Decreasing Txnip mRNA and Protein Levels in Pancreatic MIN6 Cells Reduces Reactive Oxygen Species and Restores Glucose Regulated Insulin Secretion

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Key Words

Txnip • Glucose-stimulated insulin secretion • Immunoprecipitation • ROS • siRNA • shRNA • cDNA

Abstract

Background and aims: Recently, thioredoxin-interacting protein (Txnip) expression has been implicated in a number of cellular events associated with diabetes, with increased Txnip levels associated with reduced glucose uptake into peripheral tissues, increased reactive oxygen species (ROS) in endothelial cells, beta cell glucotoxicity and apoptosis. The potential relevance of Txnip with regards to glucose-regulated insulin secretion (GSIS), a fundamentally important characteristic of beta cells and insulin-producing cells being considered as a possible cell therapy for diabetes, has not yet been investigated. **Methods:** Here, studying glucose-responsive MIN6 B1(GSIS) and cells which had significantly reduced response to glucose after time in culture i.e. MIN6 B1(Non-GSIS), using ELISAs; qRT-PCR; immunoprecipitation and Western blotting; transient and stable (siRNA/shRNA and cDNA) approaches to achieve Txnip knock-down or over-expression, respectively, we established a direct association between Txnip ex-

pression and GSIS. **Results:** Specifically, increasing Txnip levels correlate with increased intracellular ROS levels and with significant GSIS loss. Conversely, both transient and stable knock-down of Txnip expression was associated with GSIS recovery. **Conclusion:** This, we believe, is another reason in favour of targeting Txnip as a novel approach for diabetes-related therapy.

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Introduction

Thioredoxin-interacting protein (Txnip; also known as Vitamin D3 up-regulated protein 1; VDUP1) binds to the reducing agent, thioredoxin, inhibiting its activity and/or its bioavailability and thus contributing to cellular redox control. Txnip protein was initially identified in HL60 cancer cells stimulated with 1,25-dihydroxyvitamin D-3 [1]. Since then, this protein has been reported to be involved in a range of cellular functions. For example, Txnip over-expression in gastric carcinoma cell lines (SNU-620

and SNU-16) has been associated with decreased growth rate. Cell cycle arrest, induced by serum starvation or high-density culture of NIH393 and 293 cell lines, has also been associated with increased expression of Txnip. These studies implicate Txnip in inhibiting cancer cell proliferation and inducing cell cycle arrest. Furthermore, *in situ* hybridisation of representative breast, lung and stomach tumour and normal tissue, showed Txnip expression to be down-regulated in cancer [2]. Supporting a role for Txnip as a tumour suppressor gene, Sheth et al. [3] reported Txnip deficiency in knock-out mice to be sufficient to initiate the development of hepatocellular carcinoma. Other roles associated with Txnip include regulating hepatic [4] and myocardial [5] glucose uptake; controlling fatty acid utilisation (identified from studies of an animal model of Reye syndrome) [6]; and contributing to the development and differentiation of NK cells [7].

More recently, a number of studies have highlighted Txnip as of relevance to key features of diabetes. Studying healthy volunteers' primary skeletal muscle, Parikh et al. [8] reported Txnip to regulate both insulin-dependent and insulin-independent pathways of glucose uptake. In these studies, over-expression of Txnip in cultured adipocytes significantly reduced glucose uptake, while Txnip knock-down (with siRNA) enhanced glucose uptake, suggesting Txnip to be regulatory of glucose homeostasis in adipocytes and skeletal muscle. With due consideration to cardiovascular disease in diabetes, Li et al. [9] reported on the analysis of human aortic endothelial cells (HAECs) following exposure to high glucose (25 mMol) for 24 hrs.; where up-regulation of Txnip and subsequent impaired thioredoxin anti-oxidation, through p38 MAPK and FOXO1, were observed [9]. Recent analysis of RNA isolated from lymphocytes from Type 2 diabetes patients, individuals with impaired glucose tolerance (IGT) and healthy volunteers (n=10 in each group) indicated significantly higher levels of Txnip mRNA in lymphocytes from Type 2 diabetes compared to controls. Txnip mRNA levels were also higher in IGT versus controls, although significance was not reached [10]. Studying mouse models of both Type 1 and Type 2 diabetes, Minn et al. [11] reported increased expression of Txnip to be associated with beta cell apoptosis. Exposure of INS-1 cells to high glucose (25 mMol) for 24 hrs. [12] and subsequent exposure of both rat islets and INS-1E cells to high glucose (22.2 mMol) levels for extended periods (48 hrs.) was associated with increased Txnip levels and with beta cell apoptosis [13]. In keeping with this, Txnip has been identified as a mediator of beta cell glucotoxicity through carbohydrate response element-bind-

ing protein p300 and histone H4 acetylation; with lack of Txnip protecting against streptozotocin- and obesity-induced diabetes by preventing beta cell apoptosis and thus preserving beta cell mass [14].

As outlined above, a number of very interesting studies have implicated important roles for Txnip regulation in many aspects of diabetes and its management, proposing Txnip as a novel target for diabetes therapy [14]. So far, however, the relevance of Txnip in glucose-stimulated insulin secretion (GSIS), a fundamental characteristic of beta cells, has not been investigated. Here, using MIN6 and its clonal variant MIN6 B1 as cell line models which have been described as rare examples of a transformed cell line that have retained many relevant characteristics of beta cells [15-16], we investigated the functional relevance of Txnip following continuous culture of the cells, while maintaining conditions in which beta cells and islets are generally cultured; with the long-term objective of enabling future development of potential methods whereby relevant beta cell functions could be preserved in medium- to long-term culture.

Materials and Methods

Cell culture

MIN6 B1 cells were grown in DMEM with 15% heat-inactivated (56°C; 1 hr.) fetal calf serum (FCS) (Lonza, UK), 1% L-glutamine and 75 µM β-mercaptoethanol, at 37°C and 5% CO₂, as recommended by Dr. Lilla who established and generously donated these cells for this study [16]. MIN6(GSIS) and MIN6(Non-GSIS) were cultured as previously described [17-19]. Three independent stocks of all cell lines/variants were kept separately and were treated as independent cell lines for use in all biological triplicate analyses. Routine sterility checks, including screening for *Mycoplasma*, indicated that the cells were clear of contamination.

GSIS analysis

The GSIS profiles of all cells included were analysed at glucose concentrations of 3.3 and 16.7 mMol/l using methods that we have described previously [18-19].

Proliferation assays

The proliferation rates (doubling times) of cell populations were determined by monitoring their growth over consecutive 24 hrs. time periods. For this, the cells were seeded at 5X10⁴ cells/well, in 24 well plates. Cells were incubated over-night at 37°C and 5% CO₂ and 3 wells were trypsinised and counted on each of the 7 days following seeding.

Light microscopy

Cells were grown in 6 well plates and were photographed through a microscope, using phase-contrast.

qRT-PCR analysis

Total RNA was isolated using RNeasy Kit (Qiagen), following the manufacturer's instructions. In order to remove any contaminating genomic DNA that may be present in the RNA isolates, RNA was treated with 1U of RNase-free DNase (Promega, UK) per 1µg RNA in a reaction mixture of 10µl including reaction buffer (Promega, UK). Following incubation at 37°C for 30 min., DNase was inactivated by the addition of 1µl DNase stop solution (Promega, UK) and incubation at 65°C for 10 min. RNA quantity and purity were assessed spectrophotometrically at 260 nm, 280 nm and 230 nm using a NanoDrop ND-1000 (Labtech International). Gel electrophoresis and Agilent Bioanalyser were used to assess RNA qualitatively. Reverse transcription was performed for first-strand cDNA synthesis using 1µg RNA and 1µl of 500ng/µl oligo dT primers (MWG, Germany) and incubating at 72°C for 10 min. This mixture was further incubated at 37°C for 1 hr. in a reaction mixture containing 40U/µl RNasin (Sigma, UK), 10mM dNTP (Sigma, UK), 200U/µl MMLV reverse transcriptase enzyme (Sigma, UK) and 10X reverse transcriptase buffer, including nuclease-free H₂O (Ambion, UK) to make up the final volume of 15µl. cDNA was amplified by qRT-PCR (ABI 7500 Real-Time PCR System; Applied Biosystems International, UK) using Txnip-specific primers/probes. Internal housekeeping gene controls, beta-actin (4352933E; Applied Biosystems International, UK) and GAPDH (4352932E; Applied Biosystems International, UK) (established as not differing significantly in expression levels between cell populations being assessed) were included to normalise any possible differences in RNA isolation and RNA degradation, as relevant. Individual samples were analysed in triplicate. In addition to DNase-treating the RNA, to ensure exclusion of any amplification product derived from genomic DNA that could contaminate the RNA preparation, minus reverse transcriptase enzyme controls verified no DNA/pseudogene contamination of starting material.

Immunoprecipitation and Western Blotting

Cells (from approximately 80% confluent 75cm² flasks) were washed three times with PBS and suspended in a 300µl cold Lysis buffer (1M Tris pH 7.4, 3M NaCl, 1M NaF, 10% NP-40, 100 mM Na₃VO₄, 100 mM PMSF, 25x Protease Inhibitors, H₂O). This was incubated on ice for 30 min. and subsequently centrifuged at 12,000g for 10 min. at 4°C. Supernatant was then transferred to a fresh Eppendorf tubes. Anti-Txnip antibody (MBL International, USA) was added at 2µg per 200µl of cell extract. After mixing by gentle pipetting, this cocktail was incubated on a revolving apparatus for 1 hr. at 4°C. Twenty µl of 50% protein A agarose beads (Sigma, UK), re-suspended in the cold Lysis buffer, was added to the mix and was subsequently incubated with gentle agitation, as before (on revolving apparatus; 1 hr.; 4°C). This mixture was then centrifuged at 7300g for 5 min.; the beads were washed with cold Lysis buffer and were centrifuged again at 7300g. This wash step was performed 5 times. The beads were then re-suspended in 20µl of Laemmli's sample buffer (Sigma, UK); boiled for 5 min; and were centrifuged for 5 min. Protein samples (10µl/lane) were loaded onto

an SDS-polyacrylamide gel for electrophoresis. Following protein transfer, the membranes were then blocked in 10% skimmed milk in PBS (w/v) for 1 hr. at room temperature; washed in PBS (X3 times); and incubated with anti-Txnip antibody diluted in PBS (10µg/ml). After incubating over-night at 4°C, and washing as before (PBS; 5 min x 3) the membranes were incubated in the presence of secondary horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako Cytomation, Denmark) diluted with 1% skimmed milk in PBS (1:1000), for 1 hr. at room temperature. After a final wash (PBS; 5 min x 3), these were developed using the Enhanced Chemiluminescence Kit (Amersham, UK).

siRNA and shRNA transfection

To initially achieve transient knock-down of Txnip expression, siRNA studies were performed. In brief, 50 nM of 2 independent pre-designed siRNA or scrambled (Scr) control siRNA (AM4636; Ambion, UK) targeting Txnip and designated Txnip-1 (77316; Ambion, UK) and Txnip-2 (72223; Ambion, UK) were transfected using Lipofectamine 2000 (Invitrogen, Ireland). Cells (2x10⁵ per well) in a 24 well plates were used for these transfection studies. The cells were harvested for RNA extraction or assayed for GSIS 72 hrs. after transfection. For stable knock-down, three Txnip-specific shRNA in pLKO.1 lentivirus (RMM4534-NM_023719; Open Biosystem, USA) were independently introduced into the relevant cells. Cells incorporating the shRNA were selected using puromycin-containing medium (Sigma-Aldrich, Ireland). Empty shRNA plasmid was considered as control. Single-cell colonies were established for all 3 Txnip-shRNA and empty-plasmid transfected cells.

Txnip cDNA transfection

MIN6 cells were transfected with (5µg) of Txnip cDNA (MMM1013-65356; Open Biosystem, USA) sub-cloned into pCMV-SPORT6 plasmid. Empty plasmid, lacking Txnip, was transfected as negative control.

Reactive oxygen species (ROS) Analysis

For this assay, 1.875 x 10⁵ cells were seeded/well in a 24 well plate and incubated for 48 hrs. at 37°C. 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA; Calbiochem, USA) was then added to three of the six wells at a final concentration of 30 mM and incubated for 40 min. The plate was subsequently washed in PBS (X 3 times) and fluorescence was read at 485 nm excitation and 535 nm emissions in a fluorescence plate reader (Synergy HT, USA). The wells containing no DCFHDA acted as blanks for each sample. ROS production was expressed as % increase in fluorescence relative to the levels of fluorescence in empty-shRNA (*i.e.* empty plasmid) cells.

Statistical Analysis

Statistical analysis was performed in Excel. P-values were generated using Student's t-tests (paired, two tailed). P < 0.05 was considered to be statistically significant. GraphPad was used for graph generation.

Fig. 1. Loss of MIN6 B1 GSIS with passing and time in culture. Comparison of glucose stimulated insulin secretion (GSIS) in MIN6 B1 at passages 19 and 23 indicated a significant ($P=0.01$) loss of GSIS with passing and time in culture. These cellular populations were subsequently termed MIN6 B1(GSIS) and MIN6 B1(Non-GSIS), respectively. Data shown represents means $\pm$ S.E. of three independent experiments.

Fig. 2. Increased expression of Txnip mRNA is associated with loss of GSIS. qRT-PCR analysis of mRNA from MIN6 B1(GSIS) and MIN6 B1(Non-GSIS) indicated a significant ($P=0.007$) increase in Txnip mRNA expression associated with loss of GSIS. Analysis of other mRNAs including beta-actin (Actb; shown here) indicated that this was not a global effect on all mRNAs. Data shown represents means $\pm$ S.E. of three independent experiments.

Result

Glucose stimulated insulin secretion (GSIS)

MIN6 B1 cells at passage 19 (p19) produced a glucose concentration-dependent increase in insulin secretion. Fig. 1 indicates a reproducible >2 fold increase in insulin secretion when glucose concentration is increased from 3.3 mM to 16.7 mM for 1 hr. (while we were initially concerned that this fold GSIS was lower than initially cited for these cells, Dr. Lilla was able to assure us that this was as expected. These results were reproducible; *pers. comm*). These cells were subsequently termed MIN6 B1(GSIS). By contrast, following passaging to p23 involving continuous culture of cells (a total of approximately 1 month – which would be relevant when aiming to maintain beta cells/islets in culture prior to transplantation), the same increase in glucose concentration resulted in only a 1.35-fold GSIS. The latter cell population was subsequently termed MIN6 B1(Non-GSIS) (Fig. 1). Overall, in biological triplicate assays, we observed a significant loss of GSIS ($P=0.01$) in MIN6 B1(Non-GSIS) compared to MIN6 B1(GSIS).

Txnip Expression

As indicated in Fig. 2, qRT-PCR analysis of Txnip using RNA extracted from MIN6 B1(GSIS) and MIN6

B1(Non-GSIS) showed a significant up-regulation of Txnip expression (3.1-fold; $P=0.007$) in association with loss of GSIS. This was not due to a general up-regulation of mRNA levels (Beta-actin (Actb) shown as representative results). This increased level of Txnip was also observed in our microarray analysis of these cellular populations (5.25-fold higher levels MIN6 B1(Non-GSIS) versus MIN6 B1(GSIS); $P=0.003$) (manuscript in prep.).

Phenotypic Analysis of MIN6 B1(GSIS) and MIN6 B1(Non-GSIS)

Continuously sub-culturing, from p19 to p23, resulted in a noticeable increase in proliferation rate. Growth curves, plotted for 7 days (Fig. 3) after seeding at a constant density, indicated that the rate of proliferation of MIN6 B1(Non-GSIS) was significantly ($P=0.020$) higher *i.e.* approximately double that observed in MIN6 B1(GSIS). Increased cell death/apoptosis was not observed in MIN6 B1(Non-GSIS) compared to MIN6 B1(GSIS) (results not shown). In fact, there was no notable difference in morphology between the two populations, except that the MIN6 B1(GSIS) cells tended to grow on top of each other, to some extent, while the MIN6 B1(Non-GSIS) cells remained as monolayer.

Fig. 3. Phenotypic analysis shows increased proliferation rate with loss of GSIS, without a notable effects on cell viability and morphology. The proliferation rate of MIN6 B1(Non-GSIS) was found to be significantly ($P=0.02$; calculated at 168 hrs./7 day time point) higher than that of MIN6 B1(GSIS) cells. The data shown represent mean cell number $\pm$ S.E. of three independent experiments.

Transient knock-down of Txnip expression using RNA interference and associated changes in GSIS

Transient transfection, using two independent Txnip-specific siRNAs, in MIN6(Non-GSIS) (passage 47) cells resulted in a significant reduction of detectable Txnip mRNA levels, compared to non-transfected (NT) cells and cells transfected with either scrambled (Scr) or GAPDH-targeted siRNAs. Using Txnip-1 and Txnip-2 siRNAs, Txnip mRNA was reduced by approximately 43.5% and 42.4%, respectively, compared to levels in scrambled siRNA-transfected cells (Fig. 4A). This transient knock-down of Txnip expression correlated with a significant increase in GSIS in Txnip-1 siRNA ($P=0.030$) and Txnip-2 siRNA ($P=0.019$) transfected cells, compared to scrambled siRNA-transfected cells (Fig. 4B). (As expected, GSIS levels in GAPDH-siRNA transfected and non-transfected (NT) cells did not differ significantly from that in scrambled (Src) siRNA-transfected cells).

Stable knock-down of Txnip expression using RNA interference and associated changes in protein levels, proliferation and GSIS

siRNA-induced silencing is short-lived, so to study long-term effect of Txnip silencing, MIN6 cells which had lost their GSIS were stably transfected using 3 independent Txnip-shRNA; or Empty-plasmid, as control. Subsequently, two clonal populations, with maximum Txnip knock-down *i.e.* sh-1 (60.4%; compared to empty plasmid control, Em-F) and sh-9 (54.1%; compared to empty plasmid control, Em-F), were selected for further studies. The clonal population resulting from sh-3 introduction -which displayed no Txnip mRNA or protein knock-down- and a clonal population from empty-plasmid transfected cells (Em-F) were selected as negative controls (Fig. 5A). Analysis of corresponding protein levels indicated sh-1 and sh-9 clonal populations, but not sh-3 clonal population, expressed reduced levels of Txnip protein, compared to Em-F (Fig. 5B). Knock-down of Txnip expression had minimally, but not significantly, effected proliferation rates and cell viability (data not shown).

Fig. 4. Transient knock-down of Txnip expression is associated with GSIS recovery. (A) MIN6(Non-GSIS) were transfected with 2 Txnip siRNAs (Txnip-1, Txnip-2), or with Scrambled (Scr) or GAPDH- (Gapdh) targeted siRNAs as controls. Non-transfected (NT) cells were also analysed as another control. qRT-PCR analysis 72 hrs. post-transfection indicated Txnip mRNA levels to be reduced by 43.5% and 42.4%, respectively, of that in the Src-siRNA transfected cells. (B) siRNA-mediated knock-down of Txnip expression was associated with significant recovery of GSIS for these cells *i.e.* Txnip-1 siRNA; $P=0.03$; Txnip-2 siRNA; $P=0.019$. Results represent mean $\pm$ S.E from three different experiments.

Fig. 5. Stable knock-down of Txnip expression is associated with GSIS recovery and reduced intracellular ROS. (A) MIN6(Non-GSIS) (passage 47) were transfected with 3 Txnip-targeted shRNAs (namely, sh-3, sh-1, sh-9) or empty-shRNA vector (Em-F). qRT-PCR indicated Txnip mRNA level to be reduced by 60.4% and 54.1% compared to that in Em-F-transfected cells, by sh-1 sh-9 shRNAs, respectively. sh-3 showed no knock-down of Txnip mRNA; (B) the same effects were observed at the protein level, using immunoprecipitation followed by Western blotting to analyse Txnip expression; (C) the Txnip mRNA and protein knock-down was associated with a significant increase in GSIS in sh-1 (2.4-fold; $P=0.024$) clonal population. Increased GSIS was also reproducibly observed in the sh-9 population compared to that in the Em-F and sh-3 transfected populations; however, significance was not reached here ($P=0.23$); (D) corresponding intracellular ROS levels, measured by DCFHDA fluorescence staining, showed sh-1 and sh-9 clonal populations to have reduced ROS by approximately 40.5% and 35.6%, respectively, compared to that in the control populations. Results represent mean $\pm$ S.E from three different experiments.

Fig. 6. Increasing Txnip expression is associated with GSIS recovery. (A) Txnip cDNA introduction into glucose-response insulin secreting MIN6 cells resulted in an approximately 24-fold increase in Txnip mRNA levels. (B). This increased Txnip expression was associated with a significant ($P=0.013$) loss in GSIS *i.e.* GSIS was reduced to 70% that of the empty-plasmid control cell population. Results represent mean $\pm$ S.E from three different experiments.

In order to assess any functional effects of Txnip manipulation on regulated insulin secretion, GSIS analysis of Txnip-shRNA transfected clones was performed. As indicated in Fig. 5C, after normalising the mean GSIS level in the Em-F and sh-3 controls to a baseline of 1, sh-1 clonal population was found to have a significantly ($P=0.024$) higher GSIS *i.e.* 2.4-fold. While levels of GSIS were also increased (1.45-fold) in sh-9 clonal population, comparison of results from 3 biological repeat experiments indicates that statistical significance was not reached for sh-9 cells (Fig. 5C).

Analysis of reactive oxygen species (ROS) showed sh-1 and sh-9 to portray reduced ROS level *i.e.* by 40.5% and 35.6%, respectively, compared to the mean of the levels in the Em-F and the sh-3 clonal populations (Fig. 5D).

Txnip over-expression and associated GSIS

To further investigate a direct association between Txnip expression and GSIS, Txnip cDNA or the corresponding empty-plasmid (Empty) was transfected into cells with an approximately 3-fold GSIS. Analysis of mRNA levels indicated a significantly higher level (23.7-fold) of Txnip mRNA in Txnip-transfect cells compared to controls (Fig. 6A). This increase in Txnip expression was associated with a significant loss ($P=0.013$) of GSIS in Txnip over-expressing cells compared to empty-plasmid control cells *i.e.* GSIS was reduced to 70% that of empty-plasmid control cells (Fig. 6B). No changes in the total insulin content or proliferation was observed in association with increased Txnip mRNA expression.

Discussion

As summarised in the Introduction, a number of very exciting studies over the past 2 years have implicated Txnip in a number of cellular events directly or indirectly related to diabetes. These include increased Txnip expression levels being associated with reduced glucose uptake into peripheral tissue; increasing ROS in endothelial cells following exposure to high glucose levels for 24 hrs.; mediating glucotoxicity and apoptosis of beta cells; with significantly increased levels of Txnip mRNA detectable in lymphocytes from individuals with Type 2 diabetes compared to healthy volunteers. Conversely, reduced Txnip levels have been associated with enhanced glucose uptake into primary skeletal muscle and cultured adipocytes, preventing beta cell apoptosis and so preservation of beta cell mass [8]. To add to this expanding wealth of knowledge on the potential importance of Txnip control in diabetes, here we investigated the relevance of Txnip in relation to glucose-regulated insulin secretion; a fundamentally important characteristic of beta cells.

The long-term potential to routinely use replacement glucose-regulated insulin secreting (GSIS) beta cells or islets as a therapy for diabetes relies on our ability to culture such cells, *in vitro*, while maintaining their functional status. Previously, using MIN6 cells cultured over 22 passages (~6 months), we established that the GSIS phenotype is relatively unstable in long-term culture and is associated with changes in cellular morphology and apparent de-differentiation to a precursor-like cell type [19]. While these studies were important in establishing that GSIS is lost over time, we believe that shorter-term culture may be more relevant prior to transplantation in the clinical setting. In this study, we established that within

4 passages (~1 month in culture), the glucose responsive MIN6 B1 variant MIN6 B1 (GSIS) undergoes significant ($P=0.01$) loss of glucose-responsiveness. This was associated with increased expression of Txnip, at both the mRNA and protein levels. As opposed to studies reported for INS-1E cells which showed increased Txnip levels resulting from exposure to high glucose levels (25 mMol for 24 hrs. or 22.2 mMol for 48 hrs.) to be associated with cellular apoptosis [12-13], here increased Txnip expression was associated with increased proliferation of cells and was not associated with increased cell death/apoptosis. It is conceivable that this discrepancy could potentially be due to other, yet undefined, glucose-toxicity factors (aside from Txnip) contributing to cell death in the studies of INS-1E. In our study, the difference between control and glucose stimulated cells was exposure to 16.7 mMol/l compared to 3.3 mMol/l glucose for 1 hour only. This short duration of exposure could not, in itself, have induced the over-expression of Txnip and did not result in cellular toxicity.

Results from directly manipulating Txnip expression in MIN6 cell line variants support a direct role for Txnip in GSIS. Application of two independent Txnip-targeted siRNAs resulted in the specific knock-down of Txnip expression and an associated significantly increased insulin secretion in response to glucose. Advancing on this, the stable introducing of Txnip-targeting shRNA resulted in successful knock-down of Txnip expression, with expected reduced ROS levels, and associated significantly augmented GSIS. (The complete lack of effect of sh-3 on Txnip mRNA, protein, ROS and GSIS suggests that the sh-3 sequence was not successful in inducing RNA interference. However, the other 2 shRNAs clonal populations evaluated *i.e.* sh-1 and sh-9, resulted in expected effects on Txnip expression, ROS levels and also on GSIS).

To further corroborate a potential causative role for Txnip in loss of GSIS with increased passaging and time in culture, Txnip cDNA was transfected into MIN6 cells displaying a GSIS. As expected, based on our siRNA and shRNA results, exogenous Txnip expression resulted in significant ($P=0.013$) loss of glucose-regulated insulin secretion.

In summary, here we have established a direct link between expression of Txnip mRNA and protein and glucose-stimulated insulin secretion; with endogenously (due to time in culture/passaging) and exogenously (introducing cDNA) controlled increase in Txnip expression correlating with GSIS loss. Conversely, transient (siRNA) and stable (shRNA) knock-down of Txnip expression was

associated with GSIS recovery. These results, we believe, support the already reported arguments in favour of Txnip being considered as a novel target for diabetes-related therapy.

transcriptase polymerase chain reaction); Txnip (thioredoxin-interacting protein).

Acknowledgements

This work was supported by funding from Ireland's Higher Educational Authority Programme for Research in Third Level Institutions (PRTL1) and Trinity College Dublin's Start-Up Funds for New Academics 2008/2009.

Abbreviations

GSIS (glucose-stimulated insulin secretion); ROS (reactive oxygen species); qRT-PCR (quantitative reverse

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