.68, n = 2).

P53INP1 protein expression was increased after Tcf7l2 silencing

A concomitant increase in the protein expression of p53INP1 was observed by western blot. In humans, two isoforms of p53INP1 are expressed (22,23) and the size of the two bands observed in INS-1 cells corresponds to isoforms containing either all three exons (the upper band) or exons 1 and 3 (the lower band). P53INP1 was up-regulated by 61% after Tcf7l2 silencing in low glucose (Fig. 3B and C).
P53 transcriptional activity was increased after Tcf7l2 silencing

It is uncertain whether the marginal increase in Tp53 mRNA expression can explain the substantial increase in Tp53inp1 mRNA expression. However, various post-translational modifications of the p53 protein regulate its transcriptional activity and we therefore measured the p53 transcriptional activity after Tcf7l2 silencing in low and high glucose. P53 activity increased by 64% in low glucose (Fig. 4A), and by 50% in high glucose (Fig. 4B). TCF7L2 was also found to bind to the Tp53 promoter using electrophoretic mobility shift assay (EMSA). A probe in the Tp53 promoter, located 879 bp upstream from the start of exon 1 showed efficient binding by TCF7L2 (Supplementary Material, Fig. S2).

P53 is necessary for TCF7L2 regulation of Tp53inp1 expression

Since TCF7L2 directly binds the promoter of Tp53, but not the promoter of Tp53inp1, we investigated if the observed change in Tp53 expression was necessary for the Tcf7l2-induced up-regulation of Tp53inp1 expression. Tp53inp1 expression was up-regulated when only Tcf7l2 was silenced, while the combined silencing of Tp53 and Tcf7l2 attenuated Tp53inp1 expression (Fig. 5). Tp53 depletion alone led to a down-regulation of Tcf7l2 by 54%, Adams9 by 73% and Fto by 20%. A reduction in Pten expression after Tp53 depletion was observed, but did not reach statistical significance. The regulation of TCF7L2 on Adams9 was not dependent on p53. However, Tp53 depletion alone also reduced the expression of Adams9, indicating that both TCF7L2 and p53 are involved in the regulation (Fig. 5).

Tcf7l2 silencing induces apoptosis in INS-1 cells through a p53, p53inp1-dependent mechanism

In order to study whether Tp53inp1 is important for apoptosis induced by Tcf7l2 silencing, the increase in expression of Tp53inp1 after silencing of Tcf7l2 was prevented using siRNA directed against Tp53inp1. Silencing of Tp53inp1 was confirmed at both the mRNA level (44%) (Fig. 3A) and protein level (41%) (Fig. 3B and C). The p53inp1 protein level in double-silenced cells was similar to that in scrambled control cells (P = 0.248, n = 4).

Apoptosis was measured using three methods: (i) immunocytochemistry (ICC), (ii) DNA degradation and (iii) caspase-3 protein levels. For the ICC measurements, cells were triple stained using Blue Hoechst 23580 (a nucleic DNA marker), Annexin V (an early apoptosis marker) and 7-AAD (a late apoptosis marker). Tcf7l2-silenced cells showed increased staining intensity for both the early and late apoptosis markers at all time points (24, 48 and 72 h of incubation, n = 3) compared with scrambled and Tp53inp1-silenced controls (Fig. 6A). With time, increased intensity of the late apoptosis marker and decreased intensity of the early apoptosis marker were observed (24 versus 72 h incubation). In contrast, no apparent change in staining intensity for the apoptosis markers was observed in INS-1 cells with simultaneous knock-down of Tcf7l2 and Tp53inp1 compared with scrambled and Tp53inp1-silenced controls (Fig. 6A). These results indicate that blocking the Tcf7l2-induced increase in Tp53inp1 expression prevents apoptosis.

To quantify the observations obtained by ICC, the level of DNA degradation was measured using an enzyme-linked immunosorbent assay (ELISA)-based kit after 72 h of incubation with siRNA. A 74% increase in DNA degradation was observed in Tcf7l2-silenced cells when compared with the scrambled control (Fig. 6B). The Tcf7l2 and Tp53inp1 double-
silenced cells had a similar DNA degradation levels as the scrambled and \textit{Tp53inp1}-silenced controls (Fig. 4B). To further validate these results, we used a third method to assess apoptosis, i.e. measurements of the caspase-3 protein level, which represents a measure of apoptosis in cells with an intact plasma membrane. Cells incubated with 3 μM adriamycin for 4 h were used as a positive control for apoptosis and beta-actin was used as a loading control. \textit{Tcf7l2}-silenced cells had 94% lower expression of the two caspase-3 heavy bands compared with the scrambled control, indicating increased apoptosis. The double knocked down cells had a caspase-3 protein level similar to both the scrambled and \textit{Tp53inp1} knocked down controls (Fig. 6C and D).

\textit{Tcf7l2} silencing induces apoptosis and reduced viability in primary rat islets through a \textit{p53-p53INP1}-dependent mechanism.

The effect of the \textit{Tcf7l2}-p53-p53INP1 pathway on pancreatic islet survival was also examined in primary rat pancreatic islets. Islet viability was estimated by measuring cellular reducing potential after 24 h of incubation with siRNA. \textit{Tcf7l2}
silencing reduced islet viability by 41% in low glucose (Fig. 7A) and by 65% in high glucose (Fig. 7B). Islet viability was restored by silencing either Tp53 or Tp53inp1 together with Tcf7l2. Islet apoptosis was quantified by measuring caspase-3 and caspase-7 activity after 24 h of incubation with Tcf7l2 siRNA. We observed a 75% increase in low glucose and a 120% increase in high glucose (Fig. 7D). The increased apoptosis was prevented when silencing either Tp53 or Tp53inp1 together with Tcf7l2.

**DISCUSSION**

This study has identified the p53-p53INP1 pathway as a key component of the mechanism whereby TCF7L2 may induce beta-cell apoptosis. Reduced TCF7L2 activity increases expression of p53INP1, via a p53-dependent mechanism, resulting in increased apoptosis. Perturbing the TCF7L2-induced increase in p53INP1 expression inhibits the induction of apoptosis, maintaining beta-cell mass. Furthermore, these results established a molecular connection between three type 2 diabetes-associated genes, i.e. TCF7L2 (2), ADAMTS9 and TP53INP1 (16).

TCF7L2 is by far the best-replicated type 2 diabetes gene identified. However, the molecular mechanisms whereby variants in TCF7L2 increase the risk of type 2 diabetes have not been fully elucidated (24). These mechanisms seem to involve impaired beta-cell function and mass which can partially be ascribed to an impaired incretin effect (4,25). Although in islets, more supports have been presented in favor of an impairment of beta-cell function over mass, the latter has also been demonstrated (5,26–28). For example, one effect of GLP-1 is the induction of beta-cell proliferation, which is partly mediated by Wnt-signaling and TCF7L2 by regulating transcriptional programs (29,30). Another mechanism influenced by TCF7L2 is protection of beta cells from apoptosis through GLP-1 (31), this effect is probably more important than the action on beta-cell proliferation, in view of the low replication rate in human beta cells and, in fact, in all primary beta cells relative to transformed cells.

The common variant in the TCF7L2 gene rs7903146 is likely to influence expression not only of TCF7L2 itself, but also of its target genes. The risk allele increases TCF7L2 total expression (4) but also differential splicing has been suggested to be part of the molecular mechanisms (12–14). However, the consequent change in TCF7L2 activity is not clear. TCF7L2 is known to control the expression of several genes involved in oncogenesis and a list of potential TCF7L2 target genes has been identified in colorectal cancer cells using ChIP-on-chip and ChIP-seq analysis (9,10). However, similar information from pancreatic islets has been lacking. Transcription factors expressed in several tissues may regulate tissue-specific transcription, which makes it necessary to study the disease relevant tissue, e.g. for diabetes pancreatic islets. No confirmation of a Tcf7l2-dependent regulation of Fto in beta cells was obtained in this study. There could be several explanations for the lack of regulation: (i) TCF7L2 might not influence the regulation under the experimental conditions used, i.e. low and high glucose concentration at the time point chosen. (ii) Although TCF7L2 binds the promoter, the gene might not be regulated by TCF7L2 in pancreatic beta cells due to lack of expression of co-regulators in this tissue. (iii) Reduced expression of TCF7L2 without altering the proportion of different isoforms is not sufficient to interfere with the mechanism of regulation.

In this study, the ChIP-on-chip experiment was performed in a rat beta-cell line (INS-1 cells), and TCF7L2 binding in other islet cell types was not investigated. Kirkpatrick et al. (32) have shown that TCF7L2 is expressed both in alpha- and beta cells in human pancreatic islets and that TCF7L2 expression is highly expressed in alpha-cells. The potentially very different roles of TCF7L2 in different cell types in the pancreatic islet needs to be clarified in order to fully understand the influence of type 2 diabetes risk variants on islet function.
Interestingly, p53 is necessary for the observed increase in Tp53inp1 expression induced by Tcf7l2 silencing in INS-1 cells. Cells were incubated in 25 mM glucose either with scrambled siRNA (white bars), with siRNA targeting Tcf7l2 (black bars), with siRNA targeting Tcf7l2 and Tp53 (checked bars) or with siRNA targeting only Tp53 (gray bars). Tp53 depletion lead to a down-regulation of Tcf7l2 by 54% (1.00 ± 0.03 versus 0.46 ± 0.05), Adamts9 by 73% (1.00 ± 0.03 versus 0.27 ± 0.05) and Fto by 20% (1.00 ± 0.03 versus 0.80 ± 0.04) compared with scrambled control. Simultaneous silencing of Tcf7l2 and Tp53 lead to a down-regulation of Tp53 by 94% (1.14 ± 0.03 versus 0.07 ± 0.02) and of Tp53inp1 by 90% (1.65 ± 0.27 versus 0.17 ± 0.04) compared with Tcf7l2 knock-down. Data are represented as mean ± SEM, n = 6, *P < 0.05, **P < 0.01; Mann–Whitney U-tests.

In a proteome screen of pancreatic islets, it was found that pathways involved in cell cycle arrest and apoptosis were highly activated in islets from type 2 diabetic donors. In fact, the most activated was the E2F pathway, followed by Tp53 (Fig. 5). Interestingly, p53 is also a key upstream regulator of the E2F pathway (34), which positions p53 as a potential regulator of beta-cell survival and mass. In our study, Tp53inp1 was regulated by TCF7L2 in pancreatic beta-cells and variation in the TP53INP1 gene was recently reported to be associated with type 2 diabetes (16). TP53INP1 is a direct target gene for p53, and DNA damage stimulates p53 to increase the expression of Tp53inp1 (19). In turn, p53INP1 regulates Homeodomain-interacting protein kinase 2 (HIPK2), a pro-apoptotic kinase which is activated in response to DNA damage (22). HIPK2 phosphorylates serine 46 of p53, thereby creating an auto-amplification loop and inducing p53-mediated apoptosis (18,19,22). Recently, HIPK2 was also found to be able to phosphorylate TCF proteins in Xenopus embryos, thereby influencing their binding to target gene promoters (35).

Both the protein and activity level of TCF7L2 has been shown to be regulated by p53 in a human colorectal adenocarcinoma cell line (36). In line with these findings, we here show that TCF7L2 also influences the expression and activity of p53 and in particular its target gene Tp53inp1, in pancreatic beta cells. Glycogen synthase kinase-3β (GSK3β) is a negative regulator of the Wnt-signaling pathway and inactivation of GSK3β leads to activation of TCF7L2. In support of a regulation of p53 by Wnt signaling, it has previously been shown that GSK3β ablation activates p53-dependent apoptosis in colorectal cancer cells (37). Taken together, a complex regulatory pattern emerges for this system controlling cell survival, where p53 acts in a feedback-loop regulating the activity of TCF7L2, and TCF7L2 acts in a feed forward-loop influencing the activity of p53 (Fig. 8).

The current experimental data cannot easily be translated into the human situation where the risk T allele of rs7903146 leads to an open chromatin configuration in this region in the TCF7L2 gene and allows binding of trans regulatory proteins to this locus (4,38,39). The T allele functions as an enhancer leading to increased TCF7L2 mRNA expression and transcriptional activity. One possibility is that the risk T allele increases expression of an inhibitory TCF7L2 isoform with lower transcriptional activity, as shown for the exon 4-containing TCF7L2 isoform in colorectal and liver cancer cell lines (13,40). In human pancreatic islets, isoforms containing exon 4 predominate and expression of exon 4 is associated with the level of glycaemia as measured by HbA1c (12). In vitro, silencing of Tcf7l2 resulted in failure to suppress p53INP1-dependent apoptosis. It can thus be speculated that in humans, TCF7L2 might have a protective role to prevent beta cells from apoptosis by high glucose by inhibiting the p53-p53INP1 pathway. It still remains to be investigated if the rs7903146 SNP in the TCF7L2 gene in human islets is associated with impaired TCF7L2 transcriptional regulation. In vivo, the role of TCF7L2 and p53 in the link between type 2 diabetes and cancer could be TCF7L2 and p53. In this context, the strongest type 2 diabetes-associated (proposed to be the causal) SNP rs7903146 in the TCF7L2 gene has also been shown to be associated with colorectal cancer (46). On the other hand, it was recently reported that the same SNP conferred protection from prostate cancer (47). Further studies are needed to elucidate the role of TCF7L2 and p53 in the link between type 2 diabetes and cancer.

In summary, these data suggest a molecular link between the two type 2 diabetes-associated genes, Tcf7l2 and Tp53inp1, by showing that TCF7L2 regulates the p53-p53INP1 pathway to affect beta-cell survival.
MATERIALS AND METHODS

Cell culture

The rat beta-cell line INS-1 (832/13) and rat islets were cultured at 37°C, 5% CO₂ in the RPMI 1640 medium complemented with 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 10% fetal bovine serum and 100 units/ml penicillin/streptomycin.

ChIP on tiling arrays

INS-1 cells were incubated in the medium containing either 5.5 mM or 14.3 mM glucose for 24 h. ChIP was performed

Figure 6. Apoptosis measurements after siRNA treatment in INS-1 cells. (A) Apoptosis measured using ICC. Cells were triple stained using: Hoechst (DNA, blue), Annexin 5 (early apoptosis marker, green) and 7AAD (late apoptosis marker, red). Images from three time points are shown: 24, 48 and 72 h after siRNA incubation. (B) Apoptosis measured using a DNA degradation-based kit after 72 h of incubation with siRNA. Tcf7l2 silencing resulted in a 74% increased cell death (1.00 ± 0.16 versus 1.74 ± 0.25) and cells with a non-induced Tp53inp1 level, i.e. (Tp53inp1 KD) and (Tcf7l2 Tp53inp1 double KD) had similar cell death levels as control (1.02 ± 0.07 and 0.87 ± 0.09 versus 1.00 ± 0.16, P = 0.248 and P = 0.564). n = 4, *P < 0.05, Mann–Whitney U-tests. (C) Apoptosis measured using western blot analysis of caspase-3 heavy chain degradation after 72 h of incubation with siRNA. A total of 15 μg of protein lysate was run in duplicate with β-actin used as loading control. Bars represent the quantification of three independent experiments, shown together with a representative blot. Cells treated with adriamycin for 4 h (positive control) or with scrambled siRNA (negative control) were used as controls. Tcf7l2 silencing resulted in a 94% decreased caspase-3 heavy chain expression (1.00 ± 0.23 versus 0.06 ± 0.04) and cells with a non-induced Tp53inp1 level, i.e. (Tp53inp1 KD) and (Tcf7l2 Tp53inp1 double KD) had similar cell death level as the negative control (1.20 ± 0.29 and 0.84 ± 0.14 versus 1.00 ± 0.23, P = 0.275 and P = 0.827), n = 3, *P < 0.05, Mann–Whitney U-tests. Cells were incubated in 11 mM glucose. Data are represented as mean ± SEM. Cells were incubated with siRNA targeting: scrambled siRNA (control), Tcf7l2 (Tcf7l2 KD), Tp53inp1 (Tp53inp1 KD) or both Tcf7l2 and Tp53inp1 (Tcf7l2 Tp53inp1 double KD).
following the Nimblegen protocol (http://www.nimblegen.com/products/chip/tutorial.html). Briefly, cells were cross-linked with 1% formaldehyde, then burst using two lysis buffers sequentially (buffer 1: 50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.5% NP-40, 0.25% Triton X-100 and buffer 2: 200 mM NaCl, 1 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 10 mM Tris–HCl pH 7.5) and nuclear extracts were isolated and sonicated (Bioruptor, Diagenode, Belgium) to prepare chromatin fragments with high power sonic pulses, 30 s on/off mode for 15 min in 1.5 ml sonication buffer (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% Na-Deoxycholate, 0.5% N-lauroyl sarcosine). The average DNA size was 300–700 bp as estimated by agarose gel electrophoresis. The fragmented nuclear extracts were immuno-precipitated using an antibody against TCF7L2 (3.3 μg/ml, SC-8631, Santa Cruz, goat polyclonal IgG). Enriched DNA fragments were amplified using the GenomePlex Complete Whole Genome Amplification Kit (#WGA2-50RXN 088k-1728, Sigma-Aldrich, Germany). The labeling and hybridization were performed at Roche NimbleGen (Reykjavik, Iceland), using NimbelGen rat promoter tiling array 2.1M. Analysis of the ChIP data were performed using the previously described two-step TileMap approach HMM for computing probe level test-statistics and combing information from neighboring probes (15). All sequences enriched by the TCF7L2 pull-down in the ChIP-on-Chip experiment were pooled from both the low and high glucose conditions and repetitive elements were removed by RepeatMasker (Smit et al., RepeatMasker Open-3.0, 1996–2010, www.repeatmasker.org). To identify enriched binding motifs, the MDscan tool was used (41).

siRNA-mediated gene-silencing and real-time PCR

Gene silencing in INS-1 cells was performed using Lipofectamin RNAiMAX (#13778100 Invitrogen, USA) for transfection in 11 mM or 25 mM glucose siRNA targeting: rat Tcf7l2 (sense: AGUGCUCGUGGAUCAUGtt) and antisense: UCAUUAGC CAAAUCAGGACUtg. Silencer Select customer-designed siRNA against rat Tcf7l2, Tp53 (# 4390815), and scramble-siRNA number 2 (Negative Control #2 siRNA; Silencer Select Pre-designed siRNA Ambion, Applied Biosystems, USA). The sequences for scramble-siRNA number 1 were sense: 5’-GAGACCCUAUCGGUGA UUAAtt-3’ and antisense: 5’-UAUUCAGGAUGGAGCUC tt-3’ (Thermo Scientific, USA). The concentration used was 60 nM for scramble-siRNA 1, scramble-siRNA 2, Tcf7l2, Tp53 and Tp53inp1 in the single gene-silencing experiments and 15 nM for Tp53 or Tp53inp1 in the double gene-silencing experiments. The transfection complexes were prepared according to the manufacturers’ instructions. After 48 h incubation with siRNA, mRNA was extracted using the RNeasy Plus Mini kit (#74134, Qiagen, Germany). Concentration and purity of the RNA was measured using a NanoDrop ND-1000 spectrophotometer (A_{260}/A_{280} 1.8 and A_{260}/A_{230} 1.0) (NanoDrop Technologies, USA). Reverse transcription with a combination of random hexamers and oligo dT primers (1:1) was performed using the RevertAid First Strand cDNA synthesis kit (#K1622 Fermentas, Canada). Quantitative real-time polymerase chain reaction (PCR) was carried out using an ABI 7900HT sequence detection system with 20 ng cDNA in 10 μl reaction volumes and TaqMan Expression PCR Master Mix according to the manufacturer’s recommendation (Applied Biosystems). All samples for each gene were analyzed in triplicates on the same 384 well plate (maximum accepted standard deviation in Ct-value of 0.1 cycles) with three endogenous controls (Polr2a, Hprt and Ppia). The expression levels were calculated using the ΔΔCt method. TaqMan expression assays used were: Tcf7l2 (Hs01009041_g1), Tp53 (Rn01467559_g1), Tp53inp1 (Rn00710369_m1), Fto (Rn01538185_m1), Adamts9 (Rn01425211_m1) and as endogenous controls were: Hprt1 (Rn01527840_m1), Polr2a (Rn01752026_m1) and Ppia (Rn03302269_g1) (Applied Biosystems). Since all three housekeeping genes showed similar results, data were normalized against the most stable control Hprt.

TCF7L2, p53 transcriptional activity

TCF7L2 and p53 protein activity was measured using the luciferase reporter gene kit Cignal TCF/LEF or p53 Reporter...
Assay kit (#CCS-018L, #CCS-004L SABiosciences, Qiagen) following the manufacturer’s instructions. The plasmids were transfected simultaneously with siRNA against Tcf7l2 and/or Tp53, using Lipofectamin RNAiMAX (#13778100 Invitrogen) in 11 mM or 25 mM glucose condition. The luciferase activity was measured using the Dual-Glo® Reporter Assay System (#2920, Promega, UK) on Infinite M200 multi microplate reader (Tecan, Switzerland).

EMSA of TCF7L2 binding to the Tp53 promoter

Nuclear extract from INS-1 cells was prepared using Qproteome Nuclear Protein Kit (#37582 Qiagen) according to the manufacturer’s instructions. The DNA sequence in the Rattus Norvegicus Tp53 promoter region was purchased from Invitrogen Life technologies. DNA probes containing TCF7L2 binding site is: sense: 5′-ATTTCTACAGTTTTTGCCCCCCCTTGAATATCTTGCTTTGAATCCCGCAA-3′, and antisense: 5′-TTGCGGGATTCAAAGCAAGATATTCAAGGGGGGGCAAAAACTGTAGAAAT-3′. DNA probes were labeled and annealed using Biotin 3′ End DNA labeling Kit (#89818 Pierce Thermo Scientific) according to the

Figure 7. Viability and apoptosis measurements after siRNA treatment in rat primary islets. The quantification was done using five independent animals, after 24 h of incubation with siRNA in either 5.5 mM glucose condition (A and C) or 14.3 mM glucose condition (B and D). Viability was estimated by measuring islets reducing potential. Tcf7l2 KD resulted in 41% decrease (1.00 ± 0.25 versus 0.59 ± 0.12) in viability in low glucose (A) and 65% (1.00 ± 0.13 versus 0.35 ± 0.03) in high glucose (B), and islets with a non-induced Tp53inp1 or Tp53 level had similar viability as control. Apoptosis was measured quantifying active caspase-3/7 in rat islets. Tcf7l2 KD resulted in a 75% increase (1.00 ± 0.03 versus 1.75 ± 0.19) in apoptosis in low glucose (C), a 120% increase (1.00 ± 0.22 versus 2.20 ± 0.14) in apoptosis in high glucose (D). Islets with a non-induced Tp53inp1 or Tp53 level had similar apoptosis level as control. Data are represented as mean ± SEM. Wister rat islets were treated with siRNA targeting: scrambled siRNA (control), Tcf7l2 (Tcf7l2 KD), both Tcf7l2 and Tp53inp1 (Tcf7l2 Tp53inp1 double KD) or both Tcf7l2 and Tp53 (Tcf7l2 Tp53 double KD), n = 5, *P < 0.05, **P < 0.01 Mann–Whitney U-tests.

Figure 8. Schematic of the proposed mechanism of how Tcf7l2 may regulate beta-cell survival. Tp53inp1 is a direct target gene for the tumour suppressor transcription factor p53 and, for example, DNA damage or hyperglycemia may stimulate p53 to increase the expression of Tp53inp1. P53 is also able to regulate the expression level of Tcf7l2. Here we suggest that TCF7L2 regulates the expression of Tp53 and as a consequence also of Tp53inp1 in pancreatic islets thereby controlling beta-cell survival.
Assessment of INS-1 apoptosis

Imaging. Images were taken of the INS-1 cells in 11 mM glucose. Cells were washed twice with cold phosphate buffered saline followed by incubation in cold binding buffer (51-66121E, BD Pharmingen Technical, USA), then stained with FITC-Annexin V (51-65874X, BD, 1:20), 7-AAD (51-68981E, BD, 1:20) and Hoechst 23580 (1:500). FITC, 7-AAD and Hoechst 23580 were excited at 488 nm, 633 nm and 780 nm (two-photon) laser lines and emitted light was collected with filters with 500–530 nm, 650–710 nm and KP685 nm, respectively. Images were acquired using confocal microscopy with the software ZEN 2008 (Zeiss, Germany).

DNA degradation. Cell death was quantified in the 11 mM glucose condition, each measurement with ~10^5 cells using the Roche Cell Death Detection ELISA Plus kit (No. 11774425001 Roche Applied Science, Switzerland) according to the manufacturer’s instructions.

Western blot. After 72 h incubation with siRNA in the 11 mM glucose condition, cells were scraped in lysis buffer (50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% NP40 w/v, 1 mM Na-orthovanadate, 50 mM NaF, 5 mM Na-pyrophosphate, 0.27 M sucrose, complete protease inhibitor (1 tablet/50 ml) and 1 mM DTT). Protein lysates were cleared by centrifugation at 4°C before the concentration was measured using the Bradford method (48) and 15 μg of protein lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–12%) and electrotransfered to nitrocellulose membranes. Membranes were blocked in TBS-T (50 mM Tris pH 7.6, 137 mM NaCl and 0.1% (w/v) Tween-20) containing 10% (w/v) skimmed milk. Primary antibodies used were: rabbit anti-p53INP1 2 μg/ml (ab 9775 Abcam, Cambridge, UK), rabbit anti-beta-actin (Cell Signalling, Danver, MA, USA) 1/1000 dilution and rabbit anti-caspase-3 (Cell signaling 8G10 #9665) 1/1000 dilution. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) reagent (SuperSignal West Pico Chemiluminescent Substrate #34077, Thermo Scientific). Protein bands were quantified by digitizing the ECL films with a Fuji LAS 1000 charge-coupled device camera and analysis of the intensities with Image Gauge software (Fuji, Japan).

Assessment of primary rat islet apoptosis and viability

Wistar rat pancreatic islets were isolated by collagenase digestion. Islets from five individual animals were seeded in the RPMI 1640 medium for siRNA-mediated gene-silencing experiments in either 5.5 mM or 14.3 mM glucose in the medium. After 24 h incubation, primary rat islet viability was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay (# G8090, Promega, USA) and islet apoptosis was measured using Caspase-Glo® 3/7 Assay (# G3582, Promega, USA) according to the manufacturer’s instruction.

Statistical analysis

Data are presented as means and standard error of the mean. Significance was examined by two-tailed Mann–Whitney U-tests and Kruskal–Wallis tests.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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