Original Research *Highlight article*

GDF15 plays a critical role in insulin secretion in INS-1 cells and human pancreatic islets

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Impact Statement

Although several studies have linked growth differentiation factor-15 (*GDF15*) with the risk of diabetes and obesity, the question of how *GDF15* affects pancreatic β-cell physiology is yet to be answered. Herein, a series of bioinformatics, genetic, and functional studies were performed to dissect the functional role of *GDF15* in human islets and clonal INS-1 cells. The data showed that *GDF15* expression is upregulated in diabetic islets and that its expression correlates with key β-cell functional genes, including *INS*, *KCNJ11*, *GLUT1*, *MAFA*, *GCK*, and *FTO*. The data also showed that the absence of *Gdf15* suppresses the expression of *Ins*, *Pdx1*, *Mafa*, and *Glut2* genes, impairs insulin release and glucose uptake, reduces cell viability, and increases reactive oxygen species (ROS) production and cell death. Furthermore, overexpression of *GDF15* in human islets increases insulin secretion and protein expression of MAFA and GLUT1. These findings clearly show that GDF15 expression is critical for β-cell survival and function.

Abstract

Mounting evidence points to a link between growth differentiation factor-15 (*GDF15*) expression and the onset and progression of diabetes mellitus. However, the exact role of *GDF15* in pancreatic β-cell function is unclear. To examine the role of *GDF15* in β-cell function, bioinformatics analysis and functional experiments involving *GDF15* silencing and overexpression were performed in INS-1 cells and human islets. Public microarray and RNA-seq expression data showed that islets obtained from diabetic donors express high levels of *GDF15* compared to islets obtained from normal donors. Moreover, analysis of RNA-seq expression data revealed that *GDF15* expression correlates positively with that of insulin (*INS*), *KCNJ11*, *GLUT1*, *MAFA*, *INSR* and negatively with that of Glucokinase (*GCK*) and Alpha-Ketoglutarate Dependent Dioxygenase (*FTO*). No T2D-associated genetic variants in the *GDF15* were found to pass genome-wide significance in the TIGER portal. Expression silencing of *Gdf15* in INS-1 cells reduced insulin release, glucose uptake levels, increased reactive oxygen species (ROS) production and apoptosis levels. While *Gdf15*-silenced cells downregulated mRNA expression of *Ins*, *Pdx1*, *Mafa*, and *Glut2* genes, its overexpression human islets was associated with increased insulin secretion and upregulated expression of MAFA and GLUT1 but not INS or GCK. Silencing of *Pdx1* or *Mafa* in INS-1 cells did not affect the expression of GDF15. These findings suggest that *GDF15* plays a significant role in pancreatic β-cell function.

Keywords: GDF15, clonal INS-1 cell line, insulin secretion, RNA-seq, siRNA, human islets, diabetes

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Introduction

Diabetes mellitus (DM) is a polygenic metabolic disease characterized by increased blood sugar levels. Type 1 diabetes (T1D) and type 2 diabetes (T2D) are the main types of DM.[1] T1D manifests as insulin deficiency due to autoimmune β-cells destruction, whereas T2D is marked by a progressive decline in insulin secretion or insulin action.[2–4] Pancreatic β-cells secrete insulin in response to elevated levels of glucose, amino acids, and fatty acids. In addition, factors related to β-cell proliferation, reactive oxygen species (ROS) production, apoptosis, and prolonged hyperglycemia also play a crucial role in DM onset and progression.[5,6] Although the pathogenesis of DM is extensively studied, much remains to be elucidated regarding the genes and proteins involved in the disease, and uncovering novel genes involved in β-cell physiology is of great importance for developing new therapeutic strategies that could better maintain β-cell function in diabetic patients.

Growth differentiation factor-15 (GDF15), cloned and identified in the mid-90s, is a member of the transforming growth factor-beta (TGF-β) superfamily of cytokines.[7]

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Although the GDF15 protein does not share enough sequence homology with other family members, it contains the conserved cysteine domain, hence its classification as a new member of the TGF-β family.[7] Expression of GDF15 has been reported in different tissues, including the placenta,[8,9] prostate,[10] heart,[11] fat tissue,[12] the intestines, kidneys, liver, and pancreas.[13] GDF15 serum levels are typically in the 100–1200 pg/mL range but tend to significantly increase in patients with malignancies and those under nutritional stress.[14]

Mounting evidence suggests that *GDF15* plays a role in obesity and diabetes.[15–19] It has been shown that overexpressing Gdf15 in mice results in weight and fat loss under a standard chow or high-fat diet, possibly due to potential food intake reduction and high energy expenditure.[20,21] Mice overexpressing *Gdf15* also show improved glucose tolerance, possibly indicative of a role in glucose homeostasis. In contrast, *Gdf15* knockout mice were reported to gain weight relative to wild-type (WT) counterparts and that infusion of human recombinant GDF15 in *Gdf15*-knockout mice decreases body weight and food intake.[22] Similarly, antibody-mediated blocking of GDF15 was shown to increase body weight and lower insulin levels in mice on a high-fat diet.[23] GDF15 was also reported to activate neuron centers in the mouse brainstem (area postrema and the nucleus of the solitary tract) involved in energy control and body homeostasis.[24] Although the role of *GDF15* has been extensively investigated in obesity, work on its role in pancreatic β-cell physiology is begging to bear fruit. For example, GDF15 was shown to block pro-inflammatory cytokine-mediated cell death in human islets.[25] In line with this finding, *Gdf15* was reported to protect against glucotoxicity and connexin-36 downregulation in INS-1E and primary pancreatic β-cells.[26]

In this study, we investigated the role of *GDF15* in β-cell survival and function by analyzing its expression in diabetic versus non-diabetic human pancreatic islets using RNA-seq and microarray data expression data. In addition, several functional experiments involving *Gdf15* silencing and overexpression were performed in human pancreatic islets and clonal INS-1 (832/13) cells to explore its role in β-cell function.

Materials and methods

mRNA microarray and RNA-seq data

The NCBI's Gene Expression Omnibus (GEO) publicly available database was used to retrieve the expression data (RNA-seq and microarray) from isolated human islets (European ancestor) with accession numbers: GSE50398 and GSE41762. The RNA-seq was performed using Illumina's TruSeq RNA Sample Preparation Kit and the output reads were aligned to the human reference genome (hg19) using Bowtie v.0.12.8.[27] The microarrays (GeneChip® Human Gene 1.0 ST) were done using the Affymetrix standard protocol and normalized with Robust Multi-array Analysis method using the ligo package from BioConductor.[28] RNA-seq data were obtained from 89 (53 males and 35 females) islet donors; of them, 51 are non-diabetic/normoglycemic donors (HbA1c $< 5.9\%$) and 27 diabetic/hyperglycemic (HbA1c $\geq 6\%$). Microarray expression data were obtained from 45 non-diabetic donors (HbA1c $< 5.9\%$).[27,28]

Analysis of genetic variants in GDF15 for the association with T2D

The TIGER data portal (http://tiger.bsc.es)[29] was used to search the *GDF15* gene for genetic variants associated with T2D. The exploration was completed in three data sets, including the 70K-T2D project (genome-wide association study [GWAS] meta-analysis of 70,127 European ancestry subjects, of which 13,857 T2D cases and 62,126 controls), DIAGRAM 1000G (GWAS meta-analysis of 159,208 European ancestry subjects, of which 26,176 T2D patients and 132,532 controls), and DIAGRAM Diamante T2D (GWAS meta-analysis of 898,132 European ancestry subjects, of which 74,124 T2D cases and 824,006 controls).

Culture conditions of INS-1 cells and siRNA silencing

Rat INS-1 (832/13) cells (C. Newgard from Duke University, USA) were cultured in RPMI 1640 Medium as described previously.[30,31] Cells were transfected with 40 nM of a pool of two siRNA sequences for rat *Gdf15* (IDs: s131449 and s131450), *Pdx1* (ID: s131652), *Mafa* (ID: s172994), or siRNA negative control using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, USA).[32,33]

Insulin secretion

INS1 (832/13) β-cells were incubated in SAB buffer (0.2% BSA, pH 7.2.) with 2.8 mM glucose for 2 h. Next, cells were stimulated with SAB buffer with 16.7 mM glucose, 35 mM potassium chloride (KCl), or 10 mM α-KIC for 1 h. Insulin release and content were measured using insulin rat ELISA from Mercodia, Sweden.[32,33] Measurements were adjusted to protein content.

Quantitative-PCR

Expression of studied genes was analyzed by quantitative-PCR (qPCR) using TaqMan expression assays; *Gdf15* (Rn00570083_m1), *Glut2* (Rn00563565_m1), *Ins1* (Rn02121433_g1), *Ins2* (Rn01774648_g1), *Pdx1* (Rn00755591_m1), *Gck* (Rn00561265_m1), *Insr* (Rn00690703_m1), and Rat *Hprt1* (Rn01527840_m1) as endogenous control as described previously.[32,33] *Mafa* expression was analyzed using SYBR green expression using the corresponding primers: forward primer "GAGGAGGAGCGCAAGATCGG"/reverse primer "AGCAAAAGTTTCGTGCTGTCAA" and *Hprt1*: forward primer" TTGTGTCATCAGCGAAAGTGG"/reverse primer "CACAGGACTAGAACGTCTGCT." qPCR reactions were run in QuantStudio 3 system. Relative gene expression was determined by $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Western blot analysis with cells/islets was run as previously described[32,33] with the following antibodies: GDF15 (Anti-rabbit 1/1000, #AHP2468, Bio-Rad), INS (Anti-mouse; 1/1000; # 8138s, Cell signaling Technology for IN1-cells and Anti-rabbit; Cat# ab181547 [Abcam, Cambridge, United Kingdom] for human islets), GLUT2 (Anti-rabbit; 1/1000,

A12307, ABclonal, China), MAFA (Anti-rabbit; 1/1000, #ab264418, Abcam), INSRβ (Anti-mouse; 1/1000; # ab69508, Abcam), INSRα (Anti-rabbit; 1/1000; # ab5500, Abcam), GCK (Anti-rabbit; 1/500; # ab37796, Abcam), PDX1 (Anti-rabbit; 1/3000, # ab47267 [Abcam] for human islets and Anti-rabbit; 1/1000; # 5679 [Cell signaling technology] is used for INS-1 cells). β-actin (1/5000; # A5441, Sigma). The Anti-mouse (# 7076S) and anti-rabbit (# 7074S) (Cell Signaling Technology). Band intensity was analyzed using Image Lab software (Bio-Rad).

Cell viability and apoptosis assay

Cell viability was measured using MTT assay, and the annexin V-FITC/Propidium iodide (PI) test for apoptosis were used as described previously.[32,33]

ROS measurements

ROS was measured by the H_2O_2 assay (Promega, Wisconsin, USA) as described previously.[34] Briefly, 48 h post-transfection, the H_2O_2 substrate solution was added (20 µL) for 4 h at 37°C. At room temperature, 100 µL of the ROS-Glo detection solution was added for 20 min. The luminescence was examined by a plate reader, and the relative luminescence unit (RLU) was calculated.

Glucose uptake assay

The 2-NBDG assay (Invitrogen, USA) was used to investigate glucose uptake as described in a previous work.[34] First, the 2-NBDG solution was incubated with the transfected cells (100 µM/1 mL medium/well) for 1 h at 37°C. Next, cells were trypsinized, washed, and incubated in 200 µL of cold phosphate-buffered saline (PBS), and cell acquisition was performed on flow cytometry (FACS Aria™ III) using excitation 465 nm/emission 540 nm. Finally, the data analyses were done on FlowJo software (BD, USA).

Overexpression of *GDF15* in human pancreatic islets

Human pancreatic islets were obtained from three non-diabetic donors (two males and one female, 46 ± 12 years, body mass index [BMI] 25.4 ± 2.2, HbA1c% 5 ± 0.7) (Prodo Laboratories INC, CA, USA). Upon receipt, human pancreatic islets were cultured in a non-treated tissue culture grade dish containing PIM(S) media (Prodo laboratories) at 37°C. Human islets were transfected with a full-length cDNA clone of human GDF15 with C terminal GFPSpark tag (HG10936-ACG) and empty vector pCMV3-C-GFPSpark (Sino Biological, Beijing, China) using lipofectamine 3000-DNA complexes prepared in OptiMEM media as per the manufacturer's instructions. Around 600 islets were transfected with 3 µg/mL DNA of pCMV3-GDF15-GFPSpark or pCMV3-C-GFPSpark control plasmid for 72 h. After 72 h post-GFP transfection, the islets were either used for western blotting, insulin secretion, or immunofluorescence. The islets were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min, washed twice with PBS, and moved on to the slide with the mounting media. The slides were then observed under the confocal microscope (A1R Confocal Laser Microscope System, Nikon, Tokyo, Japan). After transfection, islets (10 islets/well, three replicates) were starved for 1 h in SAB buffer containing 1 mM glucose, then stimulated for 1 h with SAB buffer (2.8 and 16.7 mM glucose). Insulin measurements were determined by ELISA (Mercodia, Sweden).

Statistical analysis

Student's *t*-tests or non-parametric Mann–Whitney U tests were used for differential expression analysis between diabetic versus non-diabetic islets. Non-parametric Spearman's correlation test performed the correlation analyses. Statistical analyses were done on GraphPad Prism (8.0.0: GraphPad Software, USA). In all analyses, differences were considered significant at $P < 0.05$.

Results

Human pancreatic islets express high levels of GDF15

Using public microarray and RNA-seq expression data, we investigated the expression of *GDF15* and its receptor *GFRAL* relative to some crucial functional genes (*KCNJ11* and *GLUT1)* in human islets. Microarray expression analysis revealed that *GDF15* is expressed in normal human islets, while *GDNF* Family Receptor Alpha Like (*GFRAL*) is minimally expressed (Figure 1[A]). Notably, *GDF15* showed higher expression ($P < 0.05$) levels than *KCNJ11* or *GLUT1* genes (Figure 1[A]). RNA-seq expression data analysis confirmed these observations and showed that *GDF15*, but not *GFRAL*, is expressed in pancreatic islets (Figure 1[B]). Similarly, *GDF15* expression was significantly higher ($P < 0.05$) than that of *KCNJ11* but was comparable to that of *GLUT1* (Figure 1[B]). In terms of the bearing of hyperglycemia on *GDF15* expression, RNA-seq data showed that its expression is higher in diabetic/hyperglycemic islets (HbA1c ≥ 6%) ($P < 0.05$) relative to non-diabetic/normoglycemic (HbA1c < 6%) islets (Figure 1[C]). Moreover, pancreatic islets obtained from female donors exhibited significantly higher ($P < 0.05$) levels of expression *GDF15* relative to those obtained from male donors (Figure 1[D]). No differences in *GDF15* expression were observed in obese (BMI ≥ 30) versus lean (BMI < 26) donors or in aged (year ≥ 60) versus young (year < 40) ones (data not shown). Using Spearman's correlation test, we next analyzed the co-expression of *GDF15* with key β-cell functional genes. As shown in Figure 1(E) to (H), *GDF15* expression correlated positively ($P < 0.05$) with that of *INS*, *KCNJ11*, *GLUT1*, and *MAFA* and negatively with that of *GCK* (Figure 1[K]). However, no correlations were observed between *GDF15* and *PDX1* or *INSR* expression (Figure 1[I] and [J]). That said, a significant correlation ($P < 0.05$) was observed between *GDF15* and the obesity-related gene *FTO*[35] (Figure 1[L]), perhaps suggesting a link between the two genes *vis-à-vis* the regulation of obesity.

Genetic variants in *GDF15* and risk of T2D

Using three different data sets (70K-T2D project, DIAGRAM Diamante, and DIAGRAM 1000G), the TIGER portal was explored for the existence of *GDF15* genetic variants in relation to T2D. At $P < 0.05$, our analysis revealed the presence of

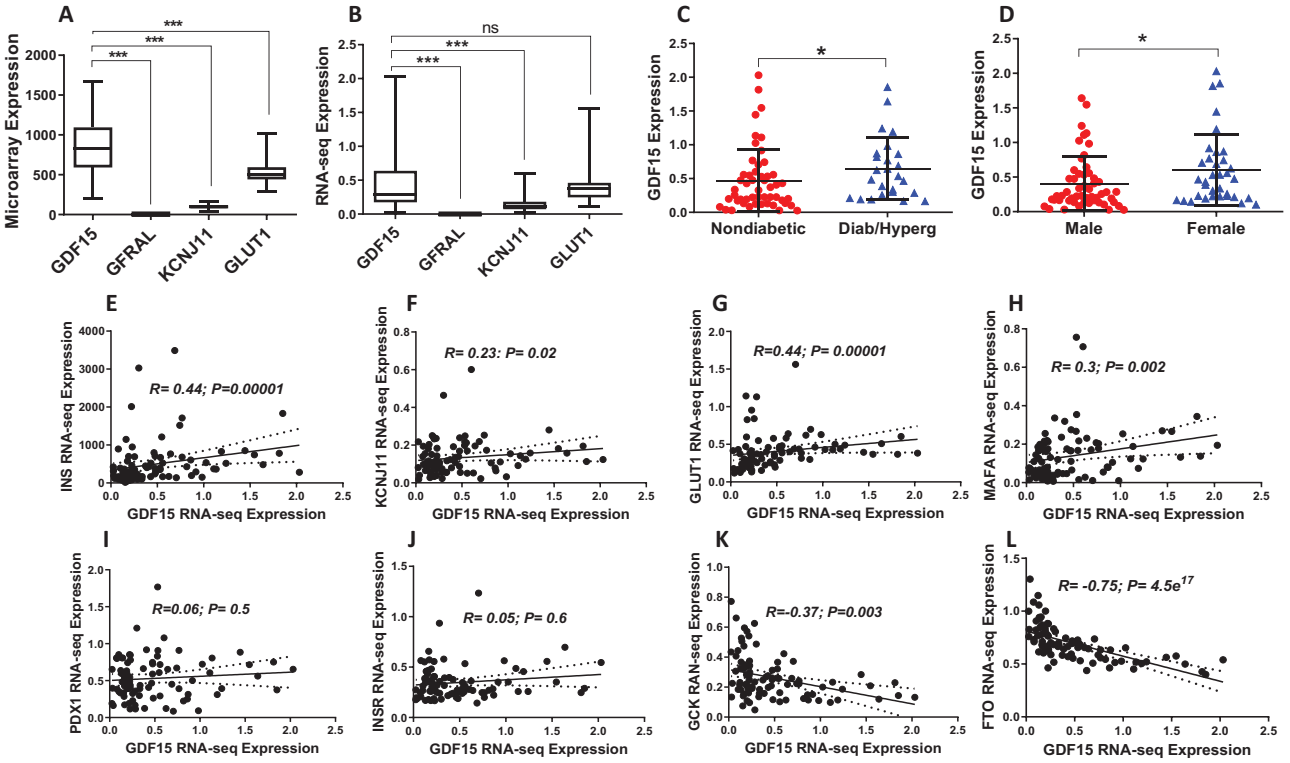

Figure 1. *GDF15* expression profile in human islets. (A) Expression of *GDF15*, *GFRAL*, *KCNJ11*, and *GLUT1* in non-diabetic (control) human islets as determined by microarray data ($n = 45$). (B) Expression of *GDF15*, *GFRAL*, *KCNJ11*, and *GLUT1* in non-diabetic (control) human islets ($n = 50$) as determined by RNA-seq. (C) *GDF15* expression analysis in diabetic/hyperglycemic islets (obtained from 27 donors) versus non-diabetic/normoglycemic islets (obtained from 50 donors) as determined by RNA-seq. (D) *GDF15* expression in male islets (obtained from 53 donors) is compared to female islets (obtained from 35 donors). Spearman's correlation of *GDF15* with (E) *INS*, (F) *KCNJ11*, (G) *GLUT1*, (H) *MAFA*, (I) *PDX1*, (J) *INSR*, (K) *GCK* and (L) *FTO* (no. of donors = 89). Bars represent mean ± SD. ns: not significant.
*$P < 0.05$. ***$P < 0.001$.

16 single-nucleotide polymorphisms (SNPs) in the 70K-T2D project, 47 SNPs in the DIAGRAM 1000G, and 120 SNPs in DIAGRAM Diamante T2D. The top three associated SNPs in GDF15 are shown in Table 1. However, none of these SNPs seem to pass the genome-wide significance threshold. Moreover, there was no expression of quantitative trait loci (eQTLs) for the tested genetic variants in the TIGER portal.

Gdf15-silencing impairs insulin secretion in INS-1 cells

To study the effect of *Gdf15* on insulin secretion, we ablated the *Gdf15* expression in INS-1 cells using a mixture of two different siRNA sequences. qPCR expression analysis of *Gdf15* 48 h post-transfection revealed a substantial reduction (~80%; $P < 0.05$) in *Gdf15*-silenced cells than the negative control (Figure 2[A]); western blot analysis further confirmed this finding (~50%; $P < 0.05$) (Figure 2[B]). Transfected cells incubated with 2.8 or 16.7 mM glucose for 1 h showed a decrease (~40%; $P < 0.05$) in glucose-stimulated insulin secretion (GSIS) at 16.7 mM glucose but not at basal level (2.8 mM glucose) compared to non-silenced cells (Figure 2[C]). To investigate the effect of *Gdf15* silencing on the exocytosis machinery or mitochondrial metabolism, transfected cells were stimulated with 35 mM KCl (a depolarizing agent) or 10 mM α-KIC (to stimulate mitochondrial metabolism). As illustrated in Figure 2(C), no significant differences were observed on stimulating with 35 mM KCl or 10 mM α-KIC between transfected and control cells. Insulin content measurements were not affected in *Gdf15*-silenced cells compared to control cells (Figure 2[D]). Next, we tested the effect of *Gdf15*-silencing on β-cell survival and function. As shown in Figure 3, silencing of *Gdf15* resulted in reduced cell viability (~18%, $P < 0.05$) (Figure 3[A]) and increased apoptosis (Figure 3[B]); both early and late apoptotic cell populations increased in transfected cells. Moreover, ROS production was significantly elevated in *Gdf15*-silenced cells (~40%; $P < 0.05$) (Figure 3[C]); increased ROS production in *Gdf15*-silenced cells was associated with a significant reduction (~20%; $P < 0.05$) in glucose uptake (Figure 3[D]).

Gdf15 silencing alters the expression of β-cell function-related genes

We next addressed whether *Gdf15* silencing influences pancreatic β-cell function-related genes at transcriptional and translational levels. As presented in Figure 4(A), silencing of *Gdf15* resulted in a significant reduction in the mRNA expression of key insulin biosynthesis-related genes, including *Ins1*, *Ins2*, *Pdx1*, and *Mafa* ($P < 0.05$); *NeuroD1* gene expression was not altered. The expression of glucose-sensing and insulin-signaling genes (*Glut2* and *Gck*) was also notably reduced. In contrast, *Insr* was upregulated ($P < 0.05$) compared to controls (Figure 4[A]). At the protein level, a significant reduction of pro/insulin (~35%; $P < 0.05$), PDX1 (~25%; $P < 0.05$), and MAFA (~25%; $P < 0.05$) was observed

Table 1. Association SNPs (the top three of each data set) in GDF15 (spanning ± 100 kb up- or downstream) and the risk of T2D using the TIGER portal.

SNP_ID	Reference allele	Alternate allele	Distance (Kb)	Type	Effect allele	Non-effect allele	OR	*p*
D. 1000G								
rs181728514	A	T	4.694	Upstream	A	T	1.65	8.9×10^{-4}
rs2112176	A	C, G	41.289	Upstream	A	C	1.17	1.7×10^{-3}
rs59356801	G	T	87.881	Upstream	T	G	0.94	3.5×10^{-3}
D. DIAMANTE								
rs569616292	G	A, C	56.052	Upstream	A	G	1.17	0.0001
rs368786619	G	A	78.443	Upstream	A	G	1.13	0.0003
rs192112149	G	A	25.145	Upstream	A	G	1.26	0.0004
70K for T2D								
rs1549143	G	T	91.617	Upstream	T	G	0.941	0.003
rs571434830	G	A	3.82	Upstream	A	G	5.96	0.007
rs202163868	CA	C	85.008	Downstream	CA	C	1.45	0.008

OR: odds ratio.

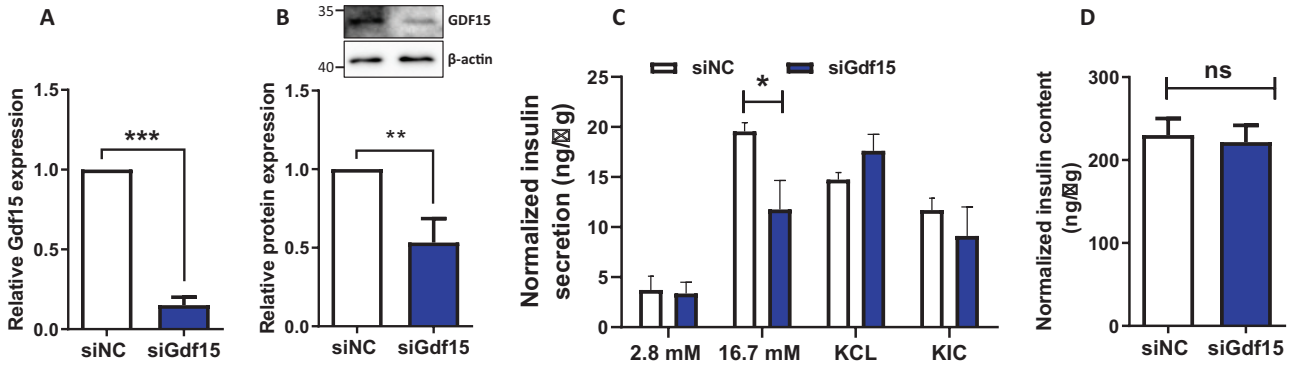

Figure 2. Silencing of *Gdf15* in INS-1 (832/13) cells. (A) Evaluation of *Gdf15* mRNA expression 48 h post-siRNA silencing. (B) Evaluation of GDF15 protein expression 48 h after siRNA silencing as determined by Western blotting (upper panel). Fold change in GDF15 protein expression is shown in the lower panel. (C) Insulin secretion measurement (normalized to protein content) in INS-1 cells stimulated with 2.8 and 16.7 mM glucose, 35 mM KCl, or 10 mM α-KIC in *Gdf15*-transfected cells compared to negative control cells. (D) Normalized insulin content measurements in transfected cells. Bars represent mean ± SD based on three independent experiments.
ns: not significant.
*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

in *Gdf15*-silenced cells relative to controls. (Figure 4[B]); GLUT2 protein expression levels were also reduced (~20%; $P < 0.05$), and those of NEUROD1, GCK, INSRα, and INSRβ proteins were not affected on Gdf15 silencing (Figure 4[B]).

Gdf15 is not regulated by *Pdx1* or *Mafa* in INS-1 cells

To determine whether the transcription factors PDX1 and MAFA are required for *Gdf15* expression, GDF15 protein expression was assessed in cells separately silenced for *Pdx1* and *Mafa*. Silencing efficiency of PDX1 and MAFA in INS-1 cells 48 h post-transfection was ~80% ($P < 0.05$) (Figure 5[A] and [B]). No significant changes in GDF15 protein expression were observed in *Pdx1*- or *Mafa*-silenced cells relative to controls (Figure 5[C]). This suggests that either PDX1 or MAFA does not regulate the expression of the Gdf15 gene.

GDF15 overexpression in human pancreatic islets enhances insulin secretion

To further investigate the role of *GDF15* in insulin secretion, *GDF15* was overexpressed in human pancreatic islets. Seventy-two hours post-transfection, *GDF15*-overexpressed islets were associated with a significant increase in insulin secretion after stimulation with glucose (16.7 mM; $P < 0.05$) (Figure 6[A]). However, no increase in insulin secretion was observed in *GDF15*-overexpressing cells stimulated with 2.8 mM glucose as compared with control cells. Moreover, the expression of MAFA and GLUT1 was significantly upregulated in *GDF15*-overexpressing cells; no change was observed regarding the expression of INS and GCK (Figure 6[B] to [E]). Taken together, these findings suggest that GDF15 is required for GSIS in human islets.

Discussion

This work showed that *GDF15* is highly expressed in human pancreatic islets. The expression of *GDF15* was elevated in diabetic islets compared to non-diabetic counterparts and correlated with the expression of *INS*, *KCNJ11*, *GLUT2*, and most importantly *FTO*. We could not find any genetic variants in *GDF15* associated with T2D in the TIGER portal. Expression reduction of *Gdf15* in INS-1 cells impaired insulin release but not content, reduced glucose uptake, elevated apoptosis/ROS levels, and modulated various functional genes in β-cells. Expression silencing of *Pdx1* or *Mafa* showed no impact on the protein expression of GDF15

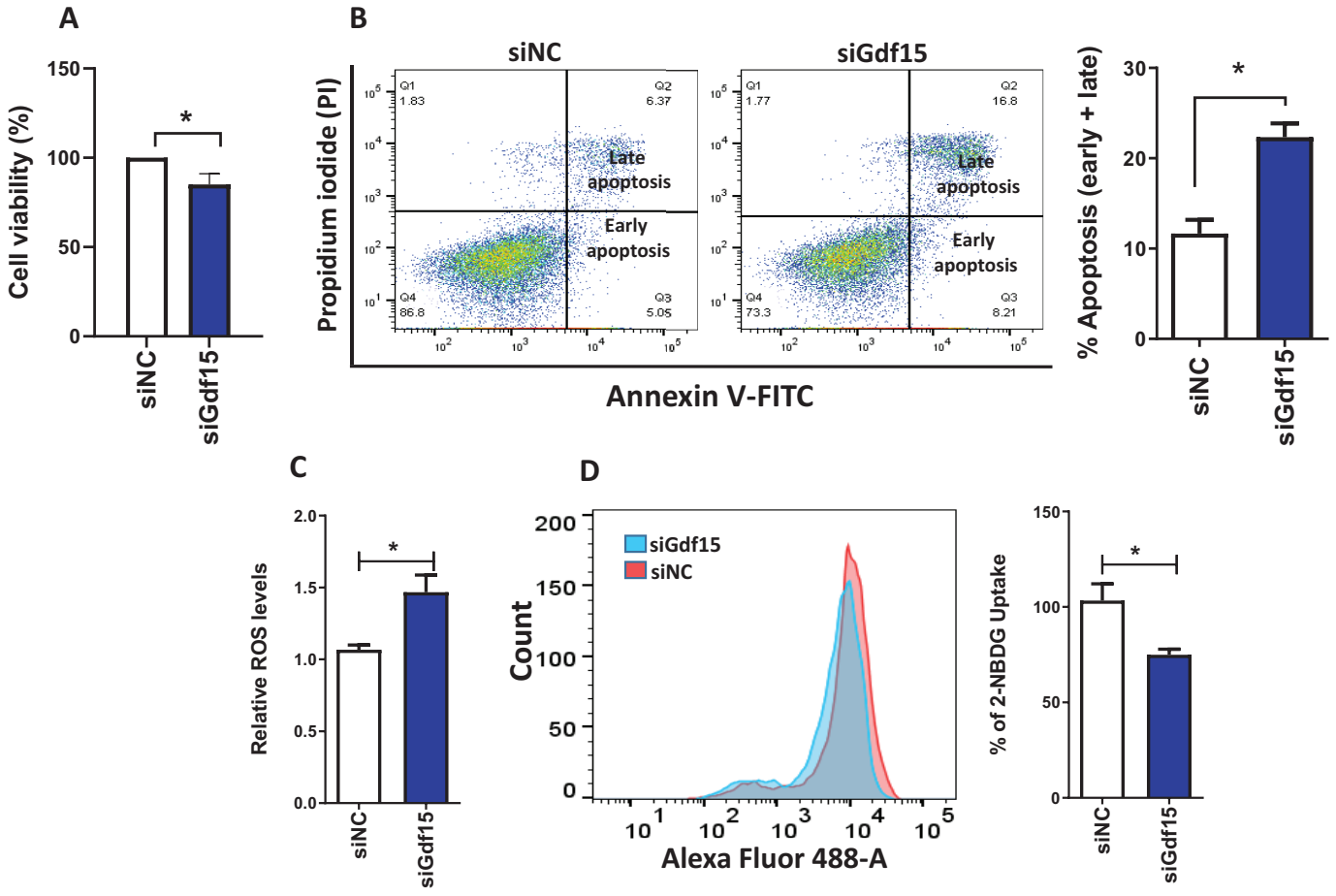

Figure 3. The role of *Gdf15* silencing on pancreatic β-cell survival and function. (A) Cell viability was evaluated by MTT assay in *Gdf15*-transfected or control cells. (B) Annexin-V-based apoptosis analysis of *Gdf15*-transfected cells compared to control cells as determined by flow cytometry. (C) ROS production in *Gdf15*-transfected and control cells as determined by fluorescence-based analysis. (D) Glucose uptake efficiency in *Gdf15*-transfected and control cells. Bars represent mean ± SD based on three independent experiments.
*$P < 0.05$.

in INS-1 cells. Interestingly, overexpression of *GDF15* in human islets increased insulin secretion and upregulated MAFA and GLUT1 expression. Collectively, these findings suggest that Gdf15 can modulate β-cells function.

The current study demonstrated that *GDF15* expression is upregulated in diabetic/hyperglycemic relative to non-diabetic/normoglycemic islets (Figure 1[C]). Such an increase in the *GDF15* expression in diabetic islets could be attributed to a compensatory response to (1) intake of glucose-lowering medications, (2) long-term exposure to high blood glucose levels (glucotoxicity), and (3) inflammation.[25] While the first assumption is supported by the finding that metformin increases the circulating levels of *GDF15* and expression,[36] the second explanation is supported by the observation that various T2D-associated genes, including *GDF15*, change their expression patterns in human islets after short-term (24 h) exposure to high glucose levels.[37] Furthermore, Asrih *et al*.[26] showed that rat INS-1E and mouse pancreatic β-cells subjected to glucolipotoxicity conditions upregulated mRNA expression of *Gdf15*. In contrast, Nakayasu *et al*.[25] found that the expression of *GDF15* was depleted in T1D islets. These inconsistent data could be explained by the fact that in T1D, very little/no insulin-producing cells will remain in pancreatic islets. In contrast, our samples are obtained from T2D that resulted from a decrease in insulin secretion and impaired insulin action. Finally, it is well documented that GDF15 levels are elevated in pathological conditions, such as inflammation or myocardial ischemia.[38]

To our knowledge, increased *GDF15* expression in female islets relative to male islets has never been reported. The finding is not surprising considering the high expression level of *GDF15* in the placenta and several other tissues irrespective of gender.[8,39] That said, a meaningful explanation of the gender bias in *GDF15* expression, especially in diabetes, is not readily available.

Data presented here highlighted the possible roles of *GDF15* in maintaining proper islet functioning and in modulating the capacity of islets to secret insulin and take up glucose. As such, functions require intracellular and extracellular interactions. In that, it is well established that *GFRAL* is expressed solely in the hindbrain.[40,41] Anatomical ablation of this area prevented the anorectic action of GDF15.[24] An axis of GDF15/GFRAL/RET has been reported to have a role in improving glucose tolerance.[21] Mice overexpressing *Gdf15* showed improved glucose tolerance on receiving an intraperitoneal injection of glucose under normal or high-fat-fed chow conditions. However, it is unclear whether

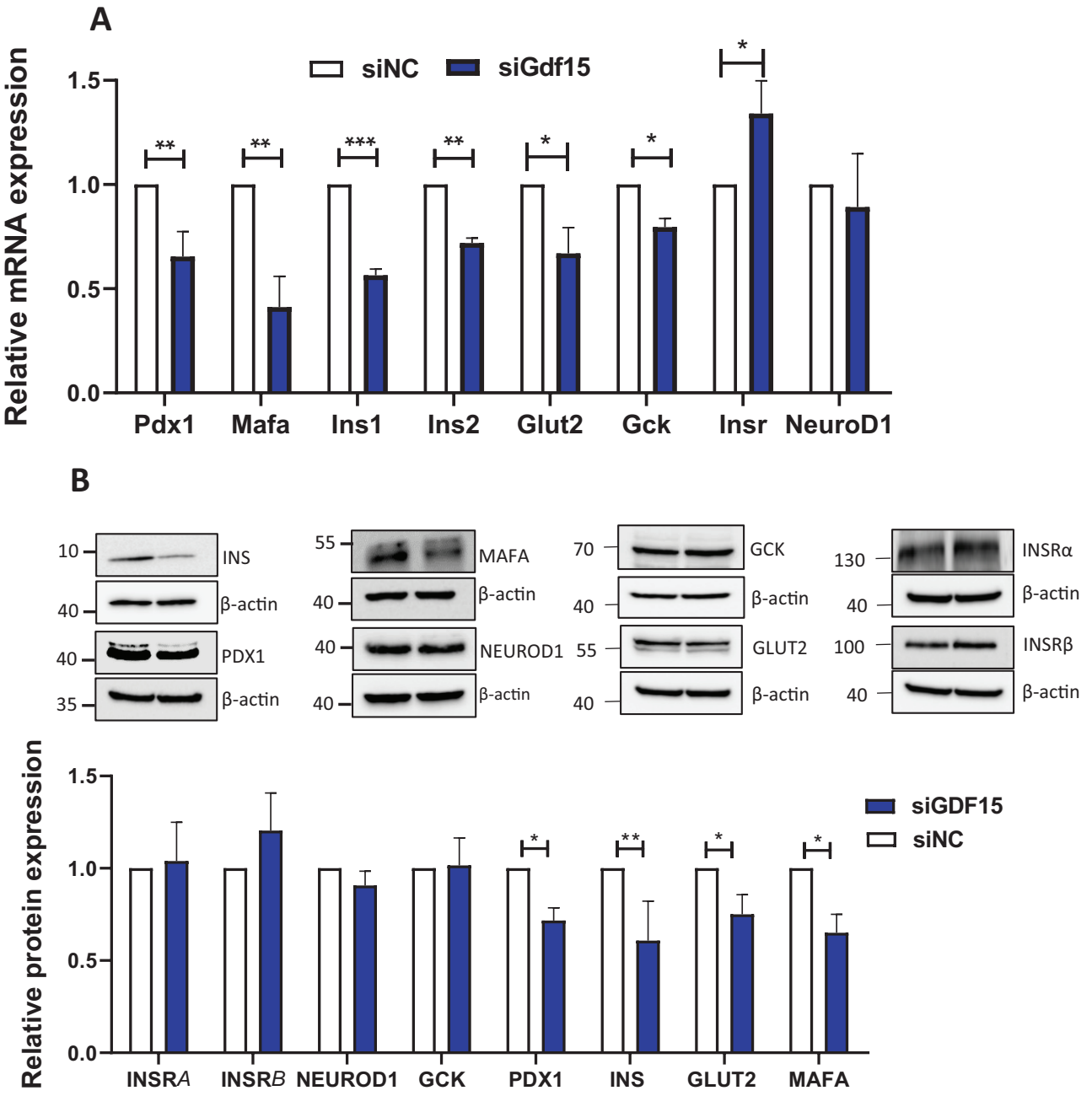

Figure 4. The role of *Gdf15* silencing on pancreatic β-cell survival and function. (A) Expression analysis of *Pdx1*, *Mafa*, *Ins1*, *Ins2*, *Glut2*, *Gck*, *Insr*, and *NeuroD1* as determined by qPCR. (B) Western blot analysis of INSRα, INSRβ, NEUROD1, GCK, PDX1, Pro/insulin (INS), GLUT2, and MAFA in *Gdf15*-silenced cells (upper panels). Bars represent mean ± SD fold change in protein expression based on three independent experiments.
*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

Gdf15 produced by β-cells exerts its effect through GDF15/GFRAL/RET or an autocrine impact on the pancreas.

Recently, silencing of *Gdf15* in mice β-cells was reported to aggravate the glucolipotoxicity impact on cell survival, whereas silencing of *Gdf15* under standard culture conditions showed no effect on cell survival.[26] In contrast, our data showed the silencing of *Gdf15* in rat INS-1 cells tends to decrease cell viability and increased apoptosis (Figure 2[E] and [F]). The discrepancy between the two studies could be due to the nature of cells and species used in the experiments; while the former used mouse primary cells, the latter used 1E cells. Furthermore, our study used a pool of two different siRNA sequences, while the other used a single siRNA sequence to silence their cells.

Several studies have documented the anti-apoptotic property of GDF15 in different cell types, such as human islet EndoC-βH1 β-cells and cardiomyocytes.[25,42] A possible explanation for such an anti-apoptotic feature is inhibiting several apoptotic pathways. Expression of GDF15 mediates a protective role against cell apoptosis through PI3K/Akt/eNOS pathway.[43] Furthermore, increased levels of GDF15 attenuate NFkb, JNK, and Caspase-3, which results in anti-apoptotic action.[44] Recently, Wang *et al*.[45] found that GDF15 significantly reversed the deregulations of the expressions of

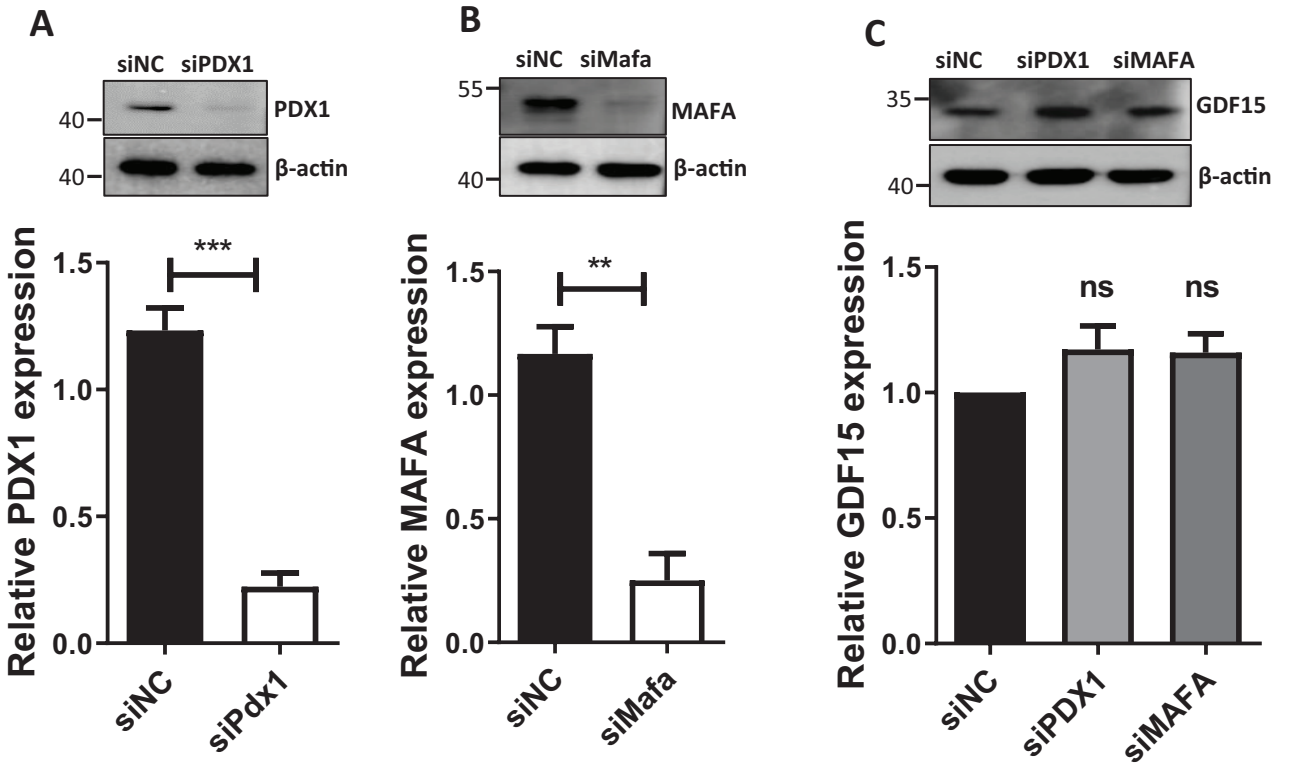

Figure 5. *Pdx1* and *Mafa* silencing in INS-1 cells. Protein expression analysis of PDX1 and MAFA in INS-1 cells 48 h post-transfection as determined by Western blotting (A and B; upper panels); Bars represent mean ± SD fold change in protein expression based on three independent experiments as shown in the lower panel. (B) Western blot analysis of GDF15 in *Pdx1*- or *Mafa*-silenced cells compared to control cells (upper panels). (B) to (C) Cganhe. Bars represent mean ± SD fold change in protein expression based on three independent experiments.
ns: not significant.
$^{**}P < 0.01$. $^{***}P < 0.001$.

cleaved caspase-3, Bax, and BCL2 in the islet of Tg mice compared to their WT mice on Streptozotocin (STZ) treatment.

As demonstrated in this study, reduced expression of *Gdf15* in INS-1 cells impairs insulin secretion at stimulation levels (16.7 mM glucose) without affecting insulin content. Our data agree with Asrih *et al.*[26] study, which showed that silencing of *Gdf15* expression in mouse pancreatic β-cells reduces insulin secretion with no impact on the insulin content. Notably, *Gdf15*-silenced INS-1 cells were linked with reduced expression of several genes involved in insulin production (*Ins1*, *Ins2*) and transcription factors (*Pdx1* and *Mafa*) that act on the promoter of insulin gene. *Pdx1* and *Mafa* genes control the maturation of β-cell function and insulin secretion through regulating the insulin gene expression.[46,47] In this study, we reported that silencing of *Pdx1* or *Mafa* does not affect the protein expression of GDF15. The finding indicates that expression of *Gdf15* is independent of PDX1 or MAFA transcriptional activity.

The elevation of ROS levels in *Gdf15*-silenced cells is not surprising. In line with our data, it has been proposed that *GDF15* attenuates endothelial cell apoptosis through ROS inhibition.[43] However, it is well established that dysregulated levels of ROS in β-cell result in the malfunction of insulin-producing cells and increase the risk of diabetes.[48] Glucose uptake was decreased in *Gdf15*-silenced INS-1 cells. This was accompanied by a downregulated expression of the glucose-sensing gene *Glut2* at both the mRNA and protein levels. GLUT2 plays a crucial role in regulating glucose uptake in β-cells through its low-affinity feature.[49] Notably, it has been reported that defects in the glucose-sensing machinery can reduce insulin secretion.[50] Hence, the downregulation of *GLUT2* might lead to insulin secretion impairment.

Similarly, the increase in insulin secretion in *GDF15*-overexpressed human islets was associated with the upregulation of GLUT1 (the main glucose transporter in human islets). The finding, in addition to the observed upregulation of MAFA, could explain the mechanism of how *GDF15* elevated insulin secretion in human islets, which could be attributed to increase glucose uptake through GLUT1. Furthermore, the transcription factor, MAFA, is well documented to regulate insulin expression by binding to the insulin gene promotor and therefore involved in insulin secretion in response to glucose induction in different experimental models.[47] Nevertheless, the underlying mechanism that linked GDF15 with GLUT1 and MAFA needs further investigation.

In conclusion, the current investigation demonstrates that *GDF15* is essential to maintain proper insulin secretion and pancreatic β-cell function. Further studies are warranted to investigate the potential use of *GDF15* as a drug target in T2D.

AUTHORS' CONTRIBUTIONS

All authors contributed to the data design, interpretation, and analysis; MGM, RS, AKM, MoH, and JT contributed in methodology; RS and AKM participated in statistical analysis; JT, RS,

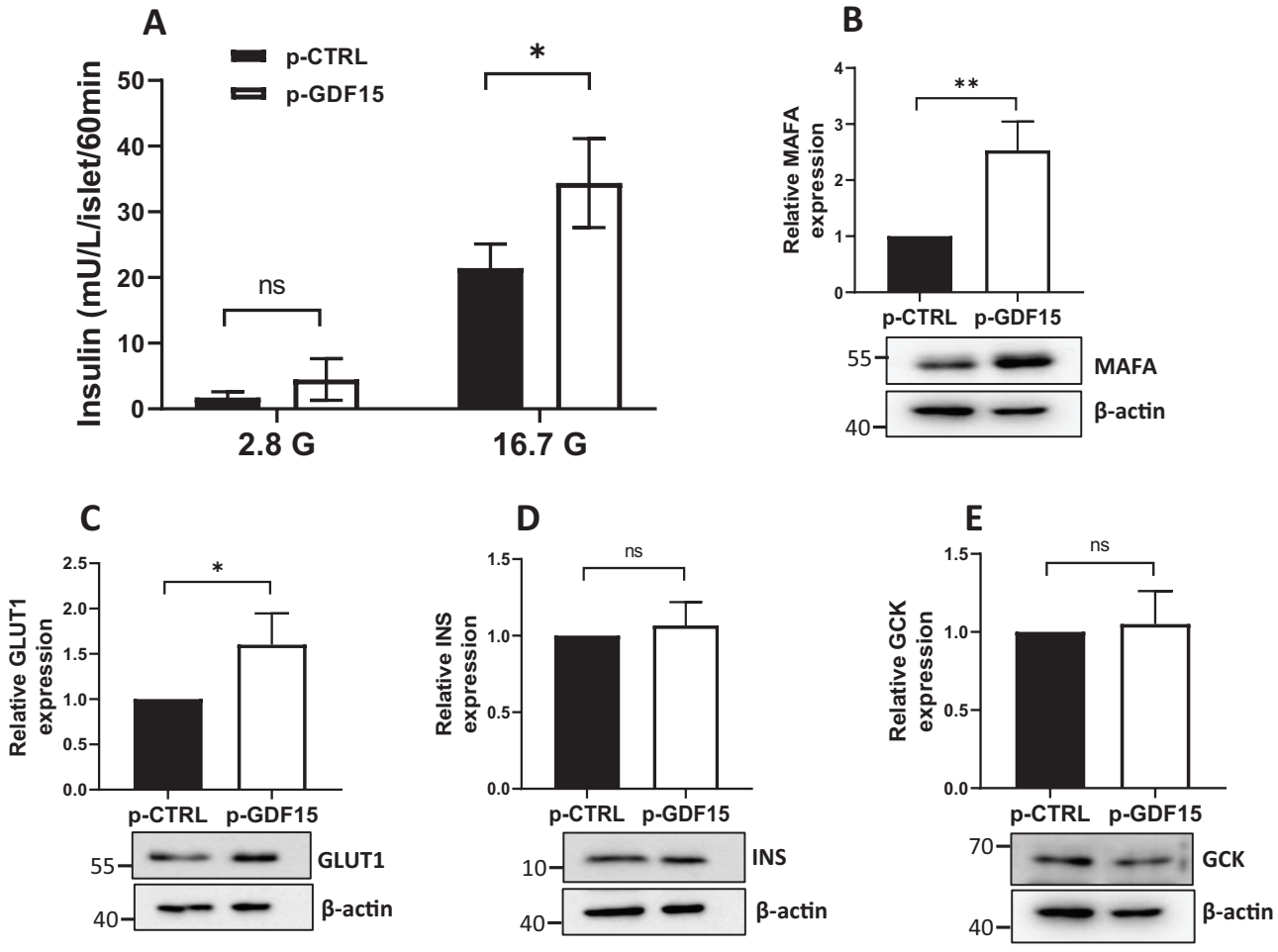

Figure 6. *GDF15* overexpression in human pancreatic islets. (A) Insulin secretion in *GDF15*-overexpressing human pancreatic islets following stimulation with 2.8 and 16.7 mM glucose. Data are based on samples from two different donors. (B to E) Western blot analysis of MAFA, GLUT1, Pro/INS, and GCK in *GDF15*-overexpressing human pancreatic islets; β-actin was used as a loading control. Data are based on samples from three different donors. Bars represent mean ± SD.
*$P < 0.05$. **$P < 0.01$.

AKM, and WE-H carried out data curation and interpretation; JT, MGM, and MaH participated in original draft preparation and draft editing. All authors have read and agreed to publish this version of the article.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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