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(Citation)

Diabetes & Metabolism Journal, 50(1):77-89

(Issue Date)

2026-01-01

(Resource Type)

journal article

(Version)

Version of Record

(Rights)

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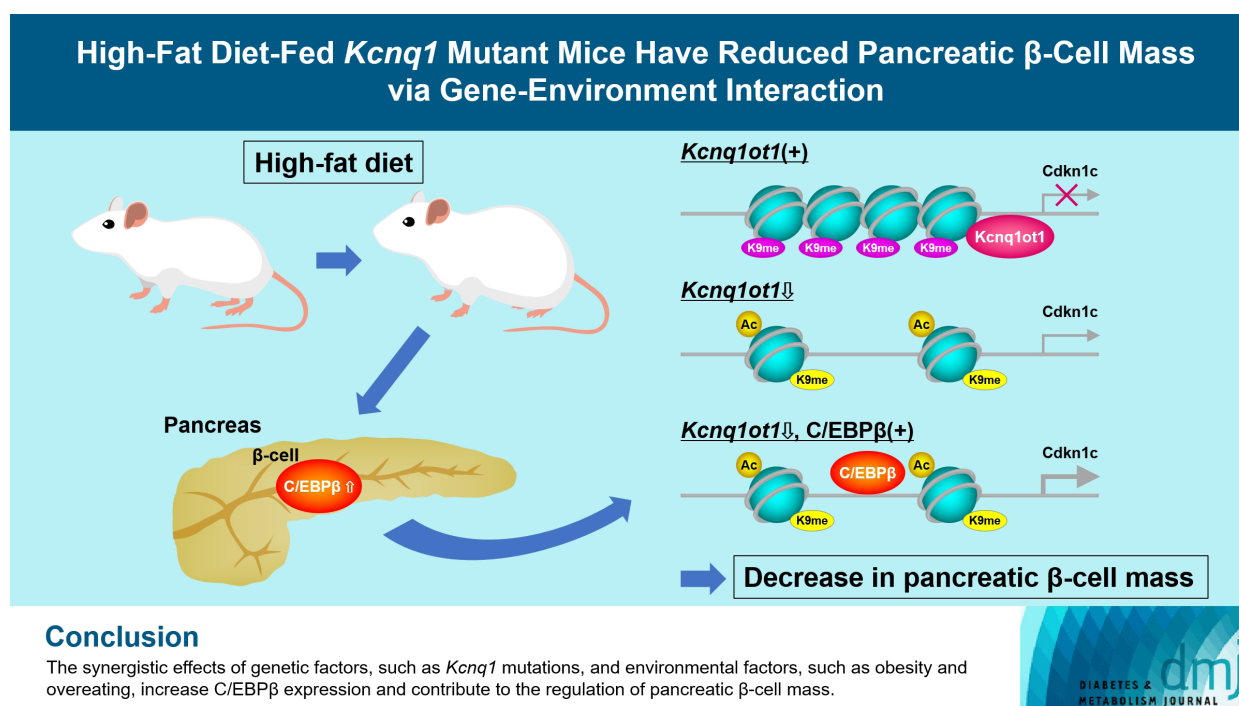
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Diabetes Metab J 2026;50:77-89 | <https://doi.org/10.4093/dmj.2024.0790>



Highlights

- Reduced *Kcnq1ot1* expression in the islets led to changes in epigenetic regulation.
- Cdkn1c was upregulated with C/EBP β expression and epigenetic changes in β -cells.
- HFD-fed *Kcnq1ot1*-reduced mice showed decreased β -cell mass.
- Cdkn1c deficiency restored β -cell mass in HFD-fed *Kcnq1ot1*-reduced mice.

How to cite this article:

Asahara S, Inoue H, Ihara Y, Teruyama K, Imai A, Hara C, et al. High-Fat Diet-Fed *Kcnq1* Mutant Mice Have Reduced Pancreatic β -Cell Mass via Gene-Environment Interaction. *Diabetes Metab J* 2026;50:77-89. <https://doi.org/10.4093/dmj.2024.0790>

High-Fat Diet-Fed *Kcnq1* Mutant Mice Have Reduced Pancreatic β -Cell Mass via Gene-Environment Interaction

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Background: The potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*) gene has recently received much attention as a candidate susceptibility gene for type 2 diabetes mellitus, especially in Asian populations. We previously reported that *Kcnq1* mutant mice exhibit reduced insulin secretion and hyperglycemia due to a decrease in pancreatic β -cell mass. Through *in vivo* and *in vitro* analyses, we ascertained that this mechanism is the result of the downregulation of the non-coding RNA '*Kcnq1ot1*,' which is expressed in the paternal allele of the *Kcnq1* gene region, causing an increase in the expression of the cell cycle inhibitor cyclin dependent kinase inhibitor 1C (Cdkn1c). It was found that decreased *Kcnq1ot1* expression resulted in pancreatic β -cell failure; however, the degree of pancreatic β -cell volume reduction was not severe.

Methods: We induced obesity in *Kcnq1ot1* truncation mice by feeding them a high-fat diet and evaluated pancreatic β -cell mass.

Results: In the present study, we reveal that CCAAT/enhancer binding protein beta (C/EBP β), which is expressed at higher levels in pancreatic β -cells in obese individuals, further increases the expression of Cdkn1c, which is upregulated by the *Kcnq1* gene mutation. We found that simultaneous Cdkn1c hypomethylation and C/EBP β overexpression in pancreatic β -cells causes a synergistic decrease in pancreatic β -cell mass.

Conclusion: This finding suggests that the synergistic effect of genetic factors such as *Kcnq1* gene mutations and environmental factors such as obesity and overeating, which lead to increased expression of C/EBP β , contribute to the regulation of pancreatic β -cell mass. This study is the first to show that the *Kcnq1* gene is related to pancreatic β -cell mass through genetic-environment interactions.

Keywords: CCAAT-enhancer-binding protein-beta; Cyclin-dependent kinase inhibitor p57; Diabetes mellitus; Gene-environment interaction; Insulin-secreting cells; KCNQ1OT1 RNA

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Received: Dec. 5, 2024; Accept: Dec. 30, 2024

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INTRODUCTION

The global population of patients with type 2 diabetes mellitus (T2DM) continues to grow at a rapid pace, presenting a serious challenge. In 2008, a genome-wide association study (GWAS) of East Asians found that single nucleotide polymorphisms (SNPs) in intron 15 of the potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*) gene on chromosome 11 were significant risk factors for T2DM [1,2]. Subsequent reports from institutions around the world have shown that populations with SNPs in the *KCNQ1* gene exhibit decreased insulin secretion [3,4]. *KCNQ1* is involved in the construction of voltage-gated channels and may also be involved in repolarization in cardiomyocytes [5]. It is known that *KCNQ1* and its neighboring genes are imprinted genes whose expression is regulated by a long non-coding RNA ‘KCNQ1 opposite strand/antisense transcript 1 (*KCNQ1OT1*)’ expressed within the *KCNQ1* gene region, and that *KCNQ1* is expressed only in the maternal allele [6].

In a previous mouse study, we found that a mutation in the paternal allele of the *Kcnq1* gene region decreased the expression of *Kcnq1ot1* in pancreatic islets and increased the expression of one of its neighboring genes, cyclin dependent kinase inhibitor 1C (*CDKN1C*) [7]. Also known as p57, *CDKN1C* is a cell cycle inhibitor, and its upregulation caused a decrease in pancreatic β -cell volume and impaired glucose tolerance [8].

It remains unclear whether there is a correlation between the *Kcnq1* mutations and SNPs we used, but it is possible that the onset of T2DM is related to changes in the expression levels of *Kcnq1ot1* and *Cdkn1c*. However, in the previous paper, the decrease in pancreatic β -cell volume in *Kcnq1* mutant mice was about 30%, which did not affect normal blood glucose levels [7]. In other words, the effect of *Cdkn1c* expression in pancreatic β -cells on glucose tolerance remains poorly understood. Recently, gene-environment interactions have been attracting increasing attention. Epigenetics is a representative phenomenon, and it has been reported that the prenatal environment and other factors can alter gene expression [9]. In adults, it has been reported that obesity and diet alter gene expression and molecular activity [10,11]. However, there are few reports on the association between disease susceptibility genes and environmental factors. Since World War II, the “westernization of food” has led to changes in the types and amounts of nutrients consumed in East Asia. Although caloric intake has not changed substantially, a large increase in lipid intake has been reported

[12,13]. The recent explosion in the population of people with T2DM in East Asia may be due in large part to this increased intake of lipids.

In this study, we focused on *Kcnq1ot1*, which regulates the expression of the *Kcnq1* and its neighboring genes, and examined the effects of a high-fat diet (HFD) on pancreatic β -cell mass and glucose tolerance using *Kcnq1ot1* truncation mice, in which *Kcnq1ot1* expression is decreased. The results revealed a significant effect on pancreatic β -cell mass and glucose tolerance. In addition, the transcription factor CCAAT/enhancer binding protein beta (C/EBP β), which accumulated in islets because of the HFD, was found to bind to the *CDKN1C* promoter with a loose chromatin structure, resulting in increased expression of p57 and a marked reduction in pancreatic β -cell volume. It was shown that the simultaneous downregulation of *Kcnq1ot1* expression (a genetic factor), and accumulation of C/EBP β (an environmental factor), synergistically reduced pancreatic β -cell volume. This mechanism of pancreatic β -cell insufficiency may have contributed to the recent explosion in the population of East Asian patients with T2DM.

METHODS

Mice

Kcnq1ot1 truncation mice and β -cell-specific p57 knockout (KO) mice were generated as previously reported [7,14–16]. JF1 mice were provided by Dr. Shiroishi of the National Institute of Genetics. Pancreatic β -cell-specific HA-tagged C/EBP β transgenic (TG) mice (Accession No. CDB0550T: <https://large.riken.jp/distribution/mutant-list.html>) were generated as follows. A pBlueScript vector containing the promoter of rat insulin 2 (*Ins2*) was provided by P.L. Herrera (University of Geneva Medical School, Geneva, Switzerland). We isolated a 3 $\times$ HA-tag and C/EBP β cDNA by polymerase chain reaction (PCR) amplification and inserted it into the *EcoRI* and *ClaI* sites of the vector downstream of the insulin gene promoter. The resulting construct was linearized and microinjected into pronuclear zygotes of C57BL/6N mice. The resulting offspring were screened for transgene transmission by PCR analysis, with forward primer (5'-CTGGTCATCATCCTGCCTTT-3'), reverse primer (5'-CACTTCCATG-GGTCTAAAGG-3'). The PCR amplicon size was approximately 200 bp. The study protocol, which conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines,

was approved by the Institutional Animal Care and Use Committee of RIKEN Kobe Branch and the Animal Ethics Committee of Kobe University Graduate School of Medicine (Approval No. P200905). For complete details regarding the mice, please see the Supplementary Methods.

Analytical procedures

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described [17]. Tail vein blood samples were obtained to determine blood glucose and plasma insulin concentrations in the mice. RNA extracted from islets pooled from three to five mice was subjected to reverse transcription and real-time PCR analysis as previously described [18]. Lysates of isolated islets were prepared as described previously [19] and probed with antibodies to CDKN1C (Santa Cruz Biotechnology, Dallas, TX, USA), C/EBP β (Santa Cruz Biotechnology), HA (Roche, Basel, Switzerland), and β -actin (Sigma-Aldrich, St. Louis, MO, USA). After sectioning of the pancreas, quantitation of β -cell mass was performed as previously described [20]. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed to detect proliferating cells. Then, proliferation and apoptosis in pancreatic β -cells were determined.

To confirm that C/EBP β binds to the *Cdkn1c* promoter *in vivo*, MIN6 cells were assayed for luciferase activity at 48 hours after transfection, as previously reported [15]. To examine DNA methylation in the *Cdkn1c* promoter region of truncated paternal (TP) mice and wild-type (WT) mice, the quality and quantity of genomic DNA was assessed as previously described [17]. MIN6 cells were cultured, and an analysis of epigenetic control and post-translational modifications was performed as previously described [7,17]. For complete details of the analytical procedures, please see the Supplementary Methods.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean. Comparisons were performed using analysis of variance followed by a two-tailed Student's *t*-test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Interaction between *Cdkn1c* promoter demethylation and increased C/EBP β expression in pancreatic β -cells

We previously investigated pancreatic β -cell mass and glucose

tolerance in mice with polyA-truncated *Kcnq1ot1* [7]. The results showed that *Kcnq1ot1* expression in pancreatic islets was decreased in TP mice with polyA inherited from the father compared with truncated maternal (TM) mice with polyA inherited from the mother as well as WT mice [14], and that they also showed decreased pancreatic β -cell volume and impaired glucose tolerance [7]. TP mice showed increased *Cdkn1c* expression in islets, which was thought to be due to a disruption in imprinting caused by decreased *Kcnq1ot1* expression. To confirm the occurrence of imprinting dysregulation in the islets of TP mice with truncated *Kcnq1ot1* in the paternal allele, we mated male C57B6 strain *Kcnq1ot1* truncation mice with female JF1 strain WT mice. Then, we extracted RNA from islets isolated from the litters and performed cDNA sequencing analysis of *Cdkn1c* (Fig. 1A). The results showed that *Cdkn1c* cDNA was reverse-transcribed from the isolated islet RNA of TM mice and that WT mice expressed maternal JF1 lineage-derived cDNA, which is originally expressed by imprinting (Fig. 1B). Meanwhile, in the *Cdkn1c* cDNA from the isolated islet RNA of TP mice, the sequencing results revealed that both paternal B6-derived and maternal JF1-derived cDNAs were expressed. In other words, imprinting regulation is disrupted in the islets of TP mice, and *Cdkn1c* is expressed in the paternal allele, which does not normally express *Cdkn1c* (Fig. 1B). Next, to analyze epigenomic regulation in the islets of *Kcnq1ot1* truncation mice, we analyzed histone modifications. *Kcnq1ot1* recruits EZH2, a histone methyltransferase responsible for H3K27 trimethylation, to the promoter region of the *Kcnq1* locus [21]. In other words, *Kcnq1ot1* functions to repress the expression of the paternal allele gene cluster via H3K27 trimethylation of the promoter in the *Kcnq1* gene region. Therefore, we used ChIP assay to examine H3K27 trimethylation at the *Cdkn1c* promoter using islets of TP, TM, and WT mice. We found that H3K27 trimethylation at the *Cdkn1c* promoter was decreased in islets from TP mice compared with TM and WT mice (Fig. 1C). Similarly, H3K9/14 acetylation, a marker of transcription promotion, was confirmed by ChIP assay, and significantly increased acetylation was observed in TP (Fig. 1C). These results indicate that *Cdkn1c* expression is upregulated in the islets of TP mice via histone modification. Furthermore, because *Kcnq1ot1* has been reported to contribute to the recruitment of DNA methyltransferase (DNMT) [22], we used bisulfite sequence analysis to examine DNA methylation in the *Cdkn1c* promoter region of TP mice and WT mice. In the islets of WT mice, DNA methyla-

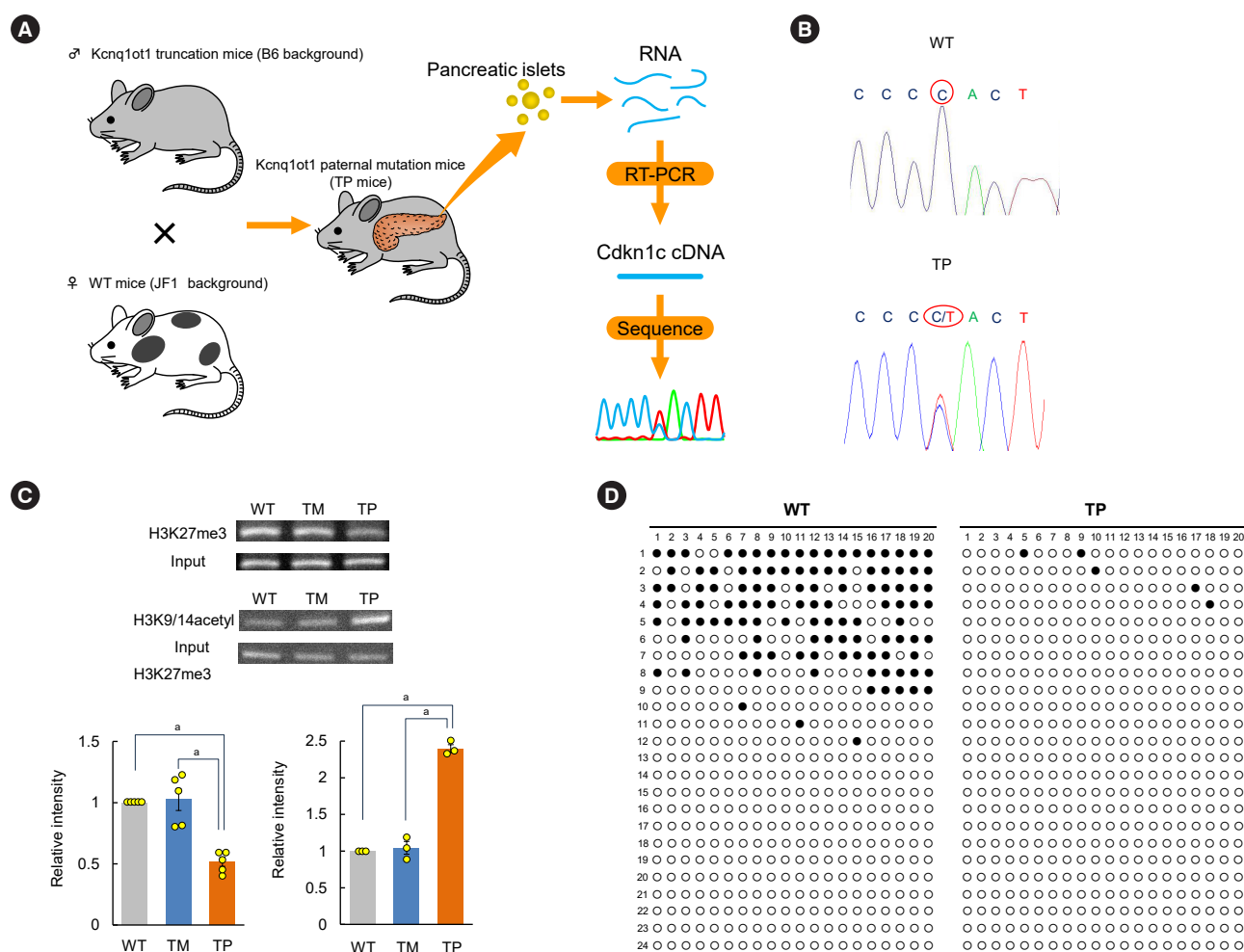


Fig. 1. Imprinting regulation in pancreatic islets of *KCNQ1* opposite strand/antisense transcript 1 (*Kcnq1ot1*) truncation mice. (A) Schema of detection of cyclin dependent kinase inhibitor 1C (*Cdkn1c*) imprinting in pancreatic islets. Embryos of *Kcnq1ot1* truncated paternal mice (TP mice) from wild-type (WT) JF1 female and *Kcnq1ot1* truncation B6 male parents have single nucleotide polymorphisms derived from their parents. (B) Sequence of *Cdkn1c* mRNA from pancreatic islets of WT mice and TP mice. (C) Chromatin immunoprecipitation (ChIP) analysis of *Cdkn1c* promoter with antibodies to trimethylated H3K27 (H3K27me3) or acetylated H3K9/14 in islets isolated from each mouse. (D) Bisulfite sequence of the *Cdkn1c* promoter from pancreatic islets isolated from each mouse. Black and white circles indicated methylated and unmethylated CpG, respectively. All quantitative data are mean $\pm$ standard error of the mean for (C) 3–5 mice in each group. RT-PCR, reverse transcription polymerase chain reaction; TM, truncated maternal. $^aP < 0.01$.

tion of the *Cdkn1c* promoter is thought to differ between paternal and maternal alleles. In fact, approximately 40% to 50% of cytosine-phosphate-guanine (CpG) regions in the islets of WT mice are methylated; these methylated CpG regions are considered paternal alleles, while the unmethylated CpG regions are considered maternal alleles (Fig. 1D). In contrast, the *Cdkn1c* promoter in the islets of TP mice has an overall unmethylated CpG region, suggesting that loss of *Kcnq1ot1* function affects DNA methylation in the paternal allele (Fig. 1D).

Cdkn1c is a cell cycle regulator and is thought to contribute to cell growth inhibition and apoptosis enhancement. It has been reported to play an important role in the regulation of pancreatic β -cell volume, and disruption of imprinting regulation by *KCNQ1* gene mutations might be an important initiator in the regulation of *Cdkn1c* expression. Sp1 is known to be a transcription factor for *Cdkn1c* expression [23], and the promoter region also contains a transcription factor the C/EBP family binding motif, the CCAAT sequence [24]. It is not known

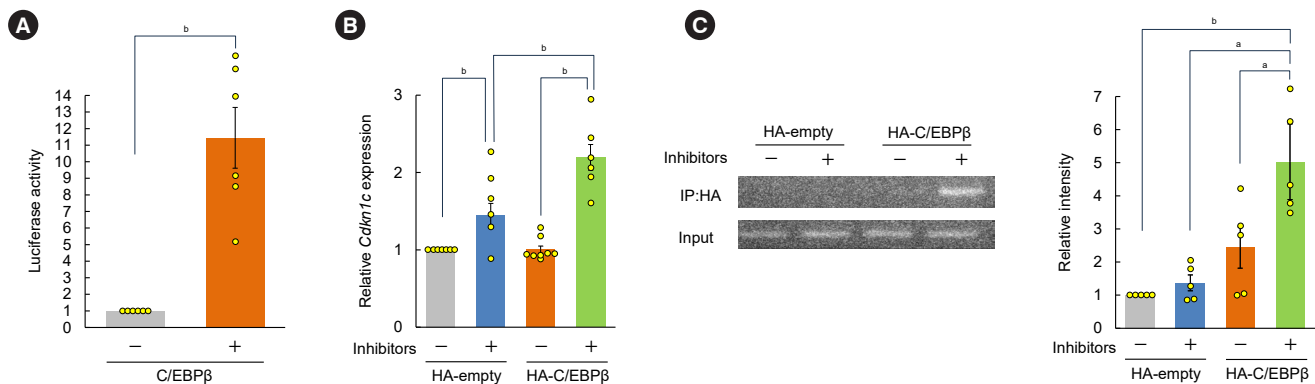


Fig. 2. Interaction of cyclin dependent kinase inhibitor 1C (*Cdkn1c*) expression between CCAAT/enhancer binding protein beta (C/EBPβ) and epigenetic regulation in pancreatic β-cell line. (A) Luciferase assays were performed to examine the effect of C/EBPβ on the transcription activation of *Cdkn1c* in MIN6 cells. (B) Quantitative reverse transcription polymerase chain reaction analysis of the effect of C/EBPβ with or without inhibitor cocktail (trichostatin A, 5-azacitidine, 3-deazaneplanocin A [Dznp]) on *Cdkn1c* expression in MIN6 cells. (C) Chromatin immunoprecipitation (ChIP) analysis of the binding of C/EBPβ to the *Cdkn1c* promoter in MIN6 cells. (Left) Representative and (right) quantitative data are shown. All quantitative data are mean ± standard error of the mean for (A) 6, (B) 6–8, or (C) 5 mice in each group. HA, hemagglutinin; IP, immunoprecipitation. ^a*P* < 0.05, ^b*P* < 0.01.

whether the C/EBP family increases *Cdkn1c* expression in pancreatic β-cells. The interaction between the *Cdkn1c* promoter and C/EBPβ was confirmed in the mouse pancreatic β-cell line MIN6 cells by luciferase assays, which showed that C/EBPβ overexpression enhanced luciferase activity (Fig. 2A). However, overexpression of C/EBPβ in MIN6 cells did not significantly alter *Cdkn1c* expression compared with controls (Fig. 2B). *Kcnq1ot1* recruits histone deacetylases (HDACs), histone methyltransferases, and DNMTs to each promoter in the *Kcnq1* gene region [21,22]. Thus, *Cdkn1c* expression was confirmed by simultaneously administering the following inhibitors of epigenome-modifying enzymes: HDAC inhibitors, histone methyltransferase inhibitors, and DNMT inhibitors. The results revealed that *Cdkn1c* expression was enhanced by administration of the inhibitor cocktail and was further enhanced by C/EBPβ overexpression (Fig. 2B). Next, we examined whether the inhibitor cocktail affected the binding of C/EBPβ to the *Cdkn1c* promoter. The ChIP assay indicated that the binding of C/EBPβ to the *Cdkn1c* promoter region was enhanced by the inhibitor cocktail (Fig. 2C). In other words, DNA demethylation and open chromatinization enhance transcription of *Cdkn1c* by the transcription factor C/EBPβ.

Effect of decreased *Kcnq1ot1* expression and increased C/EBPβ expression on pancreatic β-cell volume

In vitro studies have shown that simultaneous epigenetic dysregulation and upregulation of C/EBPβ expression leads to up-

regulation of *Cdkn1c* expression in pancreatic β-cells. Next, we tested whether the same phenomenon occurs *in vivo* by breeding TG mice. *Kcnq1ot1* truncated C/EBPβ-overexpressing (KT) mice were generated, and littermate TP mice, TG mice, and WT mice were used as controls. Blood glucose levels were measured randomly, and TG mice showed higher blood glucose levels compared with TP and WT mice. However, KT mice showed an even more pronounced hyperglycemia of >500 mg/dL despite their very light body weight (Fig. 3A and B). Next, serum insulin levels were measured at the age of 8 weeks. Consistent with the blood glucose data, TG mice showed lower serum insulin levels compared with WT and TP, while KT showed more markedly lower insulin levels (Fig. 3C). Measurements of pancreatic β-cell area and mass showed a 40% decrease in TP and TG compared with WT but an approximately 85% to 90% decrease in KT compared with WT, which may have been the cause of the decrease in insulin secretion (Fig. 3D and E). The *in vitro* analysis revealed that epigenetics modification changes mimicking decreased *Kcnq1ot1* expression increased *Cdkn1c* expression. Meanwhile, the *in vivo* analysis revealed that *Cdkn1c* expression was upregulated in KT at both the protein and mRNA levels (Fig. 3F and G). To elucidate the mechanism of the decrease in pancreatic β-cell volume in KT, we evaluated the proliferative capacity and occurrence of apoptosis in pancreatic β-cells. The results showed that Ki67-positive pancreatic β-cells were significantly decreased and TUNEL-positive pancreatic β-cells were increased

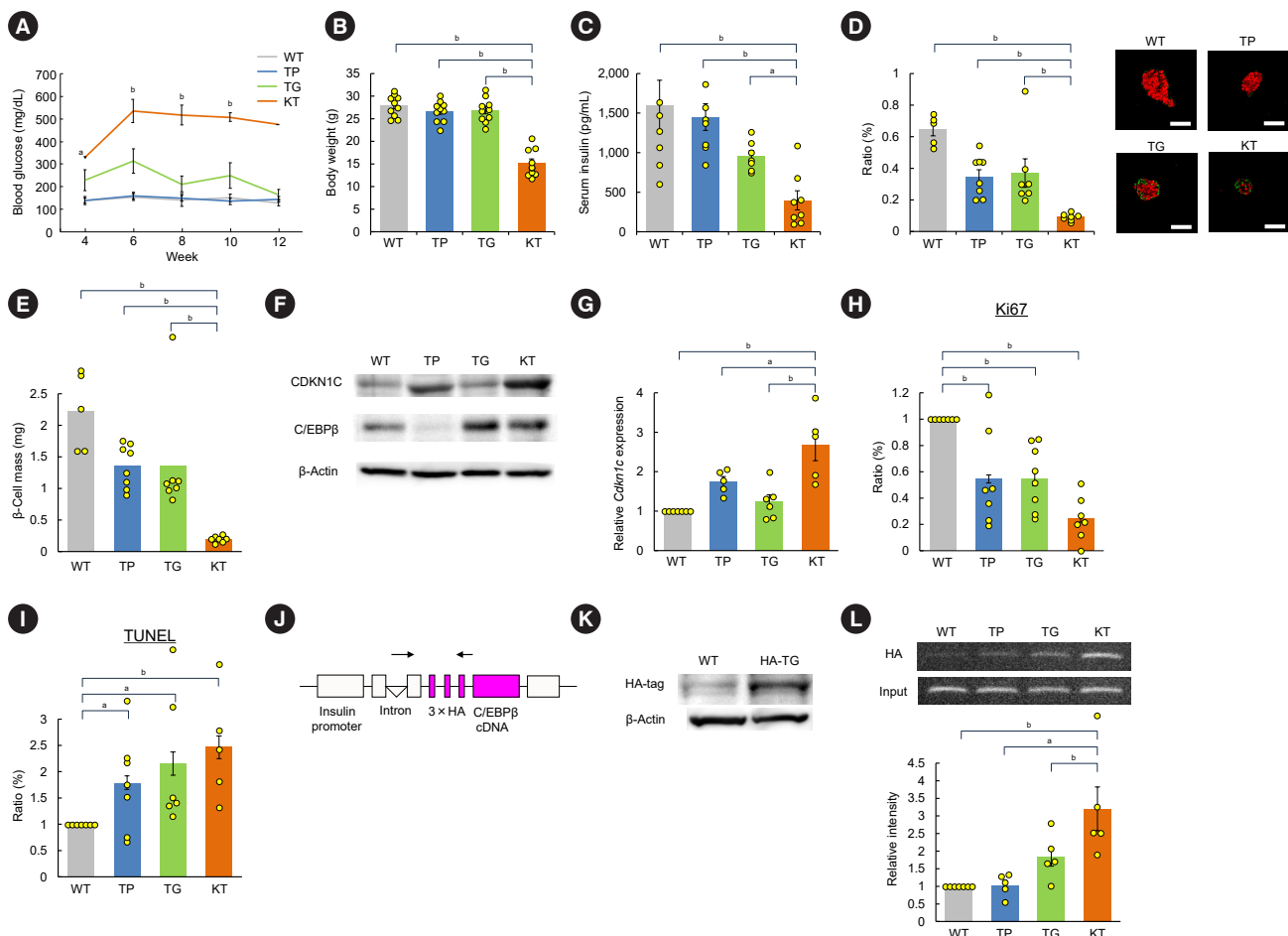


Fig. 3. Generation and analysis of KCNQ1 opposite strand/antisense transcript 1 (*Kcnq1ot1*) truncated CCAAT/enhancer binding protein beta (C/EBPβ)-overexpressing mice. (A) Blood glucose levels in the fed state for wild-type (WT), truncated paternal (TP), transgenic (TG), and *Kcnq1ot1* truncated C/EBPβ-overexpressing (KT) mice at various ages. (B) Body weight for each mouse at the age of 10 weeks. (C) Serum insulin concentrations in the fed state with normal chow diet (NCD) for each mouse at the age of 8 weeks. (D) Immunostaining of insulin (red) and glucagon (green) in pancreatic sections from each 10-week-old NCD-fed mouse. (Left) Quantitative and (Right) representative data are shown (scale bars: 50 μm). (E) Quantification of pancreatic β-cell mass in each 10-week-old mouse. (F) Immunoblot analysis of cyclin dependent kinase inhibitor 1C (CDKN1C) and C/EBPβ in islets isolated from each 10-week-old mouse. (G) Relative expression of *Cdkn1c* mRNA in islets isolated from each 10-week-old mouse. (H) Percentage of Ki67+ β-cells per islet in each 10-week-old mouse. (I) The percentage of apoptotic β-cells per islet in each mouse was determined using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. (J) Construct of pancreatic β-cell-specific hemagglutinin (HA)-tagged C/EBPβ transgenic mice. The arrows indicate sense and antisense primers. (K) Immunoblot analysis of HA in islets isolated from HA-tagged C/EBPβ transgenic mice. (L) Chromatin immunoprecipitation (ChIP) analysis of the *Cdkn1c* promoter with antibodies to HA-tag in islets isolated from each mouse. Representative (upper) and quantitative (lower) data are shown. All quantitative data are mean ± standard error of the mean for (A) 5–8, (B) 10, (C) 7–8, (D, E) 5–8, (G) 5–6, (H) 7–8, (I) 5–7, or (L) 5 mice in each group. ^a*P* < 0.05, ^b*P* < 0.01.

in KT compared with the control group (Fig. 3H and I).

Next, to confirm that C/EBPβ binds to the *Cdkn1c* promoter *in vivo*, we decided to perform a ChIP assay using isolated islets from KT mice. However, because we could not find a suitable C/EBPβ antibody to perform the ChIP assay, we generated mice

overexpressing HA-tagged C/EBPβ in pancreatic β-cells (Fig. 3J and K) and bred them with *Kcnq1ot1* truncated mice. Islets were isolated from the offspring of those mice, which we called *Kcnq1ot1* truncated HA-C/EBPβ-overexpressing (HA-KT) mice, and ChIP assay was performed using extracted chromatin

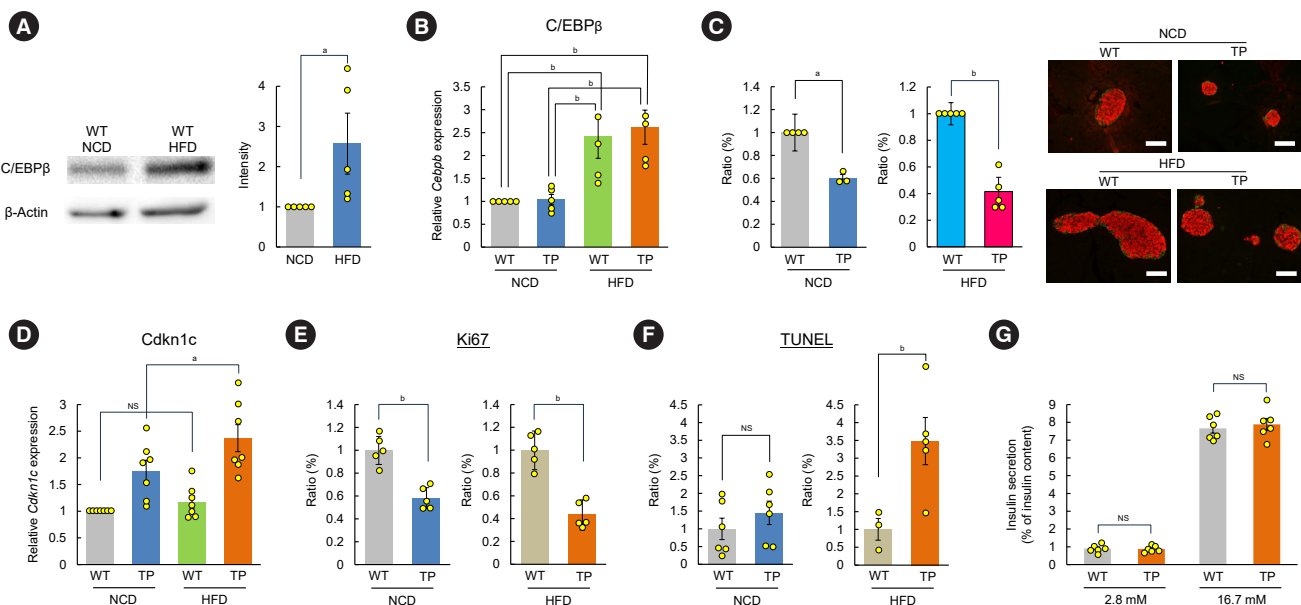


Fig. 4. Pancreatic β-cell mass in high-fat diet-fed truncated paternal (TP) mice was reduced by increased cyclin dependent kinase inhibitor 1C (*Cdkn1c*) expression via CCAAT/enhancer binding protein beta (C/EBPβ) expression. (A) Immunoblot analysis of C/EBPβ expression in pancreatic islets of high-fat diet (HFD)-fed wild-type (WT) mice. Representative (left) and quantitative (right) data are shown. (B) Relative expression of C/EBPβ mRNA in islets isolated from WT and TP with normal chow diet (NCD) or HFD. (C) Immunostaining of insulin (red) and glucagon (green) in pancreatic sections from WT and TP with NCD or HFD. Quantitative (left) and representative (right) data are shown (scale bars: 50 μm). (D) Relative expression of *Cdkn1c* mRNA in islets isolated from WT and TP with NCD or HFD. (E) Percentage of Ki67+ β-cells per islet in each 10-week-old mouse. (F) The percentage of apoptotic β-cells per islet in each mouse was determined using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. (G) Insulin secretion by pancreatic islets isolated from 10-week-old HFD-fed WT and TP mice and incubated for 30 minutes with 2.8 or 16.7 mM glucose. Data were normalized by islet insulin content. All quantitative data are mean ± standard error of the mean for (A) 5, (B) 5, (C) 4–5, (D) 7, (E) 5, (F) 3–6, or (G) 6 mice in each group. NS, not significant. ^a*P* < 0.05, ^b*P* < 0.01.

DNA. The results indicated that, similar to the *in vitro* analysis, C/EBPβ and *Cdkn1c* promoter were significantly bound in HA-KT mice compared with the control group (Fig. 3L).

HFD-fed *Kcnq1ot1* truncated mice exhibit decreased pancreatic β-cell mass via increased C/EBPβ and *Cdkn1c* expression

The pathogenesis of T2DM is reported to be caused by both genetic and environmental factors [25]. If the *KCNQ1* gene mutation is a genetic factor, then a typical environmental factor would be diet. Although we have previously shown that C/EBPβ accumulates in the islets of diabetic model mice [15], it was not clear whether C/EBPβ expression is altered in the islets of HFD-loaded mice without elevated blood glucose levels. In the present study, we confirmed the expression level by immunoblot and real-time PCR, and found that C/EBPβ expression was upregulated in the islets of HFD-fed mice at both the pro-

tein and mRNA levels (Fig. 4A and B). *Kcnq1ot1* truncated TP mice showed a 40% decrease in pancreatic β-cell volume compared with WT mice fed a normal chow diet (NCD) (Fig. 4C) [7], while TP fed a HFD showed an enhanced rate of decrease in pancreatic β-cell volume, specifically, a 60% decrease (Fig. 4C). Because *Cdkn1c* is thought to be a causative molecule for the decrease in pancreatic β-cell volume, we confirmed the expression level of *Cdkn1c* in pancreatic islets. *Cdkn1c* expression was upregulated in the islets of TP fed a NCD compared with WT mice, but *Cdkn1c* expression was further increased in the islets of TP fed a HFD (Fig. 4D). Ki67 staining revealed that the proliferative potential of HFD-fed TP islets was markedly decreased compared with the control group. Ki67 staining revealed that the islet proliferative capacity of HFD-fed TP islets was markedly decreased compared with the control group (Fig. 4E). TUNEL staining also showed increased apoptosis in the islets of TP mice fed a HFD, suggesting that the amount of

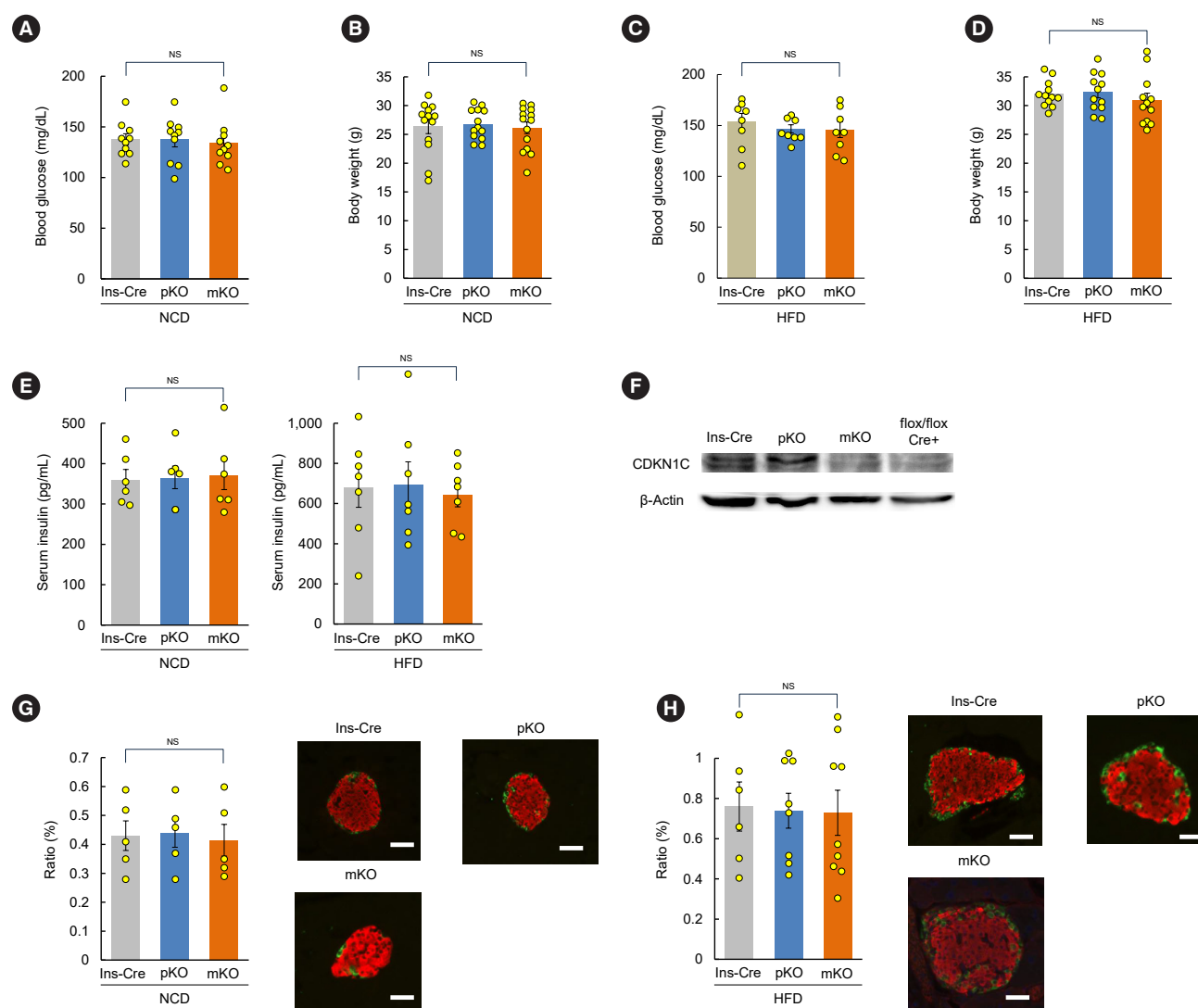


Fig. 5. Analysis of metabolic data and pancreatic β -cell mass in pancreatic β -cell-specific cyclin dependent kinase inhibitor 1C (*Cdkn1c*) knockout mice. (A) Blood glucose levels in pancreatic β -cells of paternal *Cdkn1c* heterozygous knockout (pKO) mice, maternal *Cdkn1c* heterozygous knockout (mKO) mice in pancreatic β -cells, and Ins-Cre mice fed a normal chow diet (NCD) at the age of 8 weeks. (B) Body weight for each mouse fed NCD at the age of 8 weeks. (C) Blood glucose levels for each mouse fed a high-fat diet (HFD) at the age of 10 weeks. (D) Body weight for each mouse fed a HFD at the age of 8 weeks. (E) Serum insulin concentrations in mice fed a NCD at the age of 8 weeks (left) or HFD at the age of 10 weeks (right). (F) Immunoblot analysis of CDKN1C expression in islets isolated from each mouse. (G, H) Immunostaining of insulin (red) and glucagon (green) in pancreatic sections from each mouse fed a NCD (G) or HFD (H). Quantitative (left) and representative (right) data are shown (scale bars: 50 μ m). All quantitative data are mean $\pm$ standard error of the mean for (A) 10, (B) 13–14, (C) 8, (D) 12, (E) 6–7, (G) 5, or (H) 6–9 mice in each group. NS, not significant.

pancreatic β -cells was decreased by these mechanisms (Fig. 4F). Finally, we analyzed insulin secretion using isolated islets from HFD-loaded TP and WT mice. Static incubation of pancreatic islets isolated from TP mice revealed no difference in the degree of basal or stimulated insulin secretion compared with islets from WT mice (Fig. 4G).

Decreased pancreatic β -cell mass in HFD-loaded *Kcnq1ot1* truncated mice is restored by *Cdkn1c* deletion

It is clear that when *Cdkn1c* expression is increased in pancreatic β -cells, apoptosis is induced in pancreatic β -cells, resulting in a decrease in pancreatic β -cell volume. However, the effect of decreased *Cdkn1c* expression in pancreatic β -cells is not well

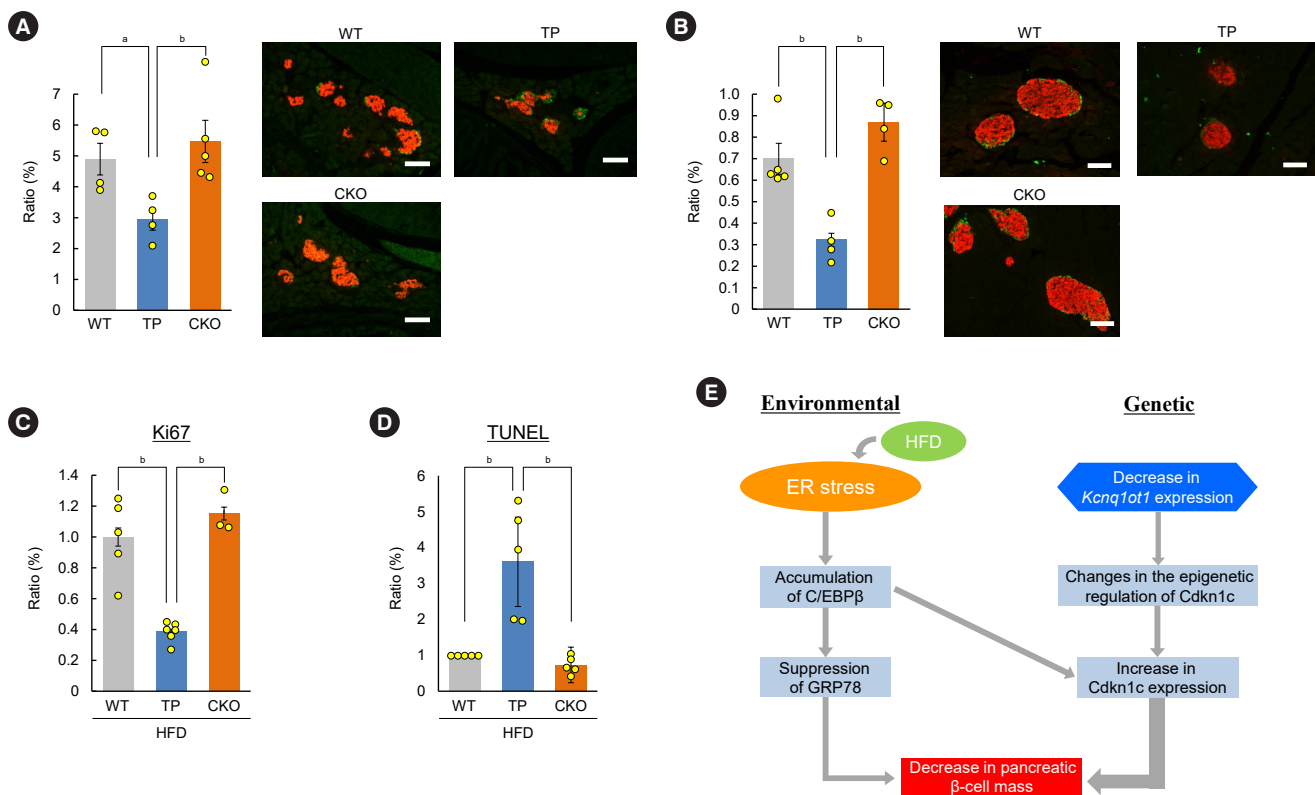


Fig. 6. Effect of reduced expression of cyclin dependent kinase inhibitor 1C (*Cdkn1c*) in pancreatic β-cells of KCNQ1 opposite strand/antisense transcript 1 (*Kcnq1ot1*) truncated mice on pancreatic β-cell mass. (A, B) Immunostaining of insulin (red) and glucagon (green) in pancreatic sections from wild-type (WT), truncated paternal (TP), and TP with *Cdkn1c* heterozygous knockout mice (CKO) at the birth stage (A) and with a high-fat diet (HFD) (B). Quantitative (left) and representative (right) data are shown (scale bars: 50 μm). (C) Percentage of Ki67+ β-cells per islet in each HFD-fed mouse. (D) Percentage of apoptotic β-cells per islet in each HFD-fed mouse was determined using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. (E) Model of the mechanism of pancreatic β-cell mass reduction by the interaction of reduced *Kcnq1ot1* expression and environmental factors. All quantitative data are mean ± standard error of the mean for (A) 4–5, (B) 4–5, (C) 3–6, or (D) 5 mice in each group. ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78. ^a*P* < 0.05, ^b*P* < 0.01.

understood. To investigate whether the decreased *Cdkn1c* expression in pancreatic β-cells affects pancreatic β-cell volume and glucose metabolism, we generated and analyzed pancreatic β-cell-specific *Cdkn1c* KO mice. *Cdkn1c* is an imprinted gene and is expressed only from maternal alleles after birth [26]. Therefore, we classified pancreatic β-cell-specific *Cdkn1c* KO mice into maternal *Cdkn1c* heterozygous KO mice (mKO), in which only the maternal allele lacks *Cdkn1c*, and paternal *Cdkn1c* heterozygous KO mice (pKO), in which only the paternal allele lacks *Cdkn1c*. Ins-Cre mice were used as controls for the analysis. Blood glucose and body weight were measured when the mice were fed a NCD, and no changes were observed among the three groups at the age of 8 weeks (Fig. 5A and B). In addition, glucose levels and body weights were also

measured in each group fed a HFD, and no significant differences were found among the three groups (Fig. 5C and D). Serum insulin levels in each group were also compared when the mice were fed a NCD and a HFD, but again no differences were found under either condition (Fig. 5E). To confirm the expression level of *Cdkn1c* in islets, Western blotting was performed using isolated islets from each mouse, and the results showed that *Cdkn1c* expression was decreased in mKO (Fig. 5F). Next, the amount of pancreatic β-cells in each group was measured when the mice were fed a NCD, and no significant differences were found among the groups (Fig. 5G). Furthermore, pancreatic β-cell volume was also measured in the three groups fed a HFD, and again no significant differences were found (Fig. 5H). These results may be due to the fact that other

cell cycle inhibitors compensate for *Cdkn1c* deficiency. To confirm that the decrease in pancreatic β -cell volume in TP was due to increased *Cdkn1c* expression, we subsequently generated TP with *Cdkn1c* heterozygous KO mice (CKO), in which *Cdkn1c* in the maternal allele of TP mice was deleted. CKO with reduced *Cdkn1c* expression in the pancreatic β -cells of TP showed recovery of pancreatic β -cell volume both at the birth stage or with a HFD (Fig. 6A and B). Finally, we confirmed proliferation and apoptosis in the pancreatic β -cells of these mice. Ki67, which was decreased in TP pancreatic β -cells, was restored to the same level as WT in CKO, while TUNEL-positive pancreatic β -cells, which were increased in TP, were predominantly decreased in CKO (Fig. 6C and D).

DISCUSSION

The global population of patients with T2DM continues to increase, exerting negative impacts on the quality of life and life expectancy for each patient as well as creating economic problems for society due to rising healthcare costs [27,28]. The pathogenesis of many diseases is believed to be due to genetic factors, environmental factors, or both. In T2DM in particular, both of these factors have a significant influence on pathogenesis, and the ratio of their contribution varies from patient to patient. Therefore, this study was conducted to elucidate the mechanism by which these factors exert a synergistic effect instead of an additive one.

Previous GWASs have reported numerous susceptibility genes for T2DM [29,30]. Given that the pathogenesis of diabetes differs significantly between Asians and Caucasians, GWASs should be performed for each ethnic group. Many previous GWASs have mixed Caucasian and Asian samples, with Caucasian samples accounting for the dominant share. With this in mind, meta-analyses conducted according to ancestry group have revealed large differences in T2DM susceptibility genes among ethnic groups [31]. In recent years, some GWASs have been reported by ethnicity, and a GWAS on 400,000 East Asians was reported in 2020 [32]. The present study shows that certain signals can cause different pathologies in different tissues, reaffirming the importance of genetic factors in the pathogenesis of T2DM. In addition, recent reports have linked GWAS data to diabetes-induced cardiovascular disease and to cell-specific phenotypes, shedding light on the heterogeneity of the pathogenesis of T2DM [33]. Thus, the continued study of T2DM susceptibility genes is expected to lead to a better un-

derstanding of the pathophysiology of individual diabetes in the future.

KCNQ1 is a voltage-gated potassium channel that contributes to cellular repolarization [34]. In 2008, Yasuda et al. [1] reported that SNPs in the 15th intron of the *KCNQ1* gene are associated with the development of T2DM in East Asians, including Japanese and Chinese. This result was subsequently replicated in a GWAS of Europeans [35], and many laboratories have also reported decreased insulin secretory capacity in groups with the corresponding risk allele [3,4]. We previously reported that *KCNQ1* mutations in pancreatic β -cells upregulate expression of the cell cycle inhibitor CDKN1C via disruption of imprinting regulation [7]. Although it remains unclear whether there is a correlation between *KCNQ1* SNP and *KCNQ1OT1* or *CDKN1C* expression in human islets, it was previously reported that *CDKN1C* expression is enhanced in the risk allele of *KCNQ1* SNP rs163184, using pancreatic beta cell lines *in vitro* [36]. In addition, although increased CDKN1C expression in pancreatic β -cells was found to decrease pancreatic β -cell volume, the decrease in pancreatic β -cell volume in *Kcnq1ot1* truncation mice on a NCD was only about 30% and there was no significant difference in blood glucose levels under normal conditions [7]. If CDKN1C is a major contributor to the pathogenesis of diabetes, there may be additional mechanisms by which CDKN1C expression is increased. For example, we noted the presence of a CCAAT motif in the CDKN1C promoter.

The CCAAT motif is a binding motif of the C/EBP family of transcription factors, and C/EBP β is reported to bind to the CCAAT motif in chondrocytes and induce differentiation via transcriptional activation of *Cdkn1c* [37]. It has been previously shown that C/EBP β expression is upregulated in pancreatic β -cells under endoplasmic reticulum stress conditions [38]. Furthermore, we previously reported that accumulation of C/EBP β in pancreatic β -cells inhibits adaptive unfolded protein response and induces apoptosis of pancreatic β -cells by down-regulating glucose-regulated protein 78 (GRP78) expression [15]. In other words, C/EBP β accumulation is thought to contribute to the decrease in pancreatic β -cell volume in diabetic pancreatic β -cells, and in fact, C/EBP β expression is upregulated in pancreatic β -cells from T2DM patients [39]. Although it has been reported that C/EBP β accumulates in pancreatic β -cells due to environmental factors, there have been no reports on the relationship between C/EBP β and genetic factors of T2DM. We therefore hypothesized that C/EBP β , which accumulates in pancreatic β -cells due to environmental factors

such as a HFD and obesity, may affect *CDKN1C* expression via the *KCNQ1* gene abnormality, which are genetic factors. Accordingly, we performed both *in vitro* and *in vivo* experiments to simultaneously induce decreased *Kcnq1ot1* expression, which regulates imprinting, as well as C/EBP β overexpression in the cells. In both experiments, the DNA demethylation state facilitated binding of C/EBP β to the *Cdkn1c* promoter, indicating that it enhances *Cdkn1c* expression. We have previously reported that, in *Kcnq1ot1*-truncated mice, *Cdkn1c* expression is upregulated specifically in pancreatic β -cells in association with reduced *Kcnq1ot1* expression [7]. Additionally, in the present study, we found that *Cdkn1c* expression was further upregulated in pancreatic β -cells of KT mice due to C/EBP β overexpression. In the future, we would like to confirm this mechanism by conducting a recovery experiment on the phenotype by crossing pancreatic β -cell-specific *Cdkn1c* KO mice with KT mice.

Cdkn1c, which is also called p57, is a cell cycle inhibitor, and its increased expression is known to decrease pancreatic β -cell mass [8]. The results of the present may suggest that the risk of developing T2DM due to the presence of SNPs in the *KCNQ1* gene may be synergistically increased by interaction with environmental factors (Fig. 6E).

Although diet and eating habits contribute substantially to environmental factors, there are not many reports on interactions with genetic factors. A GWAS of East Asians reported that polymorphisms in the aldehyde dehydrogenase 2 family member (*ALDH2*) gene, which encodes an enzyme related to alcohol metabolism, significantly increased the risk of developing T2DM only in men [32]. The *ALDH2* gene polymorphism increases alcohol tolerance, and thus heavy alcohol use increases the risk of developing T2DM. This mechanism is thought to be due not only to increased caloric intake but also to reduced glucose clearance due to decreased insulin sensitivity in the liver caused by heavy alcohol intake [40]. In addition, it has been shown that DNA methylation in adipocytes is altered when diet-induced obesity such as that caused by a HFD is present [41]. This phenomenon has been observed in both humans and mice. Integrative analysis of DNA methylation with T2DM susceptibility genes identified by GWAS identified AKT serine/threonine kinase 2 (*Akt2*) and tumor necrosis factor α -induced protein 8-like 2 (*Tnfrsf8*) as candidate molecules. These molecules are thought to contribute to the development of T2DM because they cause insulin resistance by suppressing its function in adipocytes. It is also well known that

Tcf7l2 risk alleles increase the risk of developing T2DM, and associations with decreased insulin secretion and decreased incretin secretion have been reported as mechanisms [42,43]. A cohort study reported that the increased risk of developing diabetes due to *Tcf7l2* was inversely associated with dietary fiber intake [44]. Solute carrier family 30 member 8 (*SLC30A8*) encodes a zinc transporter and was similarly identified as a susceptibility gene for T2DM by a GWAS [45], and carriers of the risk allele are reported to have elevated fasting blood glucose and decreased insulin secretion [46]. Mice lacking *ZnT8*, a protein encoded by *Slc30a8*, and overexpressing human islet amyloid polypeptide were fed a HFD and developed pancreatic β -cell failure [47]. The presence of mutations in T2DM susceptibility genes under metabolic stress is thought to dramatically increase the risk of developing diabetes.

SUPPLEMENTARY MATERIALS

Supplementary materials related to this article can be found online at <https://doi.org/10.4093/dmj.2024.0790>.

CONFLICTS OF INTEREST

Yoshiaki Kido has been an International editorial board members of the *Diabetes & Metabolism Journal* since 2022. He was not involved in the review process of this article. Otherwise, there was no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception or design: S.A., H.I., M.M., H.I., K.I.N., W.O., M.K., Y.K.

Acquisition, analysis, or interpretation of data: all authors.

Drafting the work or revising: S.A., H.I., M.M., H.I., M.K., Y.K.

Final approval of the manuscript: S.A., M.K., Y.K.

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FUNDING

This research was supported by Grants-in-Aid for Creative Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (17H01966 and 21K08579 to

Yoshiaki Kido; 17K09882, 18KK0438, and 20K08860 to Shun-ichiro Asahara; 20K08906 and 23K07990 to Maki Kimura-Koyanagi; and 20K17535 and 22K08653 to Ayumi Kanno). The funders had no role in study design, data collections and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank H. Etoh, A. Tanizawa, and K. Miyazaki for technical assistance.

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SUPPLEMENTARY METHODS

Mice

KCNQ1 opposite strand/antisense transcript 1 (*Kcnq1ot1*) truncation mice [1,2] were generated by the insertion of a poly (A) cassette 2.6 kb downstream of the *Kcnq1ot1* promoter by homologous recombination in embryonic stem cells. β -Cell-specific CCAAT/enhancer binding protein beta (C/EBP β) transgenic mice were as previously described [3]. β -Cell-specific p57 knockout mice were generated by crossing p57 floxed mice [4] with Ins-Cre mice. JF1 mice were provided by Dr. Shiroishi of the National Institute of Genetics. Pancreatic β -cell-specific hemagglutinin (HA)-tagged C/EBP β transgenic mice (Accession No. CDB0550T: <https://large.riken.jp/distribution/mutant-list.html>) were generated as follows. A pBlueScript vector containing the promoter of rat insulin 2 (Ins2) was provided by P.L. Herrera (University of Geneva Medical School, Geneva, Switzerland). We isolated a 3 $\times$ HA-tag and C/EBP β cDNA by polymerase chain reaction (PCR) amplification and inserted it into the EcoRI and ClaI sites of the vector downstream of the insulin gene promoter. The resulting construct was linearized and microinjected into pronuclear zygotes of C57BL/6N mice. The resulting offspring were screened for transgene transmission by PCR analysis. All mice were fed a normal chow diet unless indicated otherwise.

ChIP analysis

Chromatin immunoprecipitation (ChIP) analysis was performed with the use of a Magna ChIP G Kit (Merck Millipore, Burlington, MA, USA). In brief, islets were fixed with 1% formaldehyde for 30 minutes at room temperature and then subjected to ultrasonic disruption in a solution containing a protease inhibitor cocktail. The lysates were centrifuged to remove debris; diluted 1:10 with a solution containing 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl; and incubated for 2 hours at 4°C with protein G-Sepharose beads. The beads were removed by centrifugation, and the supernatants were subjected to immunoprecipitation by incubation at 4°C overnight with antibodies to trimethylated H3K27 (Merck Millipore) or acetylated H3K9/K14 (Cell Signaling Technology, Danvers, MA, USA) or HA (Roche, Basel, Switzerland), or with normal mouse immunoglobulin G (Santa Cruz Biotechnology, Dallas, TX, USA), and then for 1 hour with protein G-Sepharose. The precipitates were washed and then subjected to extraction for 4

hours at 65°C with 1% sodium dodecyl sulfate (SDS) in 100 mM NaHCO₃. Proteins were digested with proteinase K, and the remaining DNA was purified with the use of a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and subjected to PCR with cyclin dependent kinase inhibitor 1C (*Cdkn1c*)-specific primers (sense, 5'-CAGAGCTCTTTAACTCGAGC-3'; antisense, 5'-TTCA-GTTTCAACAACACCGC-3').

Analysis of metabolic parameters

Blood samples were collected from the tail vein of mice, and plasma was isolated by centrifugation of blood for 15 minutes at 4°C in a microcentrifuge. Blood glucose concentration was determined with a glucometer (Glutest Pro, Sanwa Kagaku Kenkyusho, Nagoya, Japan), and plasma insulin concentration was measured using an enzyme-linked immunosorbent assay kit with the use of a mouse insulin standard (Shibayagi, Shibukawa, Japan).

Reverse transcription and real-time PCR analysis

Total RNA was extracted from islets pooled from three to five mice, using an RNeasy kit (Qiagen). The RNA was subjected to reverse transcription and real-time PCR analysis as previously described [5]. The cDNA synthesized from the RNA was analyzed in a sequence detector (model 7500, Applied Biosystems, Foster City, CA, USA) with specific primers and SYBR Green PCR Master Mix (Qiagen). The relative abundance of each mRNA was calculated with cyclophilin mRNA as the invariant control. Primers (sense and antisense, respectively) were as follows: cyclophilin, 5'-CAGACGCCACTGTCGCTTT-3' and 5'-TGTCTTTGGAAGTTTGTCTGCAA-3'; *Cdkn1c*, 5'-CAGGACGGAATCAAGAGCAG-3' and 5'-GAAGAAGTCGTTCGCATTGGC-3'; C/EBP β , 5'-ACCGGGTTTCGGGACTTGA-3' and 5'-GTTGCGTAGTCCCGTGTCCA-3'.

Immunoblot analysis

Lysates of isolated islets were prepared as previously described [6] and probed with antibodies to CDKN1C (Santa Cruz Biotechnology), C/EBP β (Santa Cruz Biotechnology), HA (Roche), and β -actin (Sigma-Aldrich, St. Louis, MO, USA).

Determination of β -cell mass

The pancreas was immersed in Bouin's solution, embedded in paraffin, and sectioned at a thickness of 4 to 5 μ m. Sections of paraffin-embedded pancreas were immunostained at 200- μ m intervals to avoid measurement of the same islets twice. Sec-

tions were stained with antibodies to insulin and glucagon (Dako, Carpinteria, CA, USA), and immune complexes were detected with secondary antibodies conjugated with Cy3 and fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), respectively. Images of β -cells and of the entire pancreas were obtained using a fluorescence microscope (Biozero, Keyence, Osaka, Japan) and analyzed using WinROOF software (Mitani Corp., Tokyo, Japan), as described previously [7]. To determine the areas of β -cells, the entire pancreatic section was imaged, together with the total area of the pancreas, excluding the interlobular connective tissue, large blood vessels, and adipocytes. The immunoreactive area for insulin was determined, and the β -cell area was expressed as percentages of the total pancreatic area. The mass of β -cells was calculated using the following formula: $[(\beta\text{-cell area}/\text{total pancreas area}) \times \text{whole pancreas weight}]$. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using an Apoptosis *in situ* Detection Kit (Fujifilm Wako Pure Chemical Corp., Osaka, Japan). Pancreatic sections were stained with rabbit antibodies to Ki67 (Abcam, Cambridge, UK) and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) to detect proliferating cells and with insulin to detect pancreatic β -cells. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and proliferation and apoptosis in pancreatic β -cells were determined from the number of Ki67+ and TUNEL+ β -cells divided by the number of islets. Islets with a diameter of $\geq 100 \mu\text{m}$ were examined.

Luciferase assay

Based on an examination of the published sequence of human *Cdkn1c* promoter, we performed PCR to amplify a 455-bp fragment of the *Cdkn1c* promoter from genomic DNA of HeLa cells and cloned it into the KpnI-XhoI sites of the pGL3-Basic vector (Promega, Madison, WI, USA), which contains the coding sequence for firefly luciferase but lacks a eukaryotic promoter or enhancer elements. The *Cdkn1c*-luciferase reporter plasmid as well as vectors for C/EBP β were introduced into MIN6 cells. The cells were assayed for luciferase activity at 48 hours after transfection.

Bisulfite sequence

A DNeasy Blood and Tissue Kit (Qiagen) was used to extract genomic DNA from isolated islets, following the manufacturer's instructions. The quality and quantity of genomic DNA

was assessed using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). An Epitect Bisulfite Kit (Qiagen) was used to perform bisulfite treatment of DNA, following the manufacturer's instructions. For methylation-specific PCR, bisulfite-modified DNA (2 μL) was amplified at a volume of 50 μL , using specific primers targeting *Cdkn1c* promoter (including the transcriptional factor binding site) and one unit of EpiTaq HS (TaKaRa, Kusatsu, Japan), following the manufacturer's instructions. The primer sequences were 5'-TGAAATTGAAAATATTATATTATGTT-3' and 5'-CTTATCCTATCCAACTTAAACC-3'. The following amplification conditions were used: 98°C for 1 minute, followed by 40 cycles at 98°C for 10 seconds, at 48°C for 30 seconds, and 72°C for 30 seconds, and then 72°C for 7 minutes. For direct sequencing, amplified bisulfite-modified DNA was separated on 2.0% agarose gels. PCR products were purified using a QIAquick Gel Extraction kit (Qiagen) and cloned using a TOPO TA cloning kit (Thermo Fisher Scientific). The clones were sequenced on an ABI 310 Sequencer with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequenced products were analyzed with Sequence Scanner version 5.3.1 (Applied Biosystems).

Cell culture

MIN6 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 15% heat-inactivated fetal calf serum, and were cultured at 37°C with 5% CO₂. The culture medium was replaced every 3 to 4 days. For the analysis of epigenetic control and post-translational modifications, MIN6 cells were exposed to 3 μM 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich) for 72 hours, 100 nM Trichostatin A (Sigma-Aldrich) for 24 hours, and 5 μM Dznep (kindly provided by Prof. Marquez of the National Cancer Institute at Frederick) for 72 hours on the day following seeding.

Assay of insulin secretion from isolated islets

Islets were isolated from 10-week-old mice as described previously [7]. Five islets were selected manually, incubated in Krebs-Ringer solution, and stimulated at 37°C for 30 minutes with glucose. The islets were then collected by centrifugation, and the supernatant was assayed for insulin using an enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Gunma, Japan). The islets were solubilized in an acid-ethanol solution (74% [vol/vol] ethanol, 1.4% [vol/vol] hydrogen chloride [HCL]) overnight at 4°C before analysis with the insulin ELISA kit.

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