Brief Communication

MOLECULAR METABOLISM

# Overexpression of *Gjb4* impairs cell proliferation and insulin secretion in primary islet cells

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## ABSTRACT

**Objective:** Altered gene expression contributes to the development of type 2 diabetes (T2D); thus, the analysis of differentially expressed genes between diabetes-susceptible and diabetes-resistant mouse models is an important tool for the determination of candidate genes that participate in the pathology. Based on RNA-seq and array data comparing pancreatic gene expression of diabetes-prone New Zealand Obese (NZO) mice and diabetes-resistant B6.V-*ob/ob* (B6-*ob/ob*) mice, the gap junction protein beta 4 (*Gjb4*) was identified as a putative novel T2D candidate gene.
**Methods:** *Gjb4* was overexpressed in primary islet cells derived from C57BL/6 (B6) mice and INS-1 cells via adenoviral-mediated infection. The proliferation rate of cells was assessed by BrdU incorporation, and insulin secretion was measured under low (2.8 mM) and high (20 mM) glucose concentration. INS-1 cell apoptosis rate was determined by Western blotting assessing cleaved caspase 3 levels.
**Results:** Overexpression of *Gjb4* in primary islet cells significantly inhibited the proliferation by 47%, reduced insulin secretion of primary islets (46%) and INS-1 cells (51%), and enhanced the rate of apoptosis by 63% in INS-1 cells. Moreover, an altered expression of the miR-341-3p contributes to the *Gjb4* expression difference between diabetes-prone and diabetes-resistant mice.
**Conclusions:** The gap junction protein Gjb4 is highly expressed in islets of diabetes-prone NZO mice and may play a role in the development of T2D by altering islet cell function, inducing apoptosis and inhibiting proliferation.


**Keywords** Gap junction; Connexins; Type 2 diabetes; Insulin secretion; Proliferation; Beta cell

## 1. INTRODUCTION

In modern societies, the reduction in physical activity and the increase in energy intake results in a growing prevalence of obesity and its associated comorbidities, including type 2 diabetes (T2D). However, T2D is not only caused by environmental factors; it is also a polygenetic disease [1]. The role of heritability in T2D has been proven by several twin studies [2,3] and by genome-wide association studies that identified genes and loci that participate in the development of T2D [4]. Nevertheless, functional evidence connecting genetic alterations with pathogenic processes of T2D is rare. It is well accepted that T2D develops in insulin-resistant subjects who exhibit a failure of beta cell compensation and show beta cell dysfunction [5]. Mouse strains that model these pathologies are suitable for the determination of diabetes genes and clarifying their contribution to the disease. Therefore, comparing gene expression data of diabetes-resistant and diabetes-prone mice is an appropriate strategy to identify variations that can be linked to beta cell failure.

New Zealand Obese (NZO) mice represent an excellent model for polygenically derived development of T2D [6]. To induce a fast and synchronized beta cell failure in these mice, a specific feeding protocol has been established [7]. After 13 weeks on a carbohydrate-restricted diet, NZO mice are obese and insulin resistant but still normoglycemic. The switch to a carbohydrate-containing diet for at least 2 days results in a rapid increase in blood glucose levels and the induction of islet cell apoptosis [8]. By contrast, B6-*ob/ob* mice carrying a leptin mutation on the C57BL/6 background do not develop hyperglycemia under these feeding conditions [6] because of massive beta cell proliferation that contributes to high serum insulin levels [9]. Hence, diabetes-prone NZO and diabetes-resistant B6-*ob/ob* mice can serve as appropriate models to detect the genetic alterations responsible for beta cell failure.

To identify candidates differentially expressed in islets of NZO and B6-*ob/ob* mice, RNA-seq and microarray analysis were performed [7,8,10]. One of the top candidate genes that exhibited a striking difference in expression was the gap junction protein beta 4

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(*Gjb4*). *Gjb4* belongs to the family of connexins and is highly expressed in diabetes-prone NZO but not in diabetes-resistant B6-*ob/ob* islets.

The aim of this study was to investigate whether an elevated *Gjb4* expression in diabetes-prone NZO contributes to the pathogenesis of T2D. To test this hypothesis, we performed numerous assays characterizing the function of *Gjb4* in pancreatic islets and clarified the molecular cause of *Gjb4* deficiency in normoglycemic mice.

## 2. MATERIAL AND METHODS

### 2.1. Cell culture

Rat insulinoma derived INS-1 832/13 cells (INS-1 cells) were grown in RPMI 1640 (PAN-Biotech, Aidenbach, Germany) supplemented with 10% FCS, 10 mM HEPES, 2 mM 1-glutamine, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol at 37 °C in an atmosphere of humidified 5% $CO_2$ air.

### 2.2. Isolation of primary islet cells, RNA isolation, and quantitative real-time-PCR

Primary islet cells of C57BL/6J mice (B6) were isolated and cultivated as described [7]. Total RNA was extracted from mouse pancreatic islets with the RNeasy Mini Kit (Qiagen, Hilden, Germany) as described [11]. Expression levels of *Gbj4*, *Atf4*, *Atf6*, and *Chop* were detected via qRT-PCR with gene-specific primers (*Gjb4*, for: 5′-GGGTGCTGGTGTATGTGGT-3′, rev: 5′-TGAAATCCTTTTGATCGTCGT-3′; *Atf4*, for: 5′- ATCCAGCAAAGCCCCACAAC-3′, rev: 5′-CAAGCCATCATCCATAGCCG-3′; *Atf6*, for: 5′-AGGGAGAGGTGTCTGTTTCG-3′, rev: 5′-CCAAGGCATCAAATCCAAAT-3′; *Chop*, for: 5′-TTCACTACTCTTGACCCTGCGTC-3′, rev: 5′-CACTGACCACTCTGTTTCCGTTTC-3′; Sigma—Aldrich, St. Louis, USA) and SYBR Green master mix (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was calculated according to the ΔΔCt method [12] using *β-actin* (for: 5′-GCCAACCGTGAAAAGATGAC-3′, rev: 5′-TACGACCAGAGGCATACAG-3′; Sigma—Aldrich) as endogenous control.

### 2.3. Sequencing of genomic DNA

Library preparation for sequencing was performed with 1 μg of DNA from NZO for massive parallel sequencing that used two library prep protocols: Bioline JetSeq (Bioline) and Illumina PCR free TruSeq (Illumina). The DNA was loaded on an Illumina Hiseq2500 version 4 at a density of at least $\sim 240 \times 10^6$ fragments per lane (2 lanes in total), and DNA sequencing was performed by using 125 bp paired-end chemistry.

For data analysis, FastQ data of the NZO library were mapped against the *Mus musculus* mm10 genome using bwa-mem (v.0.7.13) [13]. Duplicate reads were marked by Picard-tools (v.2.4.1). Sample-wise libraries (Bioline and Illumina) were merged for further processing with GATK tools using SAMtools (v.1.3.1). Indel re-alignment and base quality score re-calibration were performed by using the GATK (v3.6) and its best practices workflow (https://www.broadinstitute.org/gatk/guide/best-practices.php). Variant calling was performed applying GATK's HaplotypeCaller in ERC mode yielding g.vcf-files ($\sim 8 \times 10^6$ variants/sample).

Next, a joint variant calling was performed by using the sample-wise g.vcf files as input for the GenotypeVCFs-tool. DbSNP (snp138 from UCSC) was used for common SNP annotation. This step yielded a multisample VCF-file with approximately $14 \times 10^6$ variants. The VCF-file was annotated by using snpeff 4.1k with the GRCm38.79 database. Single-cell transcriptomic analysis of pancreatic islets was performed according to Sachs et al., [14].

### 2.4. Overexpression of *Gjb4* in primary islet cells and INS-1 cells and BrdU proliferation assay

For *Gjb4* overexpression, primary islet cells were infected with either an adenovirus carrying cDNA from *Gjb4* (Ad-*Gjb4*, MOI 10) or the empty virus (Ad-∅, MOI 10), both under the control of a CMV promotor with a myc-tag fused to the C-terminus of the protein (Ad-m-GJB4-Myc: 20160502T#5; Ad-CMV-NULL: 20150623t#7, Vector Biolabs, Malvern, USA). Infected cells were incubated in the viral medium for 24 h and then labeled with BrdU (100 μmol/l) for 72 h. Cells were fixed with 4% paraformaldehyde. Cell membranes were permeabilized (0.2% saponin), DNA was denatured (2 M HCL), histones were eliminated (0.1% trypsin), and cells were incubated with primary antibodies against BrdU (1:500, Sigma—Aldrich, St. Louis, USA), c-myc (1:400, Santa Cruz Biotechnology, Dallas, USA), and insulin (1:100,000, Sigma—Aldrich) overnight at 4 °C. Detection was performed with fluorophore-labeled secondary antibodies (rat: Alexa Fluor546, 1:400; rabbit: Alexa Fluor488, 1:400, Invitrogen, Carlsbad, USA) and DAPI (1:1000, Roche, Basel, Switzerland) for 2 h at RT and documented with a confocal microscope (TCS SP-2-Confocal Laser scanning microscope, Leica Microsystems, Wetzlar, Germany). Statistics were performed by blinded quantification of 10—12 photographs of at least two coverslips per infection. Transduction efficiency was calculated by calculating the number of myc-(Gjb4) positive cells in relation to the total number of cells (DAPI staining).

Similarly, INS-1 cells were seeded on 24-well plates, recovered for 24 h, and infected with Ad-*Gjb4* (MOI 10) and Ad-∅ (MOI 10) for another 24 h. BrdU labeling (100 μmol/l) was performed for 2 h, and cells were fixed in 4% paraformaldehyde.

To measure protein levels of insulin after *Gjb4* overexpression, four days after adenoviral infection, primary islets were transferred to ice-cold acidic ethanol and insulin contents were determined by ELISA (80-INSMSH-E01, Alpco Diagnostics, Salem, USA). The mRNA expression of insulin was analyzed 24 h after overexpression by qRT-PCR with TaqMan probes (Mm01259683_g1, Thermo Fisher Scientific). Gene expression was calculated according to the ΔΔCt method [12] while using *Ppia* (Mm02342429_g1) as endogenous control.

### 2.5. Glucose-stimulated insulin secretion of primary islet cells and INS-1 cells

Pancreatic islets were isolated and pooled to groups of 60 islets per condition. Primary islets were infected with either Ad-*Gjb4* (MOI 10) or Ad-∅ as control (MOI 10) and recovered overnight at 37 °C (5% $CO_2$) in RPMI medium containing 11 mmol/l glucose, 100 U/ml Pen/Strep, and 10% FCS. For equilibration, islets were glucose-starved by incubation at 37 °C in KRBH buffer (pH 7.4) containing 10 mM HEPES, 20 mM $NaHCO_3$, 0.2% BSA, and 2.8 mM glucose for 1 h. To measure glucose-stimulated insulin secretion, the equilibration medium was replaced with KRBH buffer enriched with different glucose concentrations (low glucose: 2.8 mM, high glucose: 20 mM) and finally low glucose supplemented with 40 mM KCl. Secreted insulin was detected by using the Mouse High Range Insulin ELISA Kit (Alpco Diagnostics). Total DNA content of islets was measured with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, USA). The fluorescence signal was detected by the SpectraMax M4 Multi-Mode Microplate Reader (Molecular Devices, San José, USA). Secreted insulin was finally normalized to total DNA content.

For the measurement of insulin secretion in INS-1 cells, cells were seeded in 96-well plates and recovered for 72 h. The cells were then infected with either Ad-*Gjb4* or Ad-∅, and 24 h later, insulin secretion was measured under low glucose (2.8 mM) or high glucose (20 mM) concentrations.

 

### 2.6. Induction of apoptosis in INS-1 cells and Western blot analysis

INS-1 cells were seeded in a 12-well plate and infected with Ad-*Gjb4* (MOI 10) or Ad-∅ (MOI 10) for 24 h. Subsequently, cells were treated with 200 μM palmitate and 30 mM glucose for 24 h, washed with PBS and lysed in 70 μl lysis buffer per well (20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton (pH 7.4), 1× protease inhibitor cocktail (Roche), and 1× phosphatase inhibitor cocktail (2.5 mM $Na_4P_2O_7$, 1 mM $C_3H_7Na_2O_6P$, 1 mM $Na_3VO_4$, 1 mM NaF, [Roche]). Western blotting was performed by using a primary antibody against cleaved caspase 3 (1:1,000, Cell Signaling Technology, Danvers, USA) and as loading control α-tubulin (1:1,000, Sigma, St. Louis, USA) followed by application of secondary horseradish peroxidase-conjugated anti-mouse antibody (1:20,000, Dianova, Hamburg, Germany) and goat peroxidase-conjugated anti-rabbit antibody (1:20,000, Dianova).

### 2.7. Analysis of putative transcription factor binding sites (TFBS) and miR target prediction

For the identification of TFBS within the *Gjb4* promotor region, the protocol described by Saussenthaler et al. [15] was applied. In brief, regulatory elements were identified by using data of ChIP-seq of histone modifications (H3K4me3/H3K27ac) [16] and compared to TFBS from the JASPAR database [17] modeled as position weight matrices (PWMs).

To predict miRNAs targeting *Gjb4*, five prediction tools—DIANA-microT [18], miRDB [19], TarPmiR [20], TargetScan7.1 [21], and RNA22 [22]-were used as described [23]. In the case that at least three of the tools showed overlapping results, it was presumed that the examined miRNAs most likely target *Gjb4*. For identification of potential seed regions, RNA22 [22] was applied.

### 2.8. Chip-qPCR

First, pancreatic islets of B6 (2,000 islets) and NZO (1,600 islets) mice were isolated. RNA Pol II ChIP reactions were performed by using 12 μg of mouse primary islet cell chromatin and 20 μl of antibody (Active Motif, cat # 39097; Carlsbad, CA, USA). qPCR was performed by using two positive control primers (*Actb*, *Gapdh*), two sites covering the altered TFBS (P1: +2839, for: 5′-GGTACAGAGGAGAGGGGTGAC-3′, rev: 5′-TGGGGAGAAGAGCTGAGTTG-3′; and P2: +2518, for: 5′-TGGGTCATTCTTTGCATGTG-3′, rev: 5′-GCATCCTGCTTAACCTTGTG-3′), as well as a negative control primer pair that amplifies a region in a gene desert on chromosome 6 (Untr6).

### 2.9. Overexpression of miR-341

For miR-341 overexpression, primary islet cells of NZO mice were isolated (see 2.2.) and 72 h later infected with either an adenovirus expressing pre-miRNA mmu-mir-341 under an EF1a promoter and eGFP as reporter (Ad-miR-341: Ad-EF1a-mmu-mir-341-eGFP (ADV-MI0000625, Vector Biolabs), MOI 50) or the empty virus (Ad-∅: Ad-EF1a-Ctrl-miR-GFP, Vector Biolabs, MOI 50) as control. Four days after infection, total RNA was extracted and expression levels of *Gjb4* and miR-341 were detected via qRT-PCR with gene-specific primers (miR-341: MS00008127, Qiagen). Expression of miR-341 was calculated according to the ΔΔCt method [12] by using *Run6*, *Snore42b*, and *Snore69* (*Run6*: MS00033740; *Snore42b*: MS00055090; *Snore69*: MS00033733, Qiagen) as endogenous controls.

### 2.10. Statistics

All data are reported as mean ± SEM. Statistical analysis was performed by using the Student's *t* test for single comparisons and one-way ANOVA with Tukey's *post hoc* test for differences between more than two groups. Differences between two groups and different conditions were evaluated by using a two-way ANOVA with Tukey's *post hoc* correction for multiple comparisons (GraphPad Prism version 7, La Jolla, USA). Significance was accepted at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

## 3. RESULTS

### 3.1. *Gjb4* is exclusively expressed in islets of diabetes-prone NZO mice and is not regulated by glucose

Recently, we performed transcriptome analysis of isolated islets from diabetes-susceptible NZO and diabetes-resistant B6-*ob/ob* mice [7,8,10] and identified *Gjb4* as the main differently expressed gene comparing both mouse strains. *Gjb4* is expressed in islets of the NZO strain but nearly not detectable in B6-*ob/ob* islets (Figure 1A,B). *Gjb4* expression in NZO islets was not different before and two days after carbohydrate feeding (Figure 1B).

To evaluate in which type of islet cells *Gjb4* is expressed, we used single-cell data from NZO islets (unpublished data), which showed that *Gjb4* is expressed in all islet cell types and exhibits comparable expression levels in alpha and beta cells (see Suppl figure 1).

NZO mice have higher blood glucose levels than B6-*ob/ob* mice; thus, we tested whether *Gjb4* expression can be increased by elevated glucose concentrations. B6 high-fat diet mice with moderate hyperglycemia (mean BG: 10.2 mM) did not show an increase in *Gjb4* expression (Ct values > 40). Evaluation of the *Gjb4* expression in islets of six-week-old normoglycemic B6 (7.1 mM) versus NZO (7.5 mM) mice demonstrated that similar to the data from 18-week-old mice, *Gjb4* expression was only detectable in NZO islets (Suppl figure 2A). Thus, *Gjb4* expression in NZO is already high under normoglycemic conditions; it also did not correlate with blood glucose values (Suppl figure 2B). At the age of six weeks, blood glucose values were identical between B6 and NZO mice, whereas plasma insulin started to be higher in NZO than in B6. Therefore, we also calculated the correlation between insulin concentration and *Gjb4* expression but detected no effect toward elevated *Gjb4* levels in response to higher insulin values (Suppl figure 2C). We therefore conclude that the elevated *Gjb4* expression in NZO islets is based on intrinsic strain differences.

### 3.2. Overexpression of *Gjb4* inhibits proliferation of primary islet cells

As we recently described, NZO mice have a reduced capacity to induce islet cell proliferation [7]. Therefore, we tested whether the overexpression of *Gjb4* in primary islet cells affects proliferation. *Gjb4* was overexpressed via adenoviral-mediated infection (Figure 2A, upper panel), and proliferation capacity was examined by analyzing BrdU incorporation. Cells infected with an empty virus were used as control. Transfection efficiency was determined at 42.5% and notably, overexpression of *Gjb4* decreased the number of BrdU positive cells by 47% (Figure 2A; Ad-Ø: 10.62% ± 1.61, Ad-*Gjb4*: 5.62% ± 0.78), suggesting that *Gjb4* impairs the proliferation capacity of primary islet cells. In addition, we observed that cells with a positive *Gjb4* signal were rarely also positive for BrdU. The representative pictures shown in Figure 2A indicate that *Gjb4* is not only located at the plasma membrane but also in intracellular compartments of primary islet cells. To test whether the virus-mediated overexpression of *Gjb4* induces ER stress, we analyzed the expression of the ER stress markers *Atf4*, *Atf6*, and *Chop* in islet cells infected with the *Gjb4* adenovirus or the empty control. No differences were detected between the groups (Suppl figure 3), assuming that Gjb4 did not induce ER stress. Because more than 80% of islet cells are beta cells, we estimated that the reduced proliferation after *Gjb4* overexpression mostly affects pancreatic beta cells. This is supported by co-staining of BrdU and

 

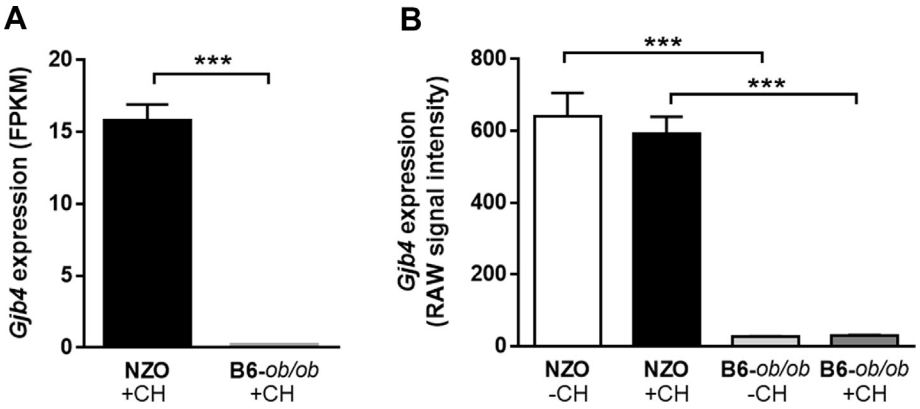


**Figure 1: Expression of *Gjb4* in islets of NZO and B6-*ob/ob* mice.** NZO and B6-*ob/ob* mice were fed a carbohydrate-restricted diet up to the age of 18 weeks and then put on either a carbohydrate-restricted (-CH) or carbohydrate-enriched diet (+CH) for 2 days. Expression of *Gjb4* was analyzed via RNA-sequencing (A, n = 5) and microarray analysis (B, n = 3). Data are presented as mean ± SEM, ***P < 0.001.

insulin (Figure 2A, lower panel), showing that Gjb4-positive cells also express insulin. Thus, Gjb4 plays a role in the proliferation of pancreatic beta cells but presumably also in the alpha cells.

To test the effect of *Gjb4* on proliferation in another pancreatic beta cell model, we overexpressed the gene in rat insulinoma cells (INS-1) and evaluated BrdU incorporation over a 2 h period. However, no change in proliferation levels could be detected (Suppl figure 4), which might be due to the high proliferation rate of immortalized INS-1 cells.

### 3.3. Overexpression of *Gjb4* inhibits insulin secretion in primary islet and INS-1 cells

To determine the impact of *Gjb4* on islet cell function, glucose-stimulated insulin secretion (GSIS) was measured in primary islet and INS-1 cells. Both cell types were infected with a virus containing *Gjb4* or an empty virus as control and subsequently exposed to basal glucose (2.8 mM) and high glucose concentrations (20 mM) and then stimulation of the primary islet cells [24] with 40 mM potassium chloride (KCl). Primary islet and INS-1 cells overexpressing *Gjb4* secreted 46% and 51% less insulin, respectively, than cells infected with the empty virus after exposure to high glucose and 38% less after KCl stimulation in primary islet cells (Figure 2B).

To clarify whether insulin secretion was reduced because of lower insulin expression or protein content, the mRNA expression and protein amount of insulin in primary islet cells were measured 24 h and four days after *Gjb4* overexpression. Both the mRNA and protein levels of insulin were identical between cells infected with the *Gjb4* virus or the respective control at both time points (Figure 2C). Thus, Gjb4 mediates a direct effect on insulin secretion.

### 3.4. Overexpression of *Gjb4* enhances apoptosis in INS-1 cells

Studies have demonstrated that loss of beta cell mass due to apoptosis plays an important role in the development of T2D [25]. Therefore, apoptosis was induced by 30 mM glucose and 200 μM palmitate in *Gjb4* overexpressing INS-1 cells. By detecting cleaved caspase 3 as a marker for apoptosis via Western blotting, *Gjb4* significantly increased apoptosis by 63% under this condition (Ad-Ø: 2.1 ± 0.2, Ad-*Gjb4*: 3.7 ± 0.6, Figure 2D).

### 3.5. *Gjb4* expression in NZO mice is possibly enhanced by a reduced miR-341-3p expression

To clarify why NZO islets exhibit much higher expression of *Gjb4* than B6-*ob/ob* islets, we first used NZO sequence data and analyzed the putative promoter region of *Gjb4* on chromosome 4 by using ChIP-seq information from mouse pancreatic beta cells [26]. Within a ChIP-seq peak region of H3K4me3 and H3K27ac, a single nucleotide polymorphism 450 bp upstream of the transcription start of *Gjb4* was detected in the NZO sequence compared to the B6 reference genome. An *in silico* approach using PWM-based TFBS prediction revealed that this SNP generates a new binding site for three transcription factors (TCF3, TCF4, and ZEB1; Figure 3A). As the B6-*ob/ob* promoter does not carry the binding site for these ubiquitously expressed transcription factors, it could be possible that this sequence alteration and a likely difference in transcription factor binding are causal for the low *Gjb4* expression in B6-*ob/ob* mice. To test this, we performed chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assays on islets from B6 and NZO mice. In Suppl figure 5, the RNA Pol II activity was high at the positive control sites (*Gapdh*, *Actb*). In contrast, the two primer pairs (P1 and P2) covering the proposed transcription factor binding site did not amplify a product, demonstrating that neither the B6 nor the NZO sequence upstream of *Gjb4* carries sites for the binding of transcription factors. Thus, an altered sequence upstream of the transcription start site of *Gjb4* is not responsible for the differential expression in B6 and NZO islets.

A second possibility for the strong difference in *Gjb4* expression in NZO compared to that of the B6-*ob/ob* islets could be the result of a specific miRNA binding to the *Gjb4* mRNA, downregulating the expression in islets. Therefore, we applied five prediction tools to identify the target sites linking *Gjb4* mRNA and specific miRNAs. Overall, two miRNAs (miR-341-3p and miR-700-3p) displayed possible seed regions that could target *Gjb4*. miR-341-3p, with two possible binding sites within the *Gjb4* target (Figure 3B), showed an elevated expression in B6-*ob/ob* mice compared to NZO islets (Figure 3C). miR-700-3p was also differently expressed, but in contrast to miR-341-3p, was lower in B6-*ob/ob*, which could not result in a lower expression of *Gjb4* in B6-*ob/ob* compared to NZO islets.

To investigate whether increased expression of miR-341-3p could be responsible for reduced expression of *Gjb4* in B6-*ob/ob* islets, miR-341-3p was overexpressed in NZO islets via adenoviral infection. Four days after infection, miR-341-3p was 3-fold overexpressed compared to control cells and the expression of *Gjb4* was significantly reduced (Figure 3D). Similar to the analysis for *Gjb4*, we tested whether the expression of the miR-341 is dependent on blood glucose levels. As shown in Suppl Figure 6, we did not detect any differences in miR-341 levels.

 

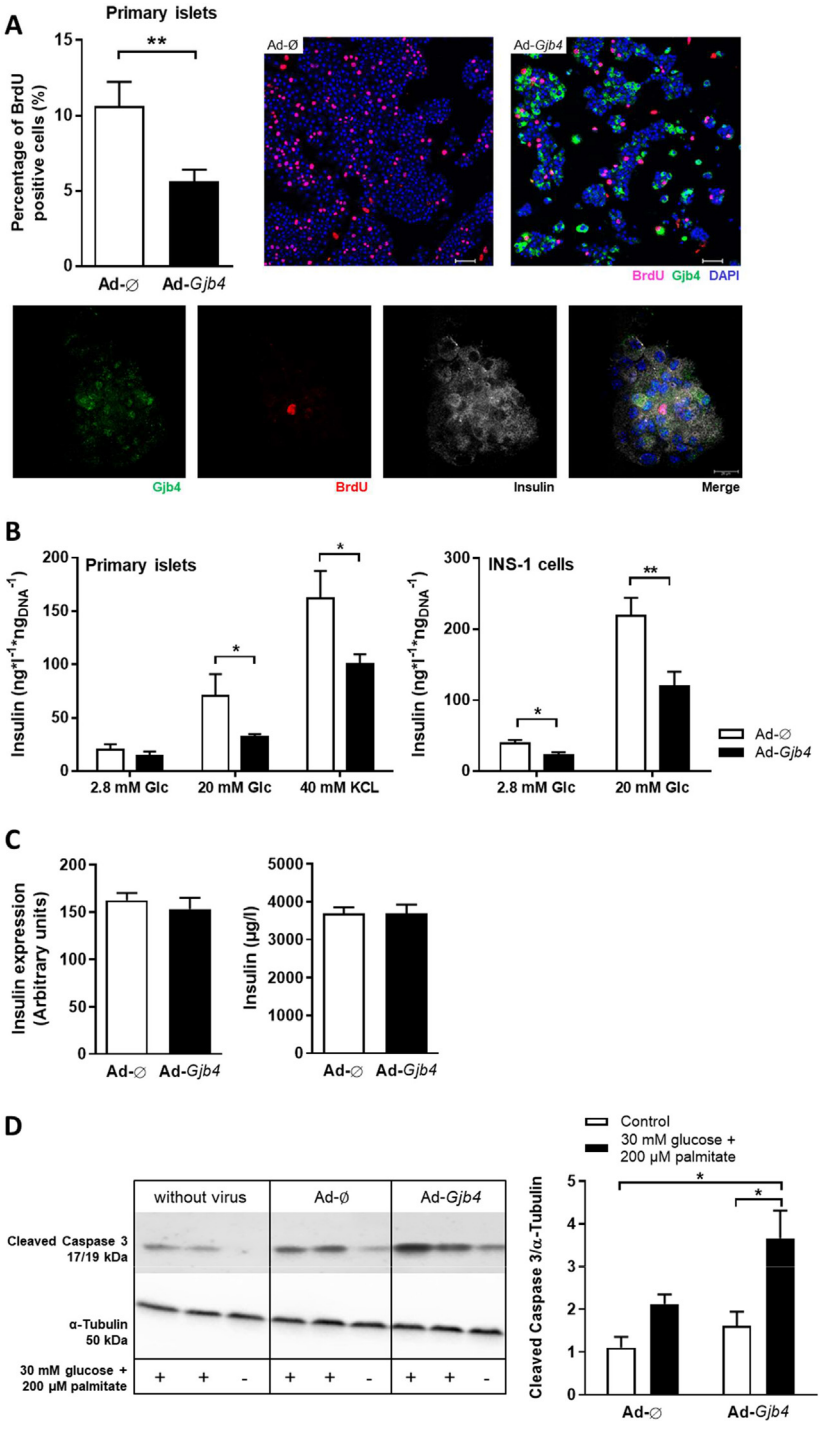


**Figure 2: *Gjb4* inhibits proliferation, insulin secretion, and apoptosis in islet cells.** (A) Isolated and dispersed islets were infected with an empty adenovirus (Ad-∅) as control or an adenovirus expressing *Gjb4* (Ad-*Gjb4*) and incubated with BrdU for 72 h. For each group, between 1,800 and 3,400 myc-expressing cells were evaluated and assessed. Representative immunocytochemical co-stainings (right panel) of BrdU (magenta), myc-tag of *Gjb4* (green), DAPI (blue), and insulin (white). Large nuclei are fibroblast-like cells that were left out in any morphometry (scale bars, 50 µm). (B) Effects of *Gjb4* overexpression on GSIS in primary islets (left panel, n = 10) and INS-1 cells (right panel, n = 20–24). Cells were equilibrated with KRBH buffer and stimulated with 2.8 mM, 20 mM glucose and with 40 mM KCl (primary islet cells). (C) mRNA expression of insulin 24 h after overexpression of *Gjb4* and protein levels of insulin at four days after *Gjb4* overexpression in primary islet cells of B6 mice. (D) Effect of *Gjb4* overexpression in INS-1 cells on protein levels of the cleaved caspase 3 as a marker for apoptosis. Infected INS-1 cells (n = 3) were stressed with 30 mM glucose and 200 µM palmitate for 24 h and protein levels of cleaved caspase 3 were analyzed and quantified by Western blotting. Data are presented as mean ± SEM, *P < 0.05; **P < 0.01; ***P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

 

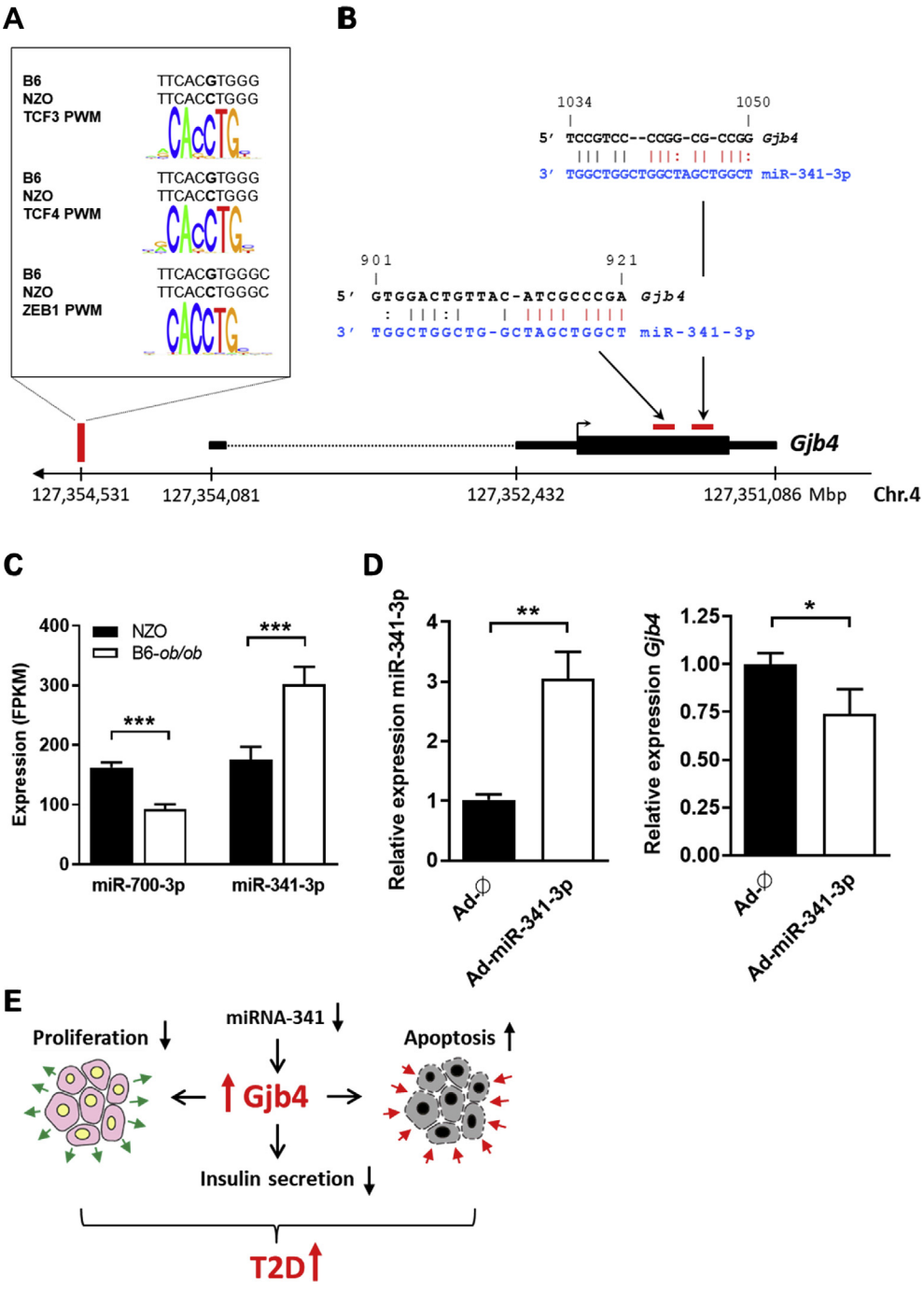


**Figure 3: Genomic organization of *Gjb4* and putative regulators affecting *Gjb4* expression.** (A) Putative transcription factor binding site in the promoter of the *Gjb4* gene in NZO mice. Promoter sequence is given as a PWM for the transcription factors TCF3, TCF4, and ZEB1 in NZO compared to B6 mice. (B) Sequence (in black) and position (in red) of two putative binding sites within the *Gjb4* gene and the miR-341-3p sequences (in blue). The corresponding putative binding site of miR-341-3p within *Gjb4* is indicated in red dashes. (C) Expression of miR-700-3p and miR-341-3p in islet cells of NZO and B6-*ob/ob* mice analyzed by RNA-seq (n = 5). (D) Expression of miR-341-3p and *Gjb4* in primary islet cells from NZO mice four days after infection with an miR-341-3p encoding adenovirus. Data are presented as mean ± SEM, *P < 0.05; **P < 0.01; ***P < 0.001. (E) Graphical summary representing the consequence of an increased *Gjb4* expression on beta cell function finally contributing to the development of T2D. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In conclusion, the decreased expression of miR-341-3p targeting the *Gjb4* gene is-at least partially-causal for the increased *Gjb4* expression in islets of diabetes-prone versus diabetes-resistant mice and finally for the altered islet cell function (Figure 3E).

## 4. DISCUSSION

In this study, we showed that *Gjb4* is a potential novel diabetes gene contributing to the pathogenesis of beta cell failure. Expression of *Gjb4*

 

reduced beta cell proliferation and insulin secretion and increased beta cell apoptosis.

Lack of the ability of beta cells to proliferate [25,27], an enhanced rate of apoptosis [28], as well as a general impaired capacity of beta cells to secrete insulin [25,29] are pathogenic hallmarks for the development of T2D. In this study we demonstrated that *Gjb4*, a gap junction protein that is expressed in islets of diabetes-prone NZO and nearly absent in diabetes-resistant B6-*ob/ob* mice, inhibits proliferation of primary islet cells, significantly reduces insulin secretion of primary islet and INS-1 cells, and enhances apoptosis in INS-1 cells. Thereby, overexpression of *Gjb4* supports all of the pathogenic hallmarks mentioned and thus could contribute to beta cell failure and the development of T2D in NZO mice.

*Gjb4* belongs to the family of connexins and is therefore also called *Cx30.3*. Connexins are proteins that oligomerize around a central hydrophilic space into a 6-protein hemichannel called connexon [30]; these connexons aggregate in the membrane to form gap junctions [31]. Gap junctions guarantee electrical and metabolic coupling between cells [32]. Thus far, no link between *Gjb4* and diabetes has been reported. Although human pancreatic islets are known to express *Gjb4* [33], limited information has been provided on *Gjb4* and islet cell function. The literature has reported an association between mutations of *Gjb4* with erythrokeratoderma variabilis [34,35], a rare skin disease, and with autosomal recessive non-syndromic hearing loss [36]. Recently, it was demonstrated that *Gjb4* is expressed in mouse embryonic stem cells and is responsive to leukemia inhibitory factor [37].

Our data is in line with a growing body of *in vivo* and *in vitro* evidence demonstrating a link between connexins and diabetes. Islet connexins are implicated in type 1 diabetes (T1D) and T2D [38], and connexin signaling has been shown to play a significant role in the regulation of the main beta cell functions including proper insulin secretion and effective glycemic control [39]. The islet gap junction protein connexin 36 (*Cx36*) seems especially important because it provides protection from the development of T1D and T2D by interfering with calcium oscillations and pulsatile insulin secretion [40–43].

However, the data on *Cx36* appear to be contradictory to our findings on *Gjb4* at first sight. A more in-depth examination of the single pathogenic processes reveals that connexins vary widely in their effects. Thus, the different connexins can be connected to both the induction and suppression of apoptosis [44–46]. Likewise, connexins display enhancing and inhibiting effects on proliferation [47–50].

The individual processes of proliferation, apoptosis, and insulin secretion also influence each other. As the role of connexins in regard to proliferation is well established, we can speculate that the impact of *Gjb4* on islet cell proliferation can finally result in defects in insulin secretion. This speculation is supported by the fact that in our study, a decrease in insulin secretion was observed at basal glucose and at stimulation levels, suggesting a beta cell function independent procedure. Because calcium elevation is responsible for insulin granule exocytosis, and because the dynamics of insulin release are tightly connected to calcium oscillations [51], differences in beta cell coupling induced by gap junctions also affect insulin secretion. By contrast, effects on insulin secretion might also cause decreased proliferation levels via the insulin/IGF signaling pathway [52]. Further studies are necessary to investigate the actual sequence of pathogenic events occurring in this case. Thus, follow-up studies including the deletion of *Gjb4* in NZO mice might be of substantial interest.

Our data also show that an increased expression of miRNA-341-3p is at least partially responsible for a reduced expression of *Gjb4* in B6-*ob/ob* compared to NZO islets. The literature has already shown that miRNAs and connexins influence insulin secretion [53,54]. This topic is of substantial interest because thus far, no reports on miRNA regulation of islet-specific or enriched connexins have been made [55,56]. Nevertheless, a strong possibility is that additional alterations, for instance, in enhancers or other regulatory regions, participate in the reduced *Gjb4* expression in diabetes-resistant mice.

## 5. CONCLUSION

In summary, this study identifies that the gap junction protein Gjb4 is highly expressed in islets of diabetes-prone NZO mice, thereby altering islet cell function, and inducing apoptosis and decreasing insulin secretion. Further studies are necessary to clarify the exact contribution of Gjb4 and the related genes on T2D development and whether they can serve as novel diabetes genes for targeted therapies and prevention strategies.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101042.

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