Original Paper

Downregulation of lncRNA TUG1 Affects Apoptosis and Insulin Secretion in Mouse Pancreatic β Cells

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Key Words

lncRNA TUG1 • Apoptosis • Insulin secretion • Insulin synthesis

Abstract

Background: Increasing evidence indicates that long noncoding RNAs (lncRNAs) perform specific biological functions in diverse processes. Recent studies have reported that lncRNAs may be involved in β cell function. The aim of this study was to characterize the role of lncRNA TUG1 in mouse pancreatic β cell functioning both *in vitro* and *in vivo*. **Methods:** qRT-PCR analyses were performed to detect the expression of lncRNA TUG1 in different tissues. RNAi, MTT, TUNEL and Annexin V-FITC assays and western blot, GSIS, ELISA and immunochemistry analyses were performed to detect the effect of lncRNA TUG1 on cell apoptosis and insulin secretion *in vitro* and *in vivo*. **Results:** lncRNA TUG1 was highly expressed in pancreatic tissue compared with other organ tissues, and expression was dynamically regulated by glucose in Nit-1 cells. Knockdown of lncRNA TUG1 expression resulted in an increased apoptosis ratio and decreased insulin secretion in β cells both *in vitro* and *in vivo*. Immunochemistry analyses suggested decreased relative islet area after treatment with lncRNA TUG1 siRNA. **Conclusion:** Downregulation of lncRNA TUG1 expression affected apoptosis and insulin secretion in pancreatic β cells *in vitro* and *in vivo*. lncRNA TUG1 may represent a factor that regulates the function of pancreatic β cells.

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Introduction

Pancreatic β cells are specialized endocrine cells located within the islets of Langerhans. A sufficient level of insulin release by pancreatic β cells is essential to maintain appropriate blood glucose levels; secretion of inappropriate amounts of insulin due to β cell dysfunction and/or loss leads to various forms of diabetes mellitus [1]. Many studies attempting to determine the cause of β cell dysfunction have focused primarily on transcription factors. However, recent studies have reported that a new class of gene-regulatory molecules in mammalian cells, long non-coding RNAs (lncRNAs), could be involved in β cell function [2, 3].

lncRNAs are arbitrarily defined as transcripts of greater than 200 nucleotides (nt) in length that lack significant open reading frames. Most lncRNAs are transcribed by RNA polymerase II in a manner similar to protein-coding mRNAs. Genome-wide analyses of the mammalian transcriptome have revealed lncRNAs to be a major class of transcripts that is pervasively transcribed [4], and these transcripts have been implicated in performing gene regulatory roles, including X inactivation, epigenetic regulation, pluripotency, cell cycle control, and cell differentiation [4-12]. Accordingly, dysregulation of lncRNAs is associated with a wide range of human diseases [13]. Recent studies have demonstrated that lncRNAs are highly enriched in pancreatic islets and/or are specifically expressed in beta cells [3, 14]. However, there are fewer studies regarding the relationship between lncRNAs and islet function.

The lncRNA taurine upregulated gene 1 (lncRNA TUG1) is a spliced, polyadenylated RNA that does not in its full-length form code for a protein in mice. lncRNA TUG1, which is highly conserved in mammals but not found in other vertebrates, was initially detected in a genomic screen for genes upregulated in response to taurine treatment in developing mouse retinal cells. lncRNA TUG1 is expressed during retinal development, and its inactivation causes loss or malformation of the outer segments of photoreceptors and affects the expression of other genes involved in eye development. Inhibition of lncRNA TUG1 results in increased apoptosis [15]. These results indicate that lncRNA TUG1 may be necessary for development and that its dysregulation may participate in human disease progression. However, the relationship between lncRNA TUG1 and β cell function has not been well characterized, prompting us to explore the role of lncRNA TUG1 in pancreatic β cells.

In this study, we explored the relationship between lncRNA TUG1 expression and the function of pancreatic β cells. We demonstrate that lncRNA TUG1 is highly expressed in mouse pancreatic tissues. Both *in vitro* and *in vivo*, downregulation of lncRNA TUG1 expression induced increased apoptosis and decreased insulin synthesis and secretion. Thus, lncRNA TUG1 may represent a factor that regulates the function of pancreatic β cells.

Materials and Methods

Animals

Adult NOD mice were purchased from the Model Animal Research Center of Nanjing University. In NOD colonies, overt diabetes appears in females at 15 weeks of age, and 60%-80% of these mice become diabetic by eight months. NOD mice that developed overt diabetes (basal non-fasting glycemia >11 mM) were used for the islet isolation experiment. BALB/c mice are non-diabetic, and their islets are often used as donors for allogeneic islet transplantation into NOD mice [16]. Adult BALB/c mice were purchased from the Research Animal Care Committee of Nanjing Medical University. The animals were treated in accordance with the guidelines set by the Research Animal Care Committee of Nanjing Medical University.

Cell culture

Min6 and Nit-1 cells are mouse insulinoma cell lines. Min6 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) containing 25 mM glucose, 10% FBS (Invitrogen), 10 mM HEPES (pH 7.4), 10.2 mM L-glutamine, 50 mM sodium pyruvate, 2.5 mM β -mercaptoethanol, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in 5% CO₂. Nit-1 cells were cultured in normal RPMI medium (Gibco)

supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol and 10 mM HEPES (pH 7.4) at 37°C in 5% CO₂.

Knockdown of lncRNA TUG1 expression via RNA interference

lncRNA TUG1-specific small interfering RNA (siRNA) and control siRNA were designed and synthesized by Invitrogen. The sequences of the three designed lncRNA TUG1 siRNAs were as follows: si-TUG1 1#, CAG UCC UGG UGA UUU AGA CAG UCU U; si-TUG1 2#, CCC AGA AGU UGU AAG UUC ACC UUG A; si-TUG1 3#, GGU AAA CGU UGG AAC UGC CUU CUU U. Non-specific siRNA (si-NC) was purchased from Invitrogen. Min6 and Nit-1 cells were transiently transfected with siRNA using the RNAi MAX reagent (Invitrogen). Twenty-four hours after transfection, the cells were harvested for real-time PCR or western blot analysis.

RNA isolation and real-time PCR

Total RNA was extracted from islets or cultured cells using the TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed in a final volume of 20 μ l under standard conditions using random primers and PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China; RR047A). After the RT reaction, 1 μ l of the complementary DNA was used for subsequent qRT-PCR reactions (SYBR Premix Ex Taq, TaKaRa) according to the manufacturer's instructions. The results were normalized to the expression level of β -actin. The primers used for qRT-PCR are presented in Table 1. qRT-PCR and data collection were performed using an ABI 7500 real-time PCR system.

MTT assays

Nit-1 and Min6 cells were transfected with lncRNA TUG1-targeted siRNA. These cells were maintained in culture for 24 h in a 96-well plate using five replicate wells at 10⁴ cells per well. After incubation for 12, 24, 48 or 72 h, MTT solution was added to the cells at a final concentration of 500 μ g/ml, followed by incubation at 37°C for 4 h. The medium was then removed, and the formazan product was solubilized using dimethyl sulfoxide. Cell viability was determined by measuring the OD at 490 nm.

Flow cytometry

Min6 and Nit-1 cells transiently transfected with lncRNA TUG1 siRNA were harvested via trypsinization at 24 h after transfection. Double-staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide was performed using a FITC-Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's recommendations; the cells were then analyzed using a flow cytometer (FACScan; BD Biosciences) equipped with Cell Quest software (BD Biosciences; ref.19). Cells were discriminated into viable cells, dead cells, early apoptotic cells and apoptotic cells, which were compared to the control-transfected cells for each experiment.

Terminal deoxy-nucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay

Apoptotic cells were detected using the TUNEL assay (In Situ Cell Death Detection Kit, Roche). Cells or tissue sections were incubated in proteinase K for 15 min at room temperature. After rinsing twice in PBS for 2 min each, the sections were incubated in 0.1% TritonX-100 in 1% sodium citrate on ice for 2 min for cells or 8 min for paraffin-embedded tissue sections. After washing twice with PBS, the sections were labeled (60

Table 1. Primers used for real time PCR

Primer Name	Sequence
TUG1 forward	5'-CAAGAAACAGCAACACCAGAAG-3'
TUG1 reverse	5'-TAAGGTCCCCATTCAAGTCAGT-3'
Insulin1 forward	5'-CCAGCTATAATCAGAGACCA-3'
Insulin1 reverse	5'-GTGTAGAAGAAGCCACGCT-3'
Insulin2 forward	5'-TCCGCTACAATCAAAAACCAT-3'
Insulin2 reverse	5'-GCTGGGTAGTGGTGGGTCTA-3'
Pdx1 forward	5'-AGCTCCCTTTCCCGTGGATGAAAT-3'
Pdx1 reverse	5'-TAGGCAGTACGGGTCCTCTTGT-3'
NeuroD1 forward	5'-CAGGGTTATGAGATCGTC-3'
NeuroD1 reverse	5'-GTTTCTGGGTCTTGGAGT-3'
MafA forward	5'-ATCATCACTCTGCCCACCAT-3'
MafA reverse	5'-AGTCGGATGACCTCCTCCTT-3'
Glut2 forward	5'-ATCGCCCTCTGCTTCCAGTAC-3'
Glut2 reverse	5'-GAACACGTAAGGCCCAAGGA-3'
β -actin forward	5'-CGCACCCTGGCATTGTCAT-3'
β -actin reverse	5'-TTCTCCTTGATGTCACGCAC-3'

min; 37°C) with the fluorescein-TUNEL reagent mixture according to the manufacturer's instructions. After rinsing three times with PBS, these sections were visualized under an Olympus (Olympus, Japan) inverted microscope, and representative images were captured using a camera. The nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI).

Islet isolation and purification

The islet isolation and culture techniques have been described previously [17]. Freshly isolated islets were transferred to sterile six-well dishes and cultured in RPMI 1640 medium containing 11.1 mM glucose supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin and 100 μ g/ml streptomycin. The islets were allowed to equilibrate for 3 h, counted and seeded into a 48-well plate and cultured overnight at 37°C in a humidified atmosphere containing 95% air and 5% CO₂.

Insulin assay

Transfected Min6 cells were plated in a 48-well plate at a density of $\sim 1 \times 10^5$ cells/well. After 16 h, the medium was removed; the cells were washed once and then incubated for 1 h in glucose free Krebs-Ringer bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄·7H₂O, 1.2 mM KH₂PO₄, 20 mM NaHCO₃, 16 mM HEPES, 2.56 mM CaCl₂, 0.2% BSA). The cells were then treated for 1 h in KRB buffer with low (5 mM) or high (25 mM) glucose. The medium was collected and stored at -20°C for ELISA (using a kit, EZRMI-13K, Millipore) to determine insulin secretion. For determining the insulin content, the cells were lysed with 0.1% Triton X-100. The insulin content and secretion were normalized to the total protein content using the bicinchoninic acid method. Changes in insulin secretion and content were then calculated with reference to the results obtained from the control group.

Western blot assay

Cells were lysed using RIPA protein extraction reagent (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride (Roche). A 50 μ g sample of the extracted protein was separated via 10% or 15% SDS-PAGE, transferred to 0.22 μ m nitrocellulose (NC) membranes (Sigma) and incubated with specific antibodies. The autoradiograms were quantified using densitometry (Quantity One software; Bio-Rad). The specific antibodies (anti-caspase-3 [#9662], -caspase-9 [#9508], -caspase-12 [#2202] and -Bcl2 antibodies [#2876] obtained from Cell Signaling Technology, Inc.) were used at a dilution of 1:1000. The secondary antibody used in these assays was horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (dilution of 1:1000; Beijing ZhongShan Biotechnology CO., Beijing, China). A β -actin antibody (#sc-47778, Santa Cruz Biotechnology, Inc., CA, USA, dilution 1:1000) was used as the control. All of the experiments were repeated at least three times.

Downregulation of lncRNA TUG1 expression in BALB/c mice using siRNA

To investigate the role of lncRNA TUG1 in insulin secretion *in vivo*, we injected lncRNA TUG1 siRNA into male BALB/c mice. The lncRNA TUG1 siRNA was obtained from Invitrogen as an annealed, predesigned *in vivo* siRNA. Scrambled siRNA was used as a negative control. All animal studies were performed according to the guidelines established by the Research Animal Care Committee of Nanjing Medical University (Nanjing, China). The mice were housed under specific pathogen-free conditions. Synthetic siRNA (100 μ g) dissolved in 0.2 mL PBS was intravenously injected hydrodynamically into the tail vein of one male BALB/c mouse, as previously reported [18-20]. To detect the knockdown efficiency, the expression of lncRNA TUG1 in the pancreas was detected by quantitative real-time PCR on day 2 after treatment.

Morphology of BALB/c mouse islets of Langerhans after downregulated lncRNA TUG1 expression

Tissue sections isolated from the pancreas on the 2nd day after siRNA injection were fixed using formalin. The tissue was then processed for paraffin embedding, and 5 μ m thick sections were sliced and mounted on glass slides. The sections were immunostained using an anti-insulin antibody and counterstained using Mayer's hematoxylin solution. Images of the pancreatic tissue were captured using a computer via a microscope connected to a charge-coupled device camera (Olympus). The ratio of the area of insulin-positive cells to the total area of the pancreatic tissue was calculated as described previously [21]. At least five pancreatic sections from each animal were analyzed.

Fig. 1. Expression of lncRNA TUG1 in mouse pancreas tissues and islets of Langerhans. A: Real-time PCR analysis of lncRNA TUG1 expression in multiple mouse tissues. B: Real-time PCR analysis of lncRNA TUG1 expression levels in the islets of Langerhans and exocrine glands of BALB/c mice. The values are expressed as the means $\pm$ SEM ($n=3$). *, $P<0.05$.

Statistics

Comparisons were performed using ANOVA for multiple groups or Student's test for two groups. The data were presented as the means $\pm$ SEM. $P<0.05$ was considered to be statistically significant, as indicated in the figures.

Results

lncRNA TUG1 is highly expressed in the mouse pancreas

To detect the expression of lncRNA TUG1 in adult BALB/c mouse tissues, including pancreatic, heart, liver, spleen, lung, muscle, colon, stomach and brain tissues, real-time PCR was performed. As shown in Fig. 1A, TUG1 appeared to be highly expressed in pancreas tissues. After islet isolation, the expression of lncRNA TUG1 was not different between mouse pancreatic endocrine and exocrine tissue. The expression of lncRNA TUG1 was also independent of sex (Fig. 1B).

To determine the potential role of lncRNA TUG1 in the pathogenesis of diabetes, we examined whether lncRNA TUG1 is abnormally expressed in mouse islets from NOD mice. We found that the expression of lncRNA TUG1 in diabetic NOD mouse islets was less than that in BALB/c mouse islets (Fig. S1). These results indicate that the expression of the lncRNA TUG1 may be associated with diabetes.

Effects of lncRNA TUG1 on β cell proliferation and apoptosis

To downregulate the expression of the lncRNA TUG1, lncRNA TUG1 siRNA (si-TUG1 1#, si-TUG1 2# or si-TUG1 3#) was transiently transfected into Min6 and Nit-1 cells. The cells were collected at 24 h after transfection for the detection of lncRNA TUG1 expression. qRT-PCR assays revealed that si-TUG1 2# or si-TUG1 3# were effective for mouse lncRNA TUG1 inhibition in both Nit-1 and Min6 cells (Fig. 2A and 2B). An MTT assay was performed to assess cell viability, and we found that the cells transiently transfected with si-TUG1 2# or si-TUG1 3# displayed reduced growth compared to the control cells (Fig. 2C and 2D).

In addition, si-TUG1 3# showed a better suppressive effect than si-TUG1 2#. We thus chose to use si-TUG1 3# in the subsequent studies. To determine whether lncRNA TUG1 RNAi resulted in increased apoptosis, a TUNEL assay was performed. As illustrated in Fig. 2E

Fig. 2. Effects of lncRNA TUG1 expression on β cell proliferation and apoptosis. Real-time PCR analysis of the expression of lncRNA TUG1 in Nit-1 (A) and Min6 (B) cells transfected with lncRNA TUG1 siRNA. MTT assays reveal that Nit-1 (C) and Min6 (D) cell proliferation was decreased in lncRNA TUG1 siRNA-treated groups compared to NC siRNA-transfected groups. Nit-1 (E) and Min6 (F) cells transfected with lncRNA TUG1 siRNA displayed a higher apoptosis ratio based on the TUNEL assay. Nit-1 (G) and Min6 (H) cells transfected with siRNA displayed a higher apoptosis ratio based on flow cytometry. I: Western blot analysis of apoptosis-related proteins in Min6 cells transfected with lncRNA TUG1 siRNA revealed an increase in caspase-3, caspase-9 and caspase-12 expression and a decrease in Bcl-2 expression. The values are expressed as the means $\pm$ SEM (n=3). *, P<0.05; **, P<0.01.

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Fig. 3. Knockdown of lncRNA TUG1 expression decreased insulin synthesis and secretion *in vitro*. A: Real-time PCR analysis of the expression of lncRNA TUG1 in response to various glucose concentrations (11.1, 16.7 or 22.2 mM). Nit-1 cells were cultured for 48 h. B: Real-time PCR analysis of insulin mRNA in Min6 cells after transfection with lncRNA TUG1 siRNA. C: Insulin content was decreased in Min6 cells after transfection with lncRNA TUG1 siRNA. D: GSIS was decreased in Min6 cells after transfection with lncRNA TUG1 siRNA. E: Real-time PCR analysis of the expression of transcription factors Pdx1, NeuroD1, MafA and GLUT2 in Min6 cells treated with lncRNA TUG1 siRNA. The values are expressed as the means $\pm$ SEM (n=3). *, P<0.05.

and 2F, an increased apoptosis ratio was detected in the lncRNA TUG1 siRNA-treated group. Consistent with our finding from the TUNEL staining assay, flow cytometry revealed that the proportion of apoptotic cells was significantly different in the si-NC and lncRNA TUG1 siRNA-treated groups (Fig. 2G and 2H).

Several genes involved in the cell death pathway are upregulated after transfection with lncRNA TUG1 siRNA. Western blot analyses indicated that the expression levels of pro-apoptotic proteins caspase-3, caspase-9 and caspase-12 increased and that the level of the anti-apoptotic protein bcl-2 decreased after transfection with lncRNA TUG1 siRNA (Fig. 2I). These results indicated that downregulation of lncRNA TUG1 reduced cell proliferation and enhanced apoptosis.

Glucose-dependent regulation of lncRNA TUG1

Mature β cells exhibit a transcriptional response to increased demand [22]. Some islet protein-coding transcripts are moderately altered in response to high-glucose conditions. To assess whether lncRNA TUG1 is also dynamically regulated in a manner similar to protein-coding transcripts, we exposed Nit-1 cells to 11.1 mM, 16.7 mM and 22.2 mM glucose for 48 h. Compared to the expression level at 11.1 mM, lncRNA TUG1 expression was downregulated at increasingly concentrations of glucose (Fig. 3A). As high glucose could inhibit lncRNA TUG1 expression, lncRNA TUG1 may play an important role in β cell functioning.

Inhibition of lncRNA TUG1 induces impairment of GSIS in Min6 cells

To directly examine the effects of lncRNA TUG1 siRNA-induced pancreatic β cell dysfunction, we used lncRNA TUG1 siRNA to knock down lncRNA TUG1 expression and

Fig. 4. Knockdown of lncRNA TUG1 expression affects apoptosis and insulin secretion *in vivo*. A: Real-time PCR analysis revealed that lncRNA TUG1 expression levels were decreased in the pancreas after siRNA injection. B: The fasting blood glucose levels in lncRNA TUG1 siRNA- and scrambled siRNA-treated BALB/c mice. Each mouse was fasted overnight. C: The serum insulin levels in lncRNA TUG1 siRNA- and scrambled siRNA-treated BALB/c mice. D: Real-time PCR analysis of the expression of Insulin1, Insulin2, Pdx1, NeuroD1, MafA and GLUT2 in lncRNA TUG1 siRNA-treated BALB/c mice. E: TUNEL analysis measuring apoptosis in the pancreas of lncRNA TUG1 siRNA-treated BALB/c mice. F: Immunohistochemistry of islets stained for insulin. The values are expressed as the means $\pm$ SEM (n=3). *, P<0.05.

evaluated its effects on insulin synthesis and secretion. First, qRT-PCR was performed to detect the expression of insulin mRNA. As shown in Fig. 3C, insulin1 and insulin2 mRNAs

were both decreased in the lncRNA TUG1 siRNA-treated group. The insulin content was also detected, and we found that the insulin content was decreased in TUG1 siRNA-treated Min6 cells (Fig. 3C). The inhibition of lncRNA TUG1 also resulted in a marked reduction in GSIS compared to control cells, supporting our hypothesis that lncRNA TUG1 is important for mediating pancreatic β cell function (Fig. 3D). The expression of the transcription factors pancreatic and duodenal homeobox 1 (Pdx1), neuronal differentiation 1 (NeuroD1), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) and glucose transporter 2 (GLUT2) has been associated with the expression of insulin. Real-time PCR analyses suggested that the expression levels of Pdx1, NeuroD1, MafA and GLUT2 were decreased in the lncRNA TUG1 siRNA-treated group (Fig. 3E).

Inhibition of the expression of lncRNA TUG1 in normal mice reduces insulin secretion

In the lncRNA TUG1 siRNA-treated group, the expression of lncRNA TUG1 in the pancreas was reduced by 60% compared to that in the scrambled siRNA-treated group (Fig. 4A). The fasting blood glucose and serum insulin levels were measured at two days after injection. The fasting blood glucose level in the lncRNA TUG1 siRNA-treated group was higher than that in the scrambled siRNA-treated groups (Fig. 4B), and the serum insulin level was significantly reduced in the lncRNA TUG1 siRNA-treated group compared to the scrambled siRNA-treated group (Fig. 4C). Real-time PCR analyses suggested that the expression levels of Pdx1, NeuroD1, MafA and GLUT2 were also decreased *in vivo* in the lncRNA TUG1 siRNA-treated group (Fig. 4D). The TUNEL assay revealed an increased apoptosis ratio in the pancreatic tissue from the lncRNA TUG1 siRNA-treated group (Fig. 4E). In addition, the relative area of insulin-positive cells was decreased in the lncRNA TUG1 siRNA-treated group compared to the scrambled siRNA-treated group (Fig. 4F).

Discussion

Recent genome-wide association studies (GWASs) have revealed that more than 98% of the total genome can be transcribed. However, most of these transcripts have limited or no protein-coding capacity and have been defined as noncoding RNA (ncRNA) [23], which includes ribosomal RNA (rRNA), transfer RNA (tRNA), microRNA (miRNA) and long non-coding RNA (lncRNA) [24]. microRNAs have been demonstrated as playing an important role in various human diseases [25], and an increasing number of studies have linked lncRNA dysregulation to a variety of human diseases [13]. In the present study, we demonstrated that lncRNA TUG1 is highly expressed in mouse pancreatic tissue compared to the mouse heart, liver, spleen, lung muscle, colon, stomach and brain. Thus, we speculated that the high expression of lncRNA TUG1 in the pancreas may be associated with islet function.

lncRNA TUG1 is expressed during retinal development, and lncRNA TUG1 downregulation induces an increase in the apoptosis ratio [15]. In our study, we downregulated lncRNA TUG1 expression in β cells, which resulted in an increased apoptosis ratio both *in vitro* and *in vivo*. Caspases play an extremely important role in the initiation and progression of apoptosis, and the mitochondria/ cytochrome C-mediated apoptosis pathway and endoplasmic reticulum pathway are both caspase-related apoptosis pathways [26, 27]. After downregulating lncRNA TUG1 expression in Min6 cells, we examined the expression levels of the apoptotic proteins caspase-3, caspase-9 and caspase-12 and the anti-apoptotic protein Bcl2. We found that caspase-3, caspase-9 and caspase-12 expression was significantly increased in the lncRNA TUG1 siRNA-treated group, whereas Bcl2 expression was significantly reduced. Thus, lncRNA TUG1 may affect the mitochondria/ cytochrome C- and endoplasmic reticulum-mediated apoptosis pathways.

In recent years, GWASs have identified many common genetic variants associated with diabetes [28]. For example, lncRNAs KCNQ1OT1 and ANRIL/CDKN2BAS were mapped to established T2D susceptibility loci [29, 30]. HI-LNC45 is decreased in human T2D islets, and HI-LNC25 negatively modulates GLIS3 mRNA in human pancreatic β cells. GLIS3 encodes

an islet transcription factor that is mutated in a form of monogenic diabetes. Additionally, lncRNA MEG3 is downregulated in human T2D islets [31]. Although there are few studies to date of the association between lncRNAs and diabetes or pancreatic β cell function, it has been demonstrated that the expression of islet-specific lncRNA can also be altered in response to different glucose concentrations [3]. In the present study, we demonstrated that the expression of lncRNA TUG1 is decreased at increasingly high glucose concentrations compared to that at 11.1 mM glucose. Thus, lncRNA TUG1 may be regulated by hyperglycemia, and glucose-dependent regulation of lncRNA TUG1 may be related to islet function.

We demonstrated that insulin synthesis and secretion were decreased after lncRNA TUG1 downregulation in β cells. Studies have revealed that the transcription factors Pdx1, NeuroD1 and MafA regulate insulin synthesis by binding to the insulin gene promoter. GLUT2 plays an important role in GSIS [32, 33]. In our study, downregulation of lncRNA TUG1 expression induced decreased insulin synthesis and secretion. Based on our examination of insulin synthesis- and secretion-related islet-specific transcription factors, Pdx1, NeuroD1, MafA and GLUT2 mRNA levels were decreased after lncRNA TUG1 downregulation in Min6 cells. Therefore, lncRNA TUG1 may mediate insulin expression by regulating the expression of Pdx1, NeuroD1 and MafA. Furthermore, studies have shown that cell apoptosis plays an important role in beta cell dysfunction [34], and an increased apoptosis ratio may also affect insulin expression. In our study, we demonstrated that lncRNA TUG1 downregulation could induce an increase in the apoptosis ratio (as shown in Fig. 2). Apoptotic beta cells can induce changes in insulin expression.

We demonstrated both *in vitro* and *in vivo* that the downregulation of lncRNA TUG1 could affect insulin expression. Beta cells and GSIS are regulated by a number of factors in addition to glucose. As shown in Fig. 1, TUG1 is expressed in many tissues, including the brain. Studies have shown that brain-selective kinase2 phosphorylation of PCTAIRE1 negatively regulates glucose-stimulated insulin secretion in pancreatic beta cells [35]. As certain signals in the brain can affect beta cells or GSIS, the *in vivo* results for blood and insulin may be due to TUG1 knockdown in tissue other than the pancreas.

To date, few mechanisms have been proposed with regard to the molecular mechanisms of lncRNAs [36, 37]. lncRNAs could exert their effects via the following pathways: (1) lncRNAs could direct chromatin-modifying complexes, such as PRC2, LSD1 and DNA methyltransferases, to specific genomic loci [38, 39]; (2) lncRNAs could bind to miRNAs and thus prevent the binding of specific miRNAs to their target mRNAs [40]; (3) lncRNAs could regulate the expression of transcription factors by directly binding to promoters [41, 42]. A recent study by Yang et al (2011) demonstrates that lncRNA TUG1 is likely to interact with polycomb complexes [43], and Zhang et al (2014) have demonstrated that lncRNA TUG1 could affect cell proliferation by binding to polycomb repressive complex 2 (PRC2) in human non-small cell lung carcinoma (NSCLC) tissues [44]. However, the mechanisms by which lncRNA TUG1 regulate pancreatic cell function remain to be further elucidated.

In summary, we demonstrated the expression of lncRNA TUG1 in multiple mouse tissues. Knockdown of lncRNA TUG1 expression in mouse β cells increased the apoptosis ratio and influenced GSIS in mouse β cells both *in vitro* and *in vivo*. Thus, downregulation of lncRNA TUG1 as a consequence of hyperglycemia may at least partially contribute to the impairment of β cell function in mice experiencing diabetes and could therefore be implicated in diabetes pathogenesis.

Disclosure Statement

The authors declare no conflict of interest.

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References

- 1 Kikuta K, Masamune A, Hamada S, Takikawa T, Nakano E, Shimosegawa T: Pancreatic stellate cells reduce insulin expression and induce apoptosis in pancreatic beta-cells. *Biochem Biophys Res Commun* 2013;433:292-297.
- 2 Guay C, Jacovetti C, Nesca V, Motterle A, Tugay K, Regazzi R: Emerging roles of non-coding rnas in pancreatic beta-cell function and dysfunction. *Diabetes Obes Metab* 2012;14:S12-21.
- 3 Moran I, Akerman I, van de Bunt M, Xie R, Benazra M, Nammo T, Arnes L, Nakic N, Garcia-Hurtado J, Rodriguez-Segui S, Pasquali L, Sauty-Colace C, Beucher A, Scharfmann R, van Arensbergen J, Johnson PR, Berry A, Lee C, Harkins T, Gmyr V, Pattou F, Kerr-Conte J, Piemonti L, Berney T, Hanley N, Gloyn AL, Sussel L, Langman L, Brayman KL, Sander M, McCarthy MI, Ravassard P, Ferrer J: Human beta cell transcriptome analysis uncovers lncrnas that are tissue-specific, dynamically regulated, and abnormally expressed in type 2 diabetes. *Cell Metab* 2012;16:435-448.
- 4 Guttman M, Donaghey J, Carey BW, Garber M, Grenier JK, Munson G, Young G, Lucas AB, Ach R, Bruhn L, Yang X, Amit I, Meissner A, Regev A, Rinn JL, Root DE, Lander ES: Lincrnas act in the circuitry controlling pluripotency and differentiation. *Nature* 2011;477:295-300.
- 5 Mattick JS: The genetic signatures of noncoding rnas. *PLoS Genet* 2009;5:e1000459.
- 6 Mattick JS, Makunin IV: Non-coding rna. *Hum Mol Genet* 2006;15:R17-29.
- 7 Qureshi IA, Mattick JS, Mehler MF: Long non-coding rnas in nervous system function and disease. *Brain Res* 2010;1338:20-35.
- 8 Wilusz JE, Sunwoo H, Spector DL: Long noncoding rnas: Functional surprises from the rna world. *Genes Dev* 2009;23:1494-1504.
- 9 Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY: Long non-coding rna hotair reprograms chromatin state to promote cancer metastasis. *Nature* 2010;464:1071-1076.
- 10 Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N: Requirement for xist in x chromosome inactivation. *Nature* 1996;379:131-137.
- 11 Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY: Functional demarcation of active and silent chromatin domains in human hox loci by noncoding rnas. *Cell* 2007;129:1311-1323.
- 12 Sleutels F, Zwart R, Barlow DP: The non-coding air rna is required for silencing autosomal imprinted genes. *Nature* 2002;415:810-813.
- 13 Wapinski O, Chang HY: Long noncoding rnas and human disease. *Trends Cell Biol* 2011;21:354-361.
- 14 Derrien T, Johnson A, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, Lagarde J, Veeravalli L, Ruan X, Ruan Y, Lassmann T, Carninci P, Brown JB, Lipovich L, Gonzalez JM, Thomas M, Davis CA, Shiekhatair R, Gingeras TR, Hubbard TJ, Notredame C, Harrow J, Guigo R: The gencode v7 catalog of human long noncoding rnas: Analysis of their gene structure, evolution, and expression. *Genome Res* 2012;22:1775-1789.
- 15 Young TL, Matsuda T, Cepko CL: The noncoding rna taurine upregulated gene 1 is required for differentiation of the murine retina. *Curr Biol* 2005;15:501-512.
- 16 Vergani A, D'Addio F, Jurewicz M, Petrelli A, Watanabe T, Liu K, Law K, Schuetz C, Carvello M, Orsenigo E, Deng S, Rodig SJ, Ansari JM, Staudacher C, Abdi R, Williams J, Markmann J, Atkinson M, Sayegh MH, Fiorina P: A novel clinically relevant strategy to abrogate autoimmunity and regulate alloimmunity in nod mice. *Diabetes* 2010;59:2253-2264.
- 17 Han X, Sun Y, Scott S, Bleich D: Tissue inhibitor of metalloproteinase-1 prevents cytokine-mediated dysfunction and cytotoxicity in pancreatic islets and beta-cells. *Diabetes* 2001;50:1047-1055.

- 18 Bradley SP, Rastellini C, da Costa MA, Kowalik TF, Bloomenthal AB, Brown M, Cicalese L, Basadonna GP, Uknis ME: Gene silencing in the endocrine pancreas mediated by short-interfering rna. *Pancreas* 2005;31:373-379.
- 19 Larson SD, Jackson LN, Chen LA, Rychahou PG, Evers BM: Effectiveness of sirna uptake in target tissues by various delivery methods. *Surgery* 2007;142:262-269.
- 20 Okamoto K, Iwasaki N, Doi K, Noiri E, Iwamoto Y, Uchigata Y, Fujita T, Tokunaga K: Inhibition of glucose-stimulated insulin secretion by *kcnj15*, a newly identified susceptibility gene for type 2 diabetes. *Diabetes* 2012;61:1734-1741.
- 21 Terauchi Y, Iwamoto K, Tamemoto H, Komeda K, Ishii C, Kanazawa Y, Asanuma N, Aizawa T, Akanuma Y, Yasuda K, Kodama T, Tobe K, Yazaki Y, Kadowaki T: Development of non-insulin-dependent diabetes mellitus in the double knockout mice with disruption of insulin receptor substrate-1 and beta cell glucokinase genes. Genetic reconstitution of diabetes as a polygenic disease. *J Clin Invest* 1997;99:861-866.
- 22 Bensellam M, Van Lommel L, Overbergh L, Schuit FC, Jonas JC: Cluster analysis of rat pancreatic islet gene mrna levels after culture in low-, intermediate- and high-glucose concentrations. *Diabetologia* 2009;52:463-476.
- 23 Kung JT, Colognori D, Lee JT: Long noncoding rnas: Past, present, and future. *Genetics* 2013;193:651-669.
- 24 Mattick JS, Taft RJ, Faulkner GJ: A global view of genomic information--moving beyond the gene and the master regulator. *Trends Genet* 2010;26:21-28.
- 25 Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W, Cui Q: An analysis of human microRNA and disease associations. *PLoS One* 2008;3:e3420.
- 26 Zou H, Li Y, Liu X, Wang X: An apaf-1.Cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 1999;274:11549-11556.
- 27 Nakagawa T, Yuan J: Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 2000;150:887-894.
- 28 Hindorf LA, Sethupathy P, Junkins HA, Ramos EM, Mehta JP, Collins FS, Manolio TA: Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proc Natl Acad Sci U S A* 2009;106:9362-9367.
- 29 Voight BF, Scott LJ, Steinthorsdottir V, Morris AP, Dina C, Welch RP, Zeggini E, Huth C, Aulchenko YS, Thorleifsson G, McCulloch LJ, Ferreira T, Grallert H, Amin N, Wu G, Willer CJ, Raychaudhuri S, McCarrroll SA, Langenberg C, Hofmann OM, Dupuis J, Qi L, Segre AV, van Hoek M, Navarro P, Ardlie K, Balkau B, Benediktsson R, Bennett AJ, Blagieva R, Boerwinkle E, Bonnycastle LL, Bengtsson Bostrom K, Bravenboer B, Bumpstead S, Burt NP, Charpentier G, Chines PS, Cornelis M, Couper DJ, Crawford G, Doney AS, Elliott KS, Elliott AL, Erdos MR, Fox CS, Franklin CS, Ganser M, Gieger C, Grarup N, Green T, Griffin S, Groves CJ, Guiducci C, Hadjadj S, Hassanali N, Herder C, Isomaa B, Jackson AU, Johnson PR, Jorgensen T, Kao WH, Klopp N, Kong A, Kraft P, Kuusisto J, Lauritzen T, Li M, Lieve A, Lindgren CM, Lyssenko V, Marre M, Meitinger T, Midtjell K, Morken MA, Narisu N, Nilsson P, Owen KR, Payne F, Perry JR, Petersen AK, Platou C, Proenca C, Prokopenko I, Rathmann W, Rayner NW, Robertson NR, Rocheleau G, Roden M, Sampson MJ, Saxena R, Shields BM, Shriver P, Sigurdsson G, Sparso T, Strassburger K, Stringham HM, Sun Q, Swift AJ, Thorand B, Tichet J, Tuomi T, van Dam RM, van Haeften TW, van Herpt T, van Vliet-Ostaptchouk JV, Walters GB, Weedon MN, Wijmenga C, Witteman J, Bergman RN, Cauchi S, Collins FS, Gloyn AL, Gyllenstein U, Hansen T, Hide WA, Hitman GA, Hofman A, Hunter DJ, Hveem K, Laakso M, Mohlke KL, Morris AD, Palmer CN, Pramstaller PP, Rudan I, Sijbrands E, Stein LD, Tuomilehto J, Uitterlinden A, Walker M, Wareham NJ, Watanabe RM, Abecasis GR, Boehm BO, Campbell H, Daly MJ, Hattersley AT, Hu FB, Meigs JB, Pankow JS, Pedersen O, Wichmann HE, Barroso I, Florez JC, Frayling TM, Groop L, Sladek R, Thorsteinsdottir U, Wilson JF, Illig T, Froguel P, van Duijn CM, Stefansson K, Altshuler D, Boehnke M, McCarthy MI: Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet* 2010;42:579-589.
- 30 Pasmant E, Sabbagh A, Vidaud M, Bieche I: Anril, a long, noncoding rna, is an unexpected major hotspot in gwas. *FASEB J* 2011;25:444-448.
- 31 Kameswaran V, Bramswig NC, McKenna LB, Penn M, Schug J, Hand NJ, Chen Y, Choi I, Vourekas A, Won KJ, Liu C, Vivek K, Naji A, Friedman JR, Kaestner KH: Epigenetic regulation of the *dlk1-meg3* microRNA cluster in human type 2 diabetic islets. *Cell Metab* 2013
- 32 Barrow J, Hay CW, Ferguson LA, Docherty HM, Docherty K: Transcription factor cycling on the insulin promoter. *FEBS Lett* 2006;580:711-715.

- 33 Matsuoka TA, Kaneto H, Stein R, Miyatsuka T, Kawamori D, Henderson E, Kojima I, Matsuhisa M, Hori M, Yamasaki Y: Mafa regulates expression of genes important to islet beta-cell function. *Mol Endocrinol* 2007;21:2764-2774.
- 34 Chen XY, Gu XT, Saiyin H, Wan B, Zhang YJ, Li J, Wang YL, Gao R, Wang YF, Dong WP, Najjar SM, Zhang CY, Ding HF, Liu JO, Yu L: Brain-selective kinase 2 (brsk2) phosphorylation on pcta1 negatively regulates glucose-stimulated insulin secretion in pancreatic beta-cells. *J Biol Chem* 2012;287:30368-30375.
- 35 Dewing AS, Rueli RH, Robles MJ, Nguyen-Wu ED, Zeyda T, Berry MJ, Bellinger FP: Expression and regulation of mouse selenoprotein p transcript variants differing in non-coding rna. *RNA Biol* 2012;9:1361-1369.
- 36 Qu Z, Adelson DL: Identification and comparative analysis of ncrnas in human, mouse and zebrafish indicate a conserved role in regulation of genes expressed in brain. *PLoS One* 2012;7:e52275.
- 37 Khalil AM, Rinn JL: Rna-protein interactions in human health and disease. *Semin Cell Dev Biol* 2011;22:359-365.
- 38 Mattick JS, Amaral PP, Dinger ME, Mercer TR, Mehler MF: Rna regulation of epigenetic processes. *Bioessays* 2009;31:51-59.
- 39 Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I: A long noncoding rna controls muscle differentiation by functioning as a competing endogenous rna. *Cell* 2011;147:358-369.
- 40 Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL: Many human large intergenic noncoding rnas associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci U S A* 2009;106:11667-11672.
- 41 Huarte M, Guttman M, Feldser D, Garber M, Koziol MJ, Kenzelmann-Broz D, Khalil AM, Zuk O, Amit I, Rabani M, Attardi LD, Regev A, Lander ES, Jacks T, Rinn JL: A large intergenic noncoding rna induced by p53 mediates global gene repression in the p53 response. *Cell* 2010;142:409-419.
- 42 Yang L, Lin C, Liu W, Zhang J, Ohgi KA, Grinstein JD, Dorrestein PC, Rosenfeld MG: Ncrna- and pc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs. *Cell* 2011;147:773-788.
- 43 Zhang EB, Yin DD, Sun M, Kong R, Liu XH, You LH, Han L, Xia R, Wang KM, Yang JS, De W, Shu YQ, Wang ZX: P53-regulated long non-coding rna tug1 affects cell proliferation in human non-small cell lung cancer, partly through epigenetically regulating hoxb7 expression. *Cell Death Dis* 2014;5:e1243.