Functional Analysis of Novel Candidate Regulators of Insulin Secretion in the MIN6 Mouse Pancreatic β Cell Line

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Abstract

Elucidating the regulation of glucose-stimulated insulin secretion (GSIS) in pancreatic β cells is important for understanding and treating diabetes. The pancreatic β cell line, MIN6, retains GSIS but gradually loses it in long-term culture. The MIN6 subclone, MIN6c4, exhibits well-regulated GSIS even after prolonged culture. We previously used DNA microarray analysis to compare gene expression in the parental MIN6 cells and MIN6c4 cells and identified several differentially regulated genes that may be involved in maintaining GSIS. Here we investigated the potential roles of six of these genes in GSIS: Tmem59l (Transmembrane protein 59 like), Scgn (Secretagogin), Gucy2c (Guanylate cyclase 2c), Slc29a4 (Solute carrier family 29, member 4), Cdhr1 (Cadherin-related family member 1), and Celsr2 (Cadherin EGF LAG seven-pass G-type receptor 2). These genes were knocked down in MIN6c4 cells using lentivirus vectors expressing gene-specific short hairpin RNAs (shRNAs), and the effects of the knockdown on insulin expression and secretion were analyzed. Suppression of Tmem59l, Scgn, and Gucy2c expression resulted in significantly decreased glucose- and/or KCl-stimulated insulin secretion from MIN6c4 cells, while the suppression of Slc29a4 expression resulted in increased insulin secretion. Tmem59l overexpression rescued the phenotype of the Tmem59l knockdown MIN6c4 cells, and immunostaining analysis indicated that the TMEM59L protein colocalized with insulin and GM130, a Golgi complex marker, in MIN6 cells. Collectively, our findings suggested that the proteins encoded by Tmem59l, Scgn, Gucy2c, and Slc29a4 play important roles in regulating GSIS. Detailed studies of these proteins and their functions are expected to provide new insights into the molecular mechanisms involved in insulin secretion.

Introduction

Glucose-stimulated insulin secretion (GSIS) from pancreatic β cells is essential for the regulation of blood glucose levels. Although GSIS in β cells has been intensively studied, the
underlying mechanisms have not been fully elucidated. As reviewed in [1], the time course of GSIS displays a biphasic pattern. The first-phase insulin release begins soon after the glucose stimulation and persists only for a few min and is followed by the second phase, which lasts for 2–3 h. This biphasic pattern is observed in vivo and in vitro. In the pathogenesis of type 2 diabetes, the earliest detectable defect in β-cell function is generally thought to be a reduction in first-phase insulin secretion [2].

The MIN6 cell line, which was established from an insulinoma of a transgenic mouse expressing the SV40 T antigen in pancreatic β cells, secretes insulin in response to physiological stimuli and is a useful tool for studying the mechanisms of insulin secretion [3]. However, since MIN6 cells in long-term culture with repeated passages lose their ability to secrete insulin in response to glucose, we isolated a subclone (MIN6c4) that retains GSIS even after long-term culture [4]. To identify genes involved in maintaining GSIS, we previously compared the gene expression profiles of four groups of MIN6 cells: parental MIN6 cells at low passage (Pr-LP) and high passage numbers (Pr-HP), and MIN6c4 cells at low passage (C4-LP) and high passage numbers (C4-HP). From this analysis, we identified a group of genes whose expression was high in the glucose-responsive Pr-LP, C4-LP, and C4-HP cells, but was extremely low in the nonresponsive Pr-HP cells, as candidate genes that may be involved in the maintenance of GSIS [4]. Analysis of these genes and their protein products is expected to extend our understanding of the molecular mechanisms of GSIS.

Other groups also performed microarray-based analyses between low-passage and high-passage MIN6 cells and between well-regulated and dysregulated MIN6 subclones, and identified a number of differentially expressed genes, which included the genes related to secretory pathway, metabolism, cell adhesion, and so forth [5,6]. These studies showed that microarray-based approach provides a useful tool for identifying genes involved in GSIS of β cells.

Neuronal cells and pancreatic β cells express many common genes involved in the mechanism of secretion [7,8], and we speculated that these commonly expressed genes might play roles in the insulin secretion from β cells. Using the databases Unigene (http://www.ncbi.nlm.nih.gov/unigene/) and T1Dbase (http://www.t1dbase.org/page/AtlasHome), we selected six of the candidate genes identified in our previous study, whose expression was high in both cell types [Tmem59l (Transmembrane protein 59 like), Scgn (Secretagogin), Gucy2c (Guanylate cyclase 2c), Slc29a4 (Solute carrier family 29, member 4), Cdhr1 (Cadherin-related family member 1), and Celsr2 (Cadherin EGF LAG seven-pass G-type receptor 2)] for further investigation. In the present study, we analyzed the effects of knockdown of these genes on GSIS in MIN6c4 cells.

**Materials and Methods**

**MIN6c4 cell culture**

MIN6c4 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 25 mM glucose, 13% heat-inactivated fetal bovine serum, and 0.1 mM 2-mercaptoethanol in humidified 5% CO₂ at 37°C, as described previously [4]. We used the MIN6c4 cells at passage 40–50.

**Quantitative RT-PCR analysis of RNA from MIN6 cells**

Total RNA was extracted from MIN6 cells by the acid guanidinium-phenol-chloroform (AGPC) method and subjected to cDNA synthesis using ReverTra Ace α (Toyobo, Tokyo, Japan). Quantitative RT-PCR analysis was carried out using FastStart Universal SYBR Green Master (RoX) (Roche, Basel, Switzerland). The reaction was performed with a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following thermal cycling
conditions: 95°C for 10 s followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The relative expression levels of the target genes were normalized to that of Rpl32 [4]. The sequences of the primers used are shown in Table 1.

RT-PCR analysis of RNA from mouse tissues

Tissues were obtained from female C57/BL6 mice and immediately homogenized in guanidine isothiocyanate solution. Islets were isolated as previously described [9]. Total RNA extraction and cDNA synthesis were performed as described above. PCR reactions were carried out with Blend Taq (Toyobo) using the following cycling conditions: 94°C for 2 min followed by 25–30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Rpl32 expression was used as an internal control [10,11]. The sequences of the primers used are shown in Table 1.

Design of short hairpin RNAs (shRNAs)

shRNAs were designed using siDirect 2.0 (http://siDirect2.RNAi.jp/) or the Public TRC Portal website (http://www.broadinstitute.org/rnai/public/seq/search). Five shRNA sequences targeting each candidate gene were selected for evaluation. The shRNA target sequences that were capable of effective knockdown were used in this study and are shown in Table 2. Each of the shRNA oligonucleotides was designed to include the mouse U6 (mU6) promoter sequence upstream of the target sequence (not shown).

Lentiviral vector production and infection of MIN6c4 cells

The lentiviral vector plasmids for shRNA expression were constructed by replacing the CMV-GFP cassette of the SIN vector (CS-CDF-CG-PRE, kindly provided by Dr. H. Miyoshi, RIKEN Tsukuba Institute) with the mU6 promoter-shRNA oligonucleotides described above. The PGK promoter-driven puromycin-resistance gene cassette was used as a selective marker and inserted upstream of the mU6 promoter in the opposite direction. The shRNA-expressing conditions: 95°C for 10 s followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The relative expression levels of the target genes were normalized to that of Rpl32 [4]. The sequences of the primers used are shown in Table 1.

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Table 1. PCR primers used in the present study.

| Gene   | Forward (5' to 3')                  | Reverse (5' to 3')                  |
|--------|------------------------------------|------------------------------------|
| Tmem59l| CCAATGCCACAGAGACAGAATG             | GCTACAGGACGATGGAAAAACAGG            |
| Scgn   | TCTCTCAAGGCGCTCATTGT              | TGGATCACAGGCGCATAGG                |
| Gucy2c | CAGGATCTTGGGGTGTTG                | CGTTCGATCTTCAATCTTACCTG            |
| SLC29a4| TTCTCGCTGCTAATGGCCGAT            | GTGCCTGTTTGAAGACGCT                |
| Cdh1   | AGCTGGACAGAGAAAGGGAAG            | CGATGAGTGTAAAGGCGCTCTG             |
| Celsr2 | TATATCCCCCTCTTGCTGAGG            | GATGAGTGGGTGGGAGCGATAG             |
| Rpl32  | CAATGTGTCCTCTAAGAACCAGAA          | CTTGGCCTTGGGATTTGG                 |

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Table 2. Target sequences of shRNA oligonucleotides used in this study.

| Gene   | Target sequences #1 | Target sequences #2 |
|--------|---------------------|---------------------|
| Tmem59l| CCTCAGAGTCGCCGAATAAC| GCTACAGGACGATGGAAAAACAGG |
| Scgn   | GGATAACAGTGTAGGTTTAT| GGATTGTGCAAGATATGATG |
| Gucy2c | GATGAAAGGACCAAGAATACA| GTCACAATGCAATCTTCAAA |
| Slc29a4| CCTCCTGCTCTTACGACATA| CGATATCTTCCACCAAGTAA |
| Cdh1   | CCCAGCAGACGGAGTCTTCTT| CCCATATGTGTACCTTTA |
| Celsr2 | GCACAGATCATGTACCAGATT| GCTCAGATTTCTTCTTTA |

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lentiviral vectors were produced by transfecting HEK293T cells with the resulting SIN vector plasmids together with packaging plasmids (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev, kindly provided by Dr. H. Miyoshi), as described previously [12,13]. The lentivirus particles were concentrated with a 4× PEG-it solution [32% (w/v) PEG-6000, 400 mM NaCl, and 40 mM HEPES, pH 7.4]. A negative control viral vector, which lacked an shRNA oligonucleotide insert, was also generated. To produce a lentiviral vector expressing Tmem59l, the CMV-GFP cassette of CS-CDF-CG-PRE was replaced with the CAG promoter-driven Tmem59l cDNA. The IRES-zeocin-resistance gene cassette was used as a selection marker and inserted downstream of the Tmem59l cDNA sequence. Similarly, a lentiviral vector expressing shRNA-resistant Tmem59l, which had a silent mutation in the shRNA target site, was also constructed.

MIN6c4 cells were seeded in a 12-well plate, cultured overnight, and infected with lentiviral vectors. The infected cells were selected with 1.2 μg/ml puromycin for shRNA expression or 200 μg/ml zeocin for Tmem59l expression for 2–3 weeks. The resulting colonies were collected and cultured for use in the experiments described below.

Measurement of insulin secretion
MIN6c4 knockdown and control cells were cultured in 24-well plates for 2–3 days. Prior to the insulin secretion assay, the cells were starved in Krebs-Ringer bicarbonate buffer (KRBB) containing 10 mM Hepes pH 7.4, 0.2% bovine serum albumin (BSA), and 3 mM glucose for 30 min, and then washed three times with KRBB. The cells were then incubated in KRBB containing Hepes and BSA with 3, 8, or 25 mM glucose, 30 mM KCl plus 3 mM glucose, or 10 nM exendin-4 plus 10 mM glucose for 1 h. The culture medium was collected, and the secreted insulin was measured using an ELISA kit (Mercodia, Uppsala, Sweden). To normalize the amount of secreted insulin, the cells in each well were lysed with RIPA buffer, and the protein concentration of the cell lysates was measured using the Bradford method (Bio-Rad, Hercules, CA, USA). The insulin secretion rate was expressed as the amount of secreted insulin (μg)/mg protein/h [14–16].

Measurement of insulin content
MIN6c4 knockdown and control cells were cultured in 6-cm dishes for 3–5 days. The cells were trypsinized and washed twice with PBS. The collected cells were then divided into two equal aliquots. One aliquot was extracted with acid-ethanol (0.18 M HCl in 75% ethanol) overnight at -20°C, followed by centrifugation and supernatant collection. The insulin level in the supernatants was determined as indicated above. The other aliquot was lysed with RIPA buffer, and the protein concentration of the extract was determined as described above. The insulin content was expressed as the amount of insulin (μg) per mg protein.

Generation of an anti-TMEM59L antibody
To raise polyclonal antibodies against TMEM59L, a fusion protein containing glutathione S-transferase (GST) and the N-terminal region of TMEM59L was produced in E. coli. In brief, a Tmem59l cDNA fragment encoding amino acids 1–222 of TMEM59L was amplified by high fidelity PCR using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA). The cDNA fragment was inserted into the pGEX-6P-3 expression vector (GE Healthcare, Buckinghamshire, UK), and the resulting plasmid was introduced into E. coli to produce the GST-TMEM59L fusion protein, which was purified using a Glutathione Sepharose 4B (GE Healthcare) column. The purified fusion protein was used to generate anti-TMEM59L polyclonal antisera in rabbits. The TMEM59L immunoreactive antisera was immunoaffinity-purified, and the resulting purified antibody was used as described below.
**Western blotting**

MIN6c4 cells were lysed in lysis buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, and 0.1% NP40) and centrifuged to remove cell debris. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out using standard techniques. The blots were incubated with either the rabbit anti-TMEM59L antibody (1:1,000) or a mouse anti-α-tubulin antibody (1:50,000, Sigma-Aldrich, Saint Louis, MO, USA), which were detected by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2,000, New England Biolabs, Cambridge, MA, USA) or HRP-conjugated goat anti-mouse IgG1 (1:2,000, Bethyl Lab, Montgomery, TX, USA), respectively. The signals were detected using an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL, USA).

**Immunofluorescence analyses**

MIN6c4 cells were fixed with 4% paraformaldehyde for 10 min and then permeabilized in PBS containing 0.2% Triton X-100 for 10 min at room temperature. The cells were blocked using Blocking One (Nacalai Tesque, Kyoto, Japan) for 60 min at room temperature followed by incubation with a primary antibody at 4°C overnight and then with a fluorescein-conjugated secondary antibody (1:400) and DAPI (4', 6-diamidino-2-phenylindole) (Sigma-Aldrich, Saint Louis, MO, USA) for 60 min at room temperature. The stained cells were examined by confocal laser scanning microscopy using a FLUOVIEW FV1000D (Olympus, Tokyo, Japan). The following primary antibodies were used in this analysis: purified rabbit anti-TMEM59L antibody (1:400), guinea pig anti-insulin antibody (1:500, DAKO, Glostrup, Denmark), mouse anti-GM130 antibody (1:500, BD Transduction Laboratories, San Jose, CA, USA), and mouse anti-β-catenin antibody (1:1,000, BD Transduction Laboratories). The following secondary antibodies were used in this analysis: Alexa Fluor 488-labeled goat anti-rabbit IgG, Alexa Fluor 594-labeled goat anti-guinea pig IgG, and Alexa Fluor 647-labeled goat anti-mouse IgG1 (Life Technologies, Grand Island, NY, USA).

**Statistical analysis**

The results are presented as the mean ± SD. Statistical analyses were performed using the Student's t-test. A value of $P<0.05$ was considered statistically significant.

**Results**

**mRNA expression analysis**

The expression patterns of the selected genes, *Tmem59l, Scgn, Gucy2c, Slc29a4, Cdhr1*, and *Celsr2* were examined in the Pr-LP, Pr-HP, C4-LP, and C4-HP cells by quantitative RT-PCR. All of these genes were highly expressed in the Pr-LP, C4-LP, and C4-HP cells, but only weakly expressed in the Pr-HP cells (Fig 1). These results were consistent with our previously reported findings [4].

The expression of these genes in various mouse tissues was examined by RT-PCR (Fig 2). Consistent with the databases we referred to (see **Introduction**), *Tmem59l, Gucy2c, Slc29a4, and Cdhr1* were expressed in both the brain and pancreatic islets. In contrast, *Scgn* was expressed in the islets, but not in the brain. The expression of these genes was not detected in the whole pancreas in this experiment, suggesting that their expression was probably restricted to the islets. The expression of *Celsr2* was much broader than that of the other genes, but was only weakly detected in the islets. Thus, most of the examined candidate genes were selectively expressed in the pancreatic islets and brain in mice.
Knockdown of the selected genes in MIN6c4 cells was performed by the lentivirus-mediated expression of specific shRNAs. Quantitative RT-PCR was used to evaluate the effectiveness of

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Fig 1. Expression of the selected candidate genes in MIN6 cells. Expression of the Tmem59l (A), Scgn (B), Gucy2c (C), Slc29a4 (D), Cdhr1 (E), and Celsr2 (F) mRNAs in the Pr-LP, Pr-HP, C4-LP, and C4-HP MIN6 cells was examined by quantitative RT-PCR. Rpl32 gene expression was used as an internal control. Values are the means ± SD (n = 3) of the gene expression levels relative to those in the Pr-LP MIN6 cells. **P<0.005.

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Effects of selected gene knockdown on GSIS

Knockdown of the selected genes in MIN6c4 cells was performed by the lentivirus-mediated expression of specific shRNAs. Quantitative RT-PCR was used to evaluate the effectiveness of

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Fig 2. Expression of the selected candidate genes in murine tissues. Expression of the Tmem59l, Scgn, Gucy2c, Slc29a4, Cdhr1, and Celsr2 mRNAs in the small intestine, liver, kidney, spleen, uterus, lung, heart, skeletal muscle (quadriceps femoris), brain, pancreas, and islets of mice was examined by RT-PCR. The PCR products were separated by electrophoresis in an agarose gel. Rpl32 gene expression was used as an internal control.

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the shRNAs, and led to the identification of one or two shRNAs that effectively knocked down each gene in the MIN6c4 cells (Fig 3). The analysis of GSIS in the knockdown cells showed that the Tmem59l and Scgn knockdown cells exhibited reduced GSIS, whereas the Slc29a4 knockdown cells exhibited enhanced GSIS, compared with the control cells (Fig 4). These data suggested that Tmem59l, Scgn, and Slc29a4 are functionally related to GSIS in MIN6c4 cells. In contrast, the Gucy2c, Cdhr1, and Celsr2 knockdowns did not show any significant effects on GSIS.

Effects of selected gene knockdown on KCl-stimulated insulin secretion

High concentrations of KCl induce β cell plasma membrane depolarization and can more potently stimulate insulin secretion than high concentrations of glucose. Thus, KCl has been used to evaluate the final process following membrane depolarization in the regulated insulin secretory pathway [17–19]. We examined insulin secretion in response to KCl using the knockdown cells. Similar to the results obtained for GSIS, the Tmem59l and Scgn knockdown cells exhibited reduced insulin secretion, whereas Slc29a4 knockdown cells showed enhanced insulin secretion compared with the control cells. Cdhr1 and Celsr2 knockdown cells showed no significant difference in insulin secretion compared with the control cells. However, while Gucy2c knockdown had no effect on GSIS, the Gucy2c knockdown cells exhibited lower insulin secretion than the control cells when stimulated with KCl (Fig 5). These results provided further evidence that Tmem59l, Scgn, and Slc29a4 are functionally involved in the insulin secretory mechanism, and suggested that Gucy2c is functionally involved in insulin secretion after β cell membrane depolarization.
Effects of selected gene knockdown on cellular insulin content

We next examined the insulin content of the knockdown cells (Fig 6). Scgn, Gucy2c, and Slc29a4 knockdown cells exhibited lower cellular insulin levels than the control cells. In contrast, the Tmem59l, Cdhr1, and Celsr2 knockdown cells showed no significant differences in insulin content compared with the control cells. These data suggested that Scgn, Gucy2c, and Slc29a4 may be functionally involved in regulating cellular insulin levels as well as insulin secretion, while Tmem59l may be specifically involved in regulating insulin secretion.

Functional analysis of Tmem59l

The initial analysis of the five Tmem59l-specific shRNA sequences indicated that only one was capable of efficiently downregulating Tmem59l expression. Thus, we could not rule out the possibility that the effects of the Tmem59l shRNA we observed were due to non-specific gene silencing, or off-target effects. To confirm that the decrease in insulin secretion was due to Tmem59l knockdown, we expressed a shRNA-resistant form of Tmem59l in the knockdown cells and evaluated its effect on insulin secretion in these cells. The resulting Tmem59l rescued cells exhibited higher levels of secreted insulin than the knockdown cells when stimulated with high concentrations of either glucose or KCl (Fig 7A and 7B). These results demonstrated that the reduced insulin secretion observed in the Tmem59l knockdown cells was due to the specific knockdown of Tmem59l mRNA. Moreover, Tmem59l-overexpressing MIN6c4 cells showed a tendency to secrete higher levels of insulin compared with the parental MIN6c4 cells. However, the insulin content was not significantly increased in the Tmem59l-overexpressing cells (Fig 7C–7E). These results provided further evidence that Tmem59l is functionally involved in regulating the insulin secretion from MIN6c4 cells.
Fig 5. KCl-stimulated insulin secretion from knockdown MIN6c4 cells. Insulin secretion from Tmem59l (A), Scgn (B), Gucy2c (C), Slc29a4 (D), Cdhr1 (E), and Celsr2 (F) knockdown MIN6c4 cells stimulated with 30 mM KCl (gray bar, shRNA#1; black bar, shRNA#2; and white bar, control). Values are means ± SD (n = 3–4). *P < 0.05 **P < 0.005.

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Fig 6. Insulin content of knockdown MIN6c4 cells. Insulin content of Tmem59l (A), Scgn (B), Gucy2c (C), Slc29a4 (D), Cdhr1 (E), and Celsr2 (F) knockdown MIN6c4 cells, compared to control cells containing empty lentiviral vectors. Values are means ± SD (n = 3–4). *P < 0.05 **P < 0.005.

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We also examined whether \textit{Tmem59l} knockdown affects the potentiation of GSIS by exendin-4, a GLP-1 analogue. Although exendin-4 potentiated insulin secretion stimulated with 10 mM glucose in both \textit{Tmem59l} knockdown and control MIN6c4 cells, the knockdown cells still exhibited reduced insulin secretion compared with the control cells (Fig 8). This result suggested that \textit{Tmem59l} is not directly involved in the potentiation of GSIS by GLP-1.

\textbf{TMEM59L protein localization in MIN6c4 cells}

Next, we generated an anti-TMEM59L antibody (see Materials and Methods) to examine the localization of TMEM59L protein in MIN6c4 cells. We demonstrated its specificity for
detecting TMEM59L protein (predicted mass: 43 kDa) by Western blotting using Tmem59l knockdown and overexpressing MIN6c4 cells (Fig 9A). Furthermore, we compared the signal intensity of immunofluorescence staining with the anti-TMEM59L antibody among Tmem59l knockdown, overexpressing, and control MIN6c4 cells. The result clearly showed that the signal intensity was decreased by Tmem59l knockdown, while greatly increased by Tmem59l overexpression (Fig 9B). Immunofluorescence analysis of MIN6c4 cells with this antibody showed that TMEM59L colocalized with insulin and with the GM130 Golgi complex marker. In contrast, TMEM59L did not colocalize with β-catenin, a plasma membrane marker (Fig 9C). These results suggested that TMEM59L localizes to insulin granules and the Golgi complex and may play a role in insulin secretion.

Discussion

We previously identified several genes that may be functionally involved in maintaining insulin secretion in MIN6c4 β cells [4]. In the present study, we used shRNA knockdown to study the functional involvement of some of these genes with insulin secretion and content in MIN6c4 cells. The knockdown of Scgn and Gucy2c resulted in decreased insulin secretion and content, while the knockdown of Tmem59L resulted in reduced insulin secretion, without affecting insulin content. In contrast, Slc29a4 knockdown resulted in increased insulin secretion and decreased cellular insulin levels. The finding that Slc29a4 knockdown resulted in increased insulin secretion was unexpected, because the selected genes were upregulated in MIN6c4 cells, a MIN6 subclone that maintains insulin secretion after long-term culture [4]. On the other
hand, 

Cdhr1 and Celsr2 knockdown did not affect the insulin secretion or insulin content (Figs 4–6).

The Sgcn cDNA was first cloned from a pancreatic β cell cDNA library and encodes a hexa EF-hand calcium-binding protein [20]. Sgcn overexpression in MIN6 cells was previously shown to enhance GSIS and it was proposed that SCGN might interact with SNAP-25 to enhance GSIS as a Ca2+-signaling protein [21–23]. These reports are consistent with our results, but the reason for the decreased insulin content seen in the Sgcn knockdown MIN6c4 cells is not clear. Gucy2c knockdown had no significant effect on GSIS, but decreased KCl-induced insulin secretion and cellular insulin content. KCl is a more potent stimulator of insulin secretion than high glucose, so that insulin secretion stimulated with KCl is likely to be more susceptible to the alternations in insulin content than that with high glucose. Therefore, it might be possible that the reduced insulin content led to a decrease in KCl-induced insulin secretion. However, the reason why Gucy2c knockdown decreased the insulin content in MIN6c4 cells is not clear. Gucy2C was reported to be expressed in the small intestine and encodes one of seven mammalian transmembrane guanylate cyclase receptors that catalyze the formation of cGMP in response to the binding of the bacterial heat-stable enterotoxin STa or of the endogenous peptides guanylin and uroguanylin [24–26]. Although the function of the Gucy2c-encoded guanylate cyclase receptor in pancreatic β cells has not been reported, guanylin has been shown to stimulate insulin secretion in a rat pancreatic β cell line [27], suggesting a potential role for its receptor in insulin secretion.

Slc29a4 knockdown enhanced insulin secretory response to glucose and KCl, while it reduced cellular insulin content. It appears that increased insulin secretion caused a decrease in insulin content. In fact, Bollheimer et al. [28] showed that exposure of rat islets to free fatty acid enhanced basal and glucose-stimulated insulin secretion and reduced insulin content. Eto et al. [29] investigated the β-cell function of phosphatidylinositol 3-kinase p85α regulatory subunit-deficient mice and showed that p85α knockout enhanced GSIS from isolated islets and reduced both insulin content and insulin secretion stimulated with diazoxide and KCl. However, in these two reports, the decrease in insulin content was attributed to lowered insulin biosynthesis. Thus, the cause-and-effect relationship between insulin content and insulin secretory capacity seems complicated. Further analysis will be required to understand the role of Slc29a4 in the regulation of insulin content and insulin secretion. Slc29a4 was identified as one of a family of equilibrative nucleoside transporter (ENT) genes. Its gene product transports not only nucleosides but also monoamines (e.g., serotonin) [30,31]. Although Slc29a4 has not been directly linked to insulin secretion, nucleosides and monoamines were shown to regulate insulin secretion in pancreatic β cells [32–36], consistent with a potential role for this solute carrier in insulin secretion.

Cdhr1 was identified as one of three non-classical cadherin genes [37] and was reported to be a candidate gene for retinal dystrophies [38]. Celsr2 was identified as a mammalian homolog of the Drosophila gene flamingo, which encodes a receptor involved in noncanonical Wnt signaling [39]. Mouse Celsr2 was reported to control the differentiation of pancreatic β cells from polarized progenitors [40]. In the present study, the downregulation of these genes had no significant effect on insulin secretion or cellular insulin content; however we cannot rule out the possibility that the low levels of Cdhr1 and Celsr2 expressed in the knockdown cells were sufficient for maintaining their functions.

Tmem59l knockdown resulted in decreases in both glucose- and KCl-stimulated insulin secretion, but did not significantly alter the cellular insulin content (Figs 4–6). Considering that the Tmem59l knockdown cells still expressed considerable levels of Tmem59l mRNA (Fig 3), the Tmem59l gene product may play an essential role in maintaining insulin secretion. We confirmed the specificity of the Tmem59l knockdown by showing that Tmem59l
overexpression could rescue the phenotype of the *Tmem59l* knockdown cells (Fig 7A and 7B). Moreover, we found that *Tmem59l*-overexpressing cells tended to exhibit enhanced insulin secretion compared with the control cells (Fig 7C–7E). These results were consistent with the notion that the *Tmem59l* gene product positively regulates insulin secretion.

The human *TMEM59L* gene is also known as *BSMAP* (brain-specific membrane-anchored protein). *BSMAP* was identified as a gene that is highly expressed in the brain and is localized to chromosome 19p12 [41]. The predicted structure of the TMEM59L protein suggests that it is a membrane-bound type 1 glycoprotein. TMEM59L was previously shown to be involved in peripheral axon extension in sensory neurons [42]. Recently, Ullrich et al. [43] identified TMEM59, a homolog of TMEM59L, as a modulator of amyloid precursor protein (APP) shedding. TMEM59 was found to be a ubiquitously expressed, Golgi-localized protein. Notably, TMEM59L was shown to have a similar inhibitory effect on APP maturation and shedding as TMEM59, suggesting that both proteins have similar functions in the brain [43]. However, the function and subcellular localization of TMEM59L in pancreatic β cells have not been reported previously. We examined the localization of TMEM59L protein in MIN6c4 cells by immunofluorescence staining and found that TMEM59L preferentially colocalized with a Golgi complex marker. We also found that TMEM59L protein colocalized with insulin granules (Fig 9C), which are known to be generated from the Golgi complex (reviewed in [44]). On the other hand, colocalization of TMEM59L with markers for mitochondria, endoplasmic reticulum (ER), or nuclei was not observed (S1 Fig). Further studies will be needed to exactly determine the localization of TMEM59L in β cells.

Our present study suggested that several of the candidate genes identified in our previous microarray analysis, which compared gene expression in MIN6c4 cells and the parental MIN6 cells and the MIN6c4 cells, are functionally involved in GSIS. We focused on *Tmem59l* and found that the TMEM59L protein localized to insulin granules and the Golgi complex, suggesting that it might play important roles in insulin transport and secretion. We expect that further analyses of these candidate genes will contribute to the elucidation of the insulin secretory pathway.

**Supporting Information**

S1 Fig. Co-staining of TMEM59L with mitochondria, endoplasmic reticulum (ER), and nucleus markers in MIN6c4 cells. Green: staining with anti-TMEM59L antibody; red: staining with MitoTracker Red CMXRos (Life Technologies), used as a mitochondria marker; blue: staining with ER-Tracker Blue-White DPX (Life Technologies), used as an ER marker; light blue: staining with DAPI, used as a nucleus marker. (TIFF)

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**Author Contributions**

Conceived and designed the experiments: MK EY JM. Performed the experiments: MK EY. Analyzed the data: MK JM. Contributed reagents/materials/analysis tools: KT FT SM. Wrote the paper: MK JM.
References

1. Henquin JC. Regulation of insulin secretion: a matter of phase control and amplitude modulation. Diabetologia. 2009; 52: 739–751. doi: 10.1007/s00125-009-1314-y PMID: 19288076

2. Pfeifer MA, Halter JB, Porte D Jr. Insulin secretion in diabetes mellitus. Am J Med. 1981; 70: 579–588. PMID: 7011013

3. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, et al. Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology. 1990; 127: 126–132. PMID: 2163307

4. Yamato E, Tashiro F, Miyazaki J. Microarray analysis of novel candidate genes responsible for glucose-stimulated insulin secretion in mouse pancreatic β cell line MIN6. PLoS One. 2013; 8: e61211. doi: 10.1371/journal.pone.0061211 PMID: 23560115

5. Lilla V, Webb G, Rickenbach K, Maturana A, Steiner DF, Halban PA, et al. Differential gene expression in well-regulated and dysregulated pancreatic beta-cell (MIN6) sublines. Endocrinology. 2003; 144: 1368–1379. PMID: 12639920

6. O’Driscoll L, Gammell P, McKiernan E, Ryan E, Jeppesen PB, Rani S, et al. Phenotypic and global gene expression profile changes between low passage and high passage MIN-6 cells. J Endocrinol. 2006; 191: 665–676. PMID: 17170223

7. Atouf F, Czernichow P, Scharfmann R. Expression of neuronal traits in pancreatic β cells. Implication of neuron-restrictive silencing factor/repressor element silencing transcription factor, a neuron-restrictive silencer. J Biol Chem. 1997; 272: 1929–1934. PMID: 8999882

8. Gerber SH, Südhof TC. Molecular determinants of regulated exocytosis. Diabetes. 2002; 51 Suppl 1: S3–11. PMID: 11815450

9. Yamato E, Ikegami H, Tahara Y, Fukuda M, Cha T, Kawaguchi Y, et al. Glyburide enhances insulin gene expression and glucose-induced insulin release in isolated rat islets. Biochem Biophys Res Commun. 1994; 199: 327–333. PMID: 8123030

10. Green CD, Olson LK. Modulation of palmitate-induced endoplasmic reticulum stress and apoptosis in pancreatic β-cells by stearoyl-CoA desaturase and Elovl6. Am J Physiol Endocrinol Metab. 2011; 300: E640–649. doi: 10.1152/ajpendo.00544.2010 PMID: 21266672

11. Marselli L, Thorne J, Daihya S, Sgroi DC, Sharma A, Bonner-Weir S, et al. Gene expression profiles of Beta-cell enriched tissue obtained by laser capture microdissection from subjects with type 2 diabetes. PLoS One. 2010; 5: e11499. doi: 10.1371/journal.pone.0011499 PMID: 20644627

12. Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. J. Virol. 1998; 72: 8150–8157. PMID: 9733856

13. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol. 1998; 72: 8463–8471. PMID: 9765382

14. Liu D, Zhen W, Yang Z, Carter JD, Si H, Reynolds KA. Genistein acutely stimulates insulin secretion in pancreatic β-cells through a cAMP-dependent protein kinase pathway. Diabetes. 2006; 55: 1043–1050. PMID: 16567527

15. Sato Y, Endo H, Okuyama H, Takeda T, Iwashashi H, Imagawa A, et al. Cellular hypoxia of pancreatic β-cells due to high levels of oxygen consumption for insulin secretion in vitro. J Biol Chem. 2011; 286: 12524–12532. doi: 10.1074/jbc.M110.194736 PMID: 21296882

16. Zhang Q, Zhu Y, Zhou W, Gao L, Yuan L, Han X. Serotonin receptor 2C and insulin secretion. PLoS One. 2013; 8: e54250. doi: 10.1371/journal.pone.0054250 PMID: 23349838

17. Grodsky GM, Bennett LL. Cation requirements for insulin secretion in the isolated perfused pancreas. Diabetes. 1966; 15: 910–913. PMID: 5957483

18. Herchuelz A, Thonnart N, Sener A, Malaisse WJ. Regulation of calcium fluxes in pancreatic islets: the role of membrane depolarization. Endocrinology. 1980; 107: 491–497. PMID: 6993189

19. Belz M, Willenborg M, Görgler N, Hamada A, Schumacher K, Rustenbeck I. Insulinotropic effect of high potassium concentration beyond plasma membrane depolarization. Am J Physiol Endocrinol Metab. 2014; 306: E697–706. doi: 10.1152/ajpendo.00362.2013 PMID: 24452455

20. Wagner L, Oliyaryk O, Gartner W, Nowotny P, Groeger M, Kaserer K, et al. Cloning and expression of secretagogin, a novel neuroendocrine- and pancreatic islet of Langerhans-specific Ca2+-binding protein. J Biol Chem. 2000; 275: 24740–24751. PMID: 10811645

21. Hasegawa K, Wakino S, Kimoto M, Minakuchi H, Fujimura K, Hosoya K, et al. The hydrolase DDHA2 enhances pancreatic insulin secretion by transcriptional regulation of secretagogin through a Sirt1-dependent mechanism in mice. FASEB J. 2013; 27: 2301–2315. doi: 10.1096/fj.12-226092 PMID: 23430976
22. Rogstam A, Linse S, Lindqvist A, James P, Wagner L, Berggård T. Binding of calcium ions and SNAP-25 to the hexa EF-hand protein secretogogin, Biochem J. 2007; 401: 353–363. PMID: 16939418

23. Nagamatsu S, Nakamichi Y, Yamamura C, Matsushima S, Watanabe T, Ozawa S, et al. Decreased expression of t-SNARE, syntaxin 1, and SNAP-25 in pancreatic beta-cells is involved in impaired insulin secretion from diabetic GK rat islets: restoration of decreased t-SNARE proteins improves impaired insulin secretion. Diabetes. 1999; 48: 2367–2373. PMID: 10580425

24. Schulz S, Green CK, Yuen PS, Garbers DL. Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell. 1990; 63: 941–948. PMID: 1701694

25. Currie MG, Fok KF, Kato J, Moore RJ, Hamra FK, Duffin KL, et al. Guanylin: an endogenous activator of intestinal guanylate cyclase. Proc Natl Acad Sci USA. 1992; 89: 947–951. PMID: 1346555

26. Kita T, Smith CE, Fok KF, Duffin KL, Moore WM, Karabatos P, et al. Characterization of human uroguanylin: a member of the guanylin peptide family. Am J Physiol. 1994; 266: F342–348. PMID: 8141334

27. Russell MA, Morgan NG. Expression and functional roles of guanylate cyclase isoforms in BRIN-BD11 β-cells. Islets. 2010; 2: 374–382. doi: 10.4161/isc.2.6.13917 PMID: 21099339

28. Bolz H, Ebermann I, Gal A. Protocadherin-21 (PCDH21), a candidate gene for human retinal dystrophies. Mol Vis. 2005; 11: 929. doi: 10.1016/j.celrep.2012.10.016 PMID: 23177622

29. Eto K, Yamashita T, Tsubamoto Y, Terauchi Y, Hirose K, Kubota N, et al. Phosphatidylinositol 3-kinase suppresses glucose-stimulated insulin secretion by affecting post-cytosolic [Ca²⁺] elevation signals. Diabetes. 2002; 51: 87–97. PMID: 11756327

30. Engel K, Zhou M, Wang J. Identification and characterization of a novel monoamine transporter in the human brain. J Biol Chem. 2004; 279: 50042–50049. PMID: 15448143

31. Zhou M, Xia L, Engel K, Wang J. Molecular determinants of substrate selectivity of a novel organic cation transporter (PMAT) in the SLC29 family. J Biol Chem. 2007; 282: 3188–3195. PMID: 17121826

32. Gylfe E. Association between 5-hydroxytryptamine release and insulin secretion. J Endocrinol. 1978; 78: 239–248. PMID: 359742

33. Paulmann N, Grohmann M, Voigt JP, Bert B, Vowinckel J, Bader M, et al. Intracellular serotonin modulates insulin secretion from pancreatic β-cells by protein serotonylation. PLoS Biol. 2009; 7: e1000229. doi: 10.1371/journal.pbio.1000229 PMID: 19859528

34. Ohara-Imaizumi M, Kim H, Yoshida M, Fujiwara T, Aoyagi K, Toyofuku Y, et al. Serotonin regulates glucose-stimulated insulin secretion from pancreatic β-cells during pregnancy. Proc Natl Acad Sci USA. 2013; 110: 19420–19425. doi: 10.1073/pnas.1310953110 PMID: 24218571

35. Ohtani M, Oka T, Ohura K. Possible involvement of A2A and A3 receptors in modulation of insulin secretion and β cell survival in mouse pancreatic islets. Gen Comp Endocrinol. 2013; 187: 86–94. doi: 10.1016/j.ygcen.2013.02.011 PMID: 23453966

36. Salehi A, Parandeh F, Fredholm BB, Grapengiesser E, Hellman B. Absence of adenosine A1 receptors unmasks pulses of insulin release and prolongs those of glucagon and somatostatin. Life Sci. 2009; 85: 470–476. doi: 10.1016/j.lfs.2009.08.001 PMID: 19682463

37. Nakajima D, Nakayama M, Kikuno R, Hirosawa M, Nagase T, Ohara O. Identification of three novel non-classical cadherin genes through comprehensive analysis of large cDNAs. Brain Res Mol Brain Res. 2001; 94: 85–89. PMID: 11597768

38. Bolz H, Ebermann I, Gal A. Protocadherin-21 (PCDH21), a candidate gene for human retinal dystrophies. Mol Vis. 2005; 11: 929–933. PMID: 16288196

39. Usui T, Shima Y, Shimada Y, Hirano S, Burgess RW, Schwarz TL, et al. Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell. 1999; 98: 585–595. PMID: 10490098

40. Cortijo C, Gouzi M, Tissir F, Grapin-Botton A. Planar cell polarity controls pancreatic β cell differentiation and glucose homeostasis. Cell Rep. 2012; 2: 1593–1606. doi: 10.1016/j.celrep.2012.10.016 PMID: 23177622

41. Elson GC, de Coignac AB, Aubry JP, Delneste Y, Magistrelli G, Holzwarth J, et al. BSMAP, a novel protein expressed specifically in the brain whose gene is localized on chromosome 19p12. Biochem Biophys Res Commun. 1999; 264: 55–62. PMID: 10527841

42. Aoki M, Segawa H, Naito M, Okamoto H. Identification of possible downstream genes required for the extension of peripheral axons in primary sensory neurons. Biochem Biophys Res Commun. 2014; 445: 357–362. doi: 10.1016/j.bbrc.2014.01.193 PMID: 24513284
43. Ullrich S, Münch A, Neumann S, Kremmer E, Tatzelt J, Lichtenthaler SF. The novel membrane protein TMEM59 modulates complex glycosylation, cell surface expression, and secretion of the amyloid precursor protein. J Biol Chem. 2010; 285: 20664–20674. doi:10.1074/jbc.M109.055608 PMID: 20427278

44. Suckale J, Solimena M. The insulin secretory granule as a signaling hub. Trends Endocrinol Metab. 2010; 21: 599–609. doi:10.1016/j.tem.2010.06.003 PMID: 20609596