Paternal allelic mutation at the *Kcnq1* locus reduces pancreatic β-cell mass by epigenetic modification of *Cdkn1c*

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Genetic factors are important determinants of the onset and progression of diabetes mellitus. Numerous susceptibility genes for type 2 diabetes, including potassium voltage-gated channel, KQT-like subfamily Q, member1 (*KCNQ1*), have been identified in humans by genome-wide analyses and other studies. Experiments with genetically modified mice have also implicated various genes in the pathogenesis of diabetes. However, the possible effects of the parent of origin for diabetes susceptibility alleles on disease onset have remained unclear. Here, we show that a mutation at the *Kcnq1* locus reduces pancreatic β-cell mass in mice by epigenetic modulation only when it is inherited from the father. The noncoding RNA *KCNQ1* overlapping transcript1 (*Kcnq1ot1*) is expressed from the *Kcnq1* locus and regulates the expression of neighboring genes on the paternal allele. We found that disruption of *Kcnq1* results in reduced *Kcnq1ot1* expression as well as the increased expression of cyclin-dependent kinase inhibitor 1C (*Cdkn1c*), an imprinted gene that encodes a cell cycle inhibitor, only when the mutation is on the paternal allele. Furthermore, histone modification at the *Cdkn1c* promoter region in pancreatic islets was found to contribute to this phenomenon. Our observations suggest that the *Kcnq1* genomic region directly regulates pancreatic β-cell mass and that genomic imprinting may be a determinant of the onset of diabetes mellitus.

pancreatic β-cells | imprinting | Kcnq1

Genetic and environmental factors are important determinants of the development of type 2 diabetes. Recent large-scale studies, including genome-wide association studies, have identified many susceptibility genes for this disease (1–3). However, the mechanisms by which these genes contribute to the pathogenesis of type 2 diabetes remain unclear.

Potassium voltage-gated channel, KQT-like subfamily Q, member1 (*KCNQ1*) was identified as a susceptibility gene for type 2 diabetes in 2008 (3, 4). Exonic mutations in *KCNQ1*, which encodes a voltage-gated K^+ channel, have been implicated in familial atrial fibrillation and long QT syndrome (5, 6). KCNQ1 contributes to cell repolarization and plays a role in both gastric acid secretion and secretion in the respiratory tract (7, 8). Intronic single nucleotide polymorphism (SNPs) in the *KCNQ1* genomic region have also been associated with reduced insulin secretion by pancreatic β-cells in individuals with diabetes mellitus (9, 10), although the mechanism underlying this association has remained unclear. SNPs of *KCNQ1* have been associated with diabetes mellitus in the Icelandic population in a manner dependent on parental origin (11). Although the underlying mechanism remains unknown, this finding indicates that SNPs of *KCNQ1* influence imprinting control of this genomic region.

With the use of genetically engineered mutant mice, we have now found that a paternal allelic mutation at the *Kcnq1* locus resulted in an abnormality of imprinting control at this locus and an associated decrease in pancreatic β-cell mass. Our results suggest that defective imprinting control at the *KCNQ1* locus might contribute to the pathogenesis of pancreatic β-cell failure and type 2 diabetes by affecting the expression of neighboring genes.

Results

Insulin Secretion by Pancreatic β-Cells Is Not Impaired in *Kcnq1* Homozygous KO Mice. To investigate whether loss of function of KCNQ1 affects insulin secretion, we evaluated this process in mice in which exon 2 of *Kcnq1* on chromosome 7 has been replaced by a

Significance

Recently, the potassium voltage-gated channel, KQT-like subfamily Q, member1 (*KCNQ1*) gene has received much attention as a candidate susceptibility gene for type 2 diabetes in Asian, European, and other populations. The molecular mechanism underlying the association of *KCNQ1* with the onset of type 2 diabetes has remained unclear; however, we have now found that a paternal allelic mutation of *Kcnq1* results in the up-regulation of the neighboring imprinted gene cyclin-dependent kinase inhibitor 1C (*Cdkn1c*), a cell cycle inhibitor, in pancreatic β-cells of mice, with this effect being mediated by epigenetic modification of the *Cdkn1c* promoter. These changes seem to be responsible for the reduced pancreatic β-cell mass and impaired glucose tolerance characteristics of *Kcnq1* mutant mice.

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neomycin resistance gene (12). Static incubation of pancreatic islets isolated from *Kcnq1* homozygous KO (*Kcnq1*$^{-/-}$) mice revealed no difference in the extent of basal or stimulated insulin secretion compared with islets from WT mice (Fig. 1*A*). In addition, perfusion of the pancreas revealed no difference in glucose-induced insulin secretion between *Kcnq1*$^{-/-}$ and WT mice (Fig. S1). Together, these results showed that loss of KCNQ1 did not affect the insulin-secreting capacity of pancreatic β-cells in the mice used in this study.

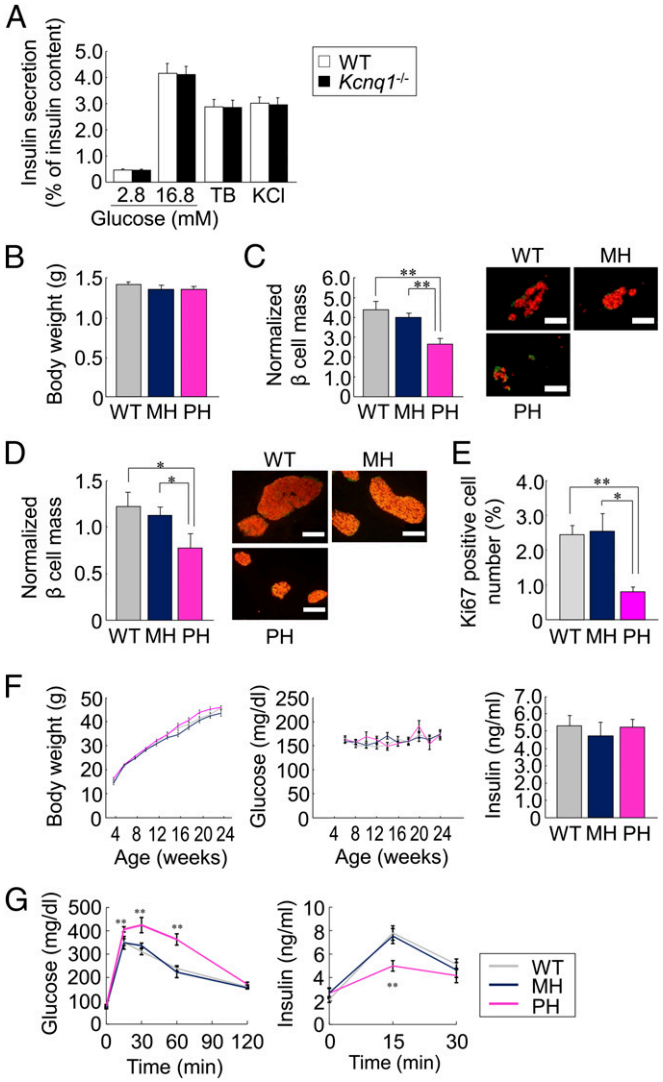

Fig. 1. Effect of *Kcnq1* mutation on pancreatic β-cell mass and glucose tolerance. (*A*) Insulin secretion by pancreatic islets isolated from 12-wk-old WT or Kcnq1$^{-/-}$ mice and incubated for 30 min with 2.8 or 16.8 mM glucose, 500 μM tolbutamide (TB), or 30 mM KCl. Data were normalized by islet insulin content. (*B*) Birth weight of WT and *Kcnq1* heterozygous KO (MH or PH) mice. (*C*, *Left* and *D*, *Left*) Normalized β-cell mass for WT, MH, and PH mice at (*C*) birth and (*D*) 24 wk of age after maintenance on a high-fat diet after weaning. The β-cell mass was determined as the ratio of the area positive for insulin immunostaining (red) to the total pancreatic area in sections similar to those shown in *C*, *Right* and *D*, *Right*. The pancreatic sections were also stained with antibodies to glucagon (green). (Scale bars: 50 μm.) (*E*) Fraction of Ki67-positive cells in the insulin-positive cells observed in 24-wk-old WT, MH, and PH mice. (*F*) Body weight as well as blood glucose and plasma insulin concentrations in the fed state for WT, MH, and PH mice at various ages or 12 wk of age (plasma insulin). (*G*) Blood glucose and plasma insulin levels of 24-wk-old WT, MH, and PH mice during an OGTT. All quantitative data are means ± SEM for (*A*) 7, (*B*) 6–20, (*C*) 5–9, (*D*) 8–11, (*E*) 4–5, (*F*) 5–37, or (*G*) 7–9 mice of each group. **P* < 0.05; ***P* < 0.01 for the indicated comparisons or vs. the corresponding value for WT or MH mice.

Effect of *Kcnq1* Mutation on Pancreatic β-Cell Mass Depends on the Parent from Which the Mutant Allele Was Inherited. *Kcnq1* is an imprinted gene that is expressed exclusively from the maternal allele during fetal development (13). However, although imprinting of *Kcnq1* is lost after birth (14), neighboring genes are also imprinted and expressed exclusively from the maternal allele even after birth (15). The noncoding RNA *KCNQ1* overlapping transcript1 (*Kcnq1ot1*) is expressed from the paternally derived *Kcnq1* genomic region and regulates the imprinted expression of neighboring target genes by silencing them on the paternal allele (16). The *Kvdmr1* locus, which is located in intron 10 of *Kcnq1* and has been referred to as an imprinting control region, includes the *Kcnq1ot1* promoter. Methylation of DNA in the *Kvdmr1* region of the maternal allele inhibits *Kcnq1ot1* expression, thereby allowing expression of the gene cluster at the *Kcnq1* locus on this allele. Mice with a deletion of the *Kvdmr1* region on the paternal allele show biallelic expression of the imprinted gene cluster at the *Kcnq1* locus, resulting in systemic growth deficiency during fetal development. This growth defect is attributable in large part to the increased expression of the cyclin-dependent kinase inhibitor 1C (*Cdkn1c*) gene (17), which encodes a cell cycle regulator and is located on the telomeric side of *Kcnq1*.

We hypothesized that mutations in the *Kcnq1* region might affect pancreatic islets by altering the expression of imprinted genes. Therefore, we categorized *Kcnq1* heterozygous KO (*Kcnq1*$^{+/-}$) mice as either paternal heterozygous (PH) or maternal heterozygous (MH) depending on whether they inherited the mutant allele from their father or mother, respectively. The birth weight of these two types of KO mice did not differ from that of WT littermates (Fig. 1*B*). However, pancreatic β-cell mass at both birth (Fig. 1*C*) and 24 wk of age (Fig. 1*D*) was significantly reduced in PH mice compared with WT and MH mice. Furthermore, the number of Ki67-positive pancreatic β-cells was significantly lower in PH mice than in the other two groups of mice (Fig. 1*E*). Although body weight as well as blood glucose and plasma insulin concentrations in the fed state did not differ significantly among the three groups of mice at various ages (Fig. 1*F*), an oral glucose tolerance test (OGTT) performed at 24 wk revealed a phenotype characterized by hyperglycemia and impaired insulin secretion after glucose loading for PH mice (Fig. 1*G*).

Decreased Expression of *Kcnq1ot1* Leads to Loss of Imprinting Control in Pancreatic β-Cells. Comparison of WT and *Kcnq1*$^{+/-}$ mice without regard to the parental origin of the mutant allele revealed that, whereas the abundance of KCNQ1 in pancreatic islets was reduced in the *Kcnq1*$^{+/-}$ mice, there was no significant difference in blood glucose or plasma insulin levels either in the fed state or during an OGTT between the two groups of animals (Fig. S2). These observations together with our findings that pancreatic β-cell mass was reduced and glucose tolerance was impaired in PH mice but not in MH mice suggested that the phenotype of PH mice might result from failed regulation of an imprinted gene. However, the systemic growth deficiency described previously for mice with a deletion of the *Kvdmr1* region on the paternal allele (17) was not apparent in our PH mice. Truncation of each *Kcnq1ot1* allele separately by the insertion of a poly(A) sequence in mice in which *Kvdmr1* was intact revealed that *Cdkn1c* was expressed in a biallelic and tissue-specific manner only in the animals in which *Kcnq1ot1* was truncated on the paternal allele (18). Therefore, we examined whether *Kcnq1ot1* expression might be affected in pancreatic islets of PH mice. Indeed, *Kcnq1ot1* RNA levels were reduced in PH mice but not in MH mice compared with its levels in WT animals (Fig. 2*A*).

We next determined whether the expression of other imprinted genes at the *Kcnq1* locus might be affected by the attenuated expression of *Kcnq1ot1* in the islets of PH mice. Whereas the

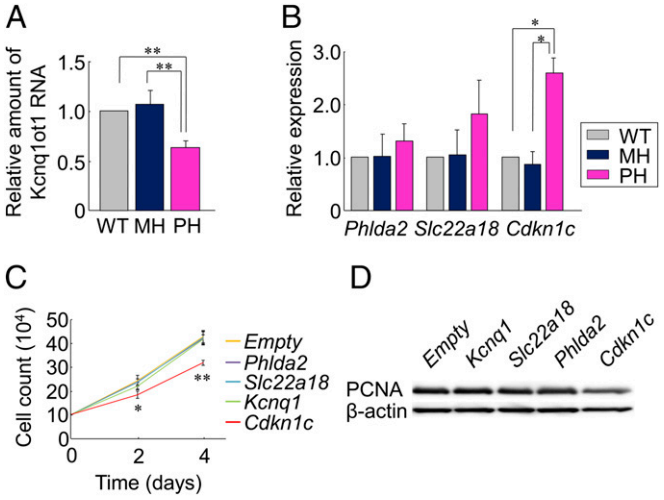

Fig. 2. Analysis and effects of gene imprinting in allele-specific *Kcnq1* heterozygous KO mice. (*A*) Relative abundance of *Kcnq1ot1* RNA in islets isolated from WT, MH, or PH mice at 6 wk of age. (*B*) Relative expression of genes in the *Kcnq1* region in islets isolated from mice at 6 wk of age. (*C*) Cell proliferation and (*D*) immunoblot analysis of proliferating cell nuclear antigen (PCNA) and β-actin as loading controls for INS-1 cells overexpressing *Kcnq1*, *Phlda2*, *Slc22a18*, or *Cdkn1c* or those infected with the corresponding empty retrovirus. All quantitative data are means ± SEM for (*A*) five or (*B*) three to four mice of each group or (*D*) six independent experiments. $*P < 0.05$; $**P < 0.01$ for the indicated comparisons or vs. the corresponding value for cells infected with the empty virus.

expressions of pleckstrin homology-like domain family A member 2 (*Phlda2*) and solute carrier family 22 member 18 (*Slc22a18*) tended to be higher in PH mice, only *Cdkn1c* expression was significantly increased compared with WT and MH mice (Fig. 2*B*). This differential effect might reflect a dependence of the regulatory action of *Kcnq1ot1* RNA on the proximity of genes to *Kvdmr1* (19). To examine the potential effect of the up-regulation of these imprinted genes on pancreatic β-cell mass, we infected INS-1 cells with corresponding recombinant retroviruses. Cells overexpressing *Cdkn1c* but not those overexpressing *Kcnq1*, *Phlda2*, or *Slc22a18* showed a decrease in both their proliferation rate (Fig. 2*C*) and the expression of proliferating cell nuclear antigen (Fig. 2*D*).

Decreased Expression of *Kcnq1ot1* Leads to a Tissue-Specific Increase in CDKN1C Expression in the Islets. CDKN1C (also known as $p57^{Kip2}$) is a cell cycle inhibitor that restrains the proliferation of tumor cells (20), and our results suggested that it also inhibits the proliferation of pancreatic β-cells. We next measured the expression of *Cdkn1c* in islets of PH, MH, and WT mice shortly after birth. *Cdkn1c* expression was markedly increased at both the mRNA and protein level in PH mice compared with WT or MH mice (Fig. 3 *A* and *B*). Previous studies have examined the mechanism by which *Kcnq1ot1* RNA inhibits gene expression on the paternal allele of WT mice (21, 22). The *Kcnq1ot1* transcript recruits the histone methyltransferase EZH2 to the promoter regions of the gene cluster at the *Kcnq1* locus (22). EZH2 catalyzes the trimethylation of lysine-27 of histone H3 (H3K27) and thereby, inhibits gene expression. The expression of *Cdkn1c* in mammary tumor cell lines was found to be strongly influenced by the extent of H3K27 trimethylation (23). The recruitment of EZH2 to the promoter region of *Cdkn1c* might, thus, be expected to be inhibited as a result of the down-regulation of *Kcnq1ot1* expression, leading to a reduced level of H3K27 trimethylation and attenuated inhibition of gene expression. We examined the extent of H3K27 trimethylation at the *Cdkn1c*

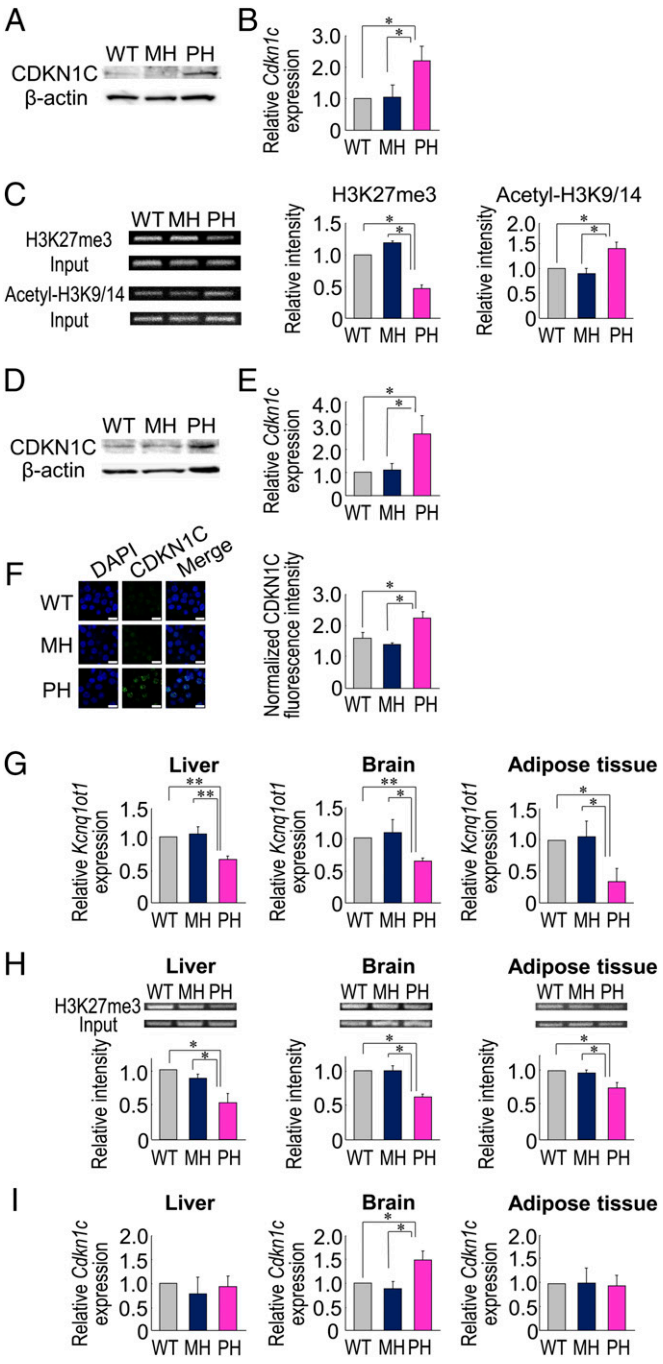

Fig. 3. Effect of paternal *Kcnq1* mutation on the expression of *Cdkn1c*. (*A*) Immunoblot analysis of CDKN1C in islets isolated from 1-wk-old WT, MH, or PH mice. (*B*) Relative expression of *Cdkn1c* in islets from mice of three groups at 1 wk of age. (*C*) ChIP analysis of the *Cdkn1c* promoter with antibodies to trimethylated H3K27 (H3K27me3) or acetylated H3K9/K14 in islets isolated from 6-wk-old mice. (*Left*) Representative and (*Center* and *Right*) quantitative data are shown. (*D* and *E*) Expression of *Cdkn1c* at the (*D*) protein and (*E*) mRNA levels in islets from 24-wk-old mice fed a high-fat diet. (*F*, *Left*) Representative immunostaining of CDKN1C and staining of nuclei with DAPI in islets from 24-wk-old mice fed a high-fat diet. (Scale bars: 10 μm.) (*F*, *Right*) The fluorescence intensity for CDKN1C normalized by that of DAPI was also determined. (*G*) Relative expression of *Kcnq1ot1* in organs of three groups of mice. (*H*) ChIP analysis of the *Cdkn1c* promoter with antibodies to H3K27me3 in liver, brain, and epididymal white adipose tissue isolated from 6-wk-old mice. (*I*) Relative expression of *Cdkn1c* in liver, brain, and adipose tissue from 6-wk-old mice. All quantitative data are means ± SEM for (*B*) three to five, (*C*) six, (*E*) three to five, (*F*) four to eight, or (*G*–*I*) five to six mice of each group. $*P < 0.05$; $**P < 0.01$.

promoter region in islets of PH, MH, and WT mice by ChIP analysis. H3K27 trimethylation was significantly reduced in PH mice compared with WT and MH mice (Fig. 3*C*). Similar analysis showed that the extent of H3K9/K14 acetylation, an epigenetic mark indicative of active transcription, was significantly increased in islets of PH mice (Fig. 3*C*). These data, thus, suggested that the increased *Cdkn1c* expression associated with the loss of *Kcnq1ot1* transcripts in islets of PH mice might result from altered histone modification. *Cdkn1c* expression in islets of PH mice was also increased at both the mRNA and protein level at 24 wk of age (Fig. 3 *D* and *E*). Immunostaining further showed a marked accumulation of CDKN1C in the nuclei of islet cells in PH mice at this age (Fig. 3*F*).

We also assessed the relationship between the expression of *Cdkn1c* and *Kcnq1ot1* in various organs of WT, PH, and MH mice at 6 wk of age. Whereas *Kcnq1ot1* expression (Fig. 3*G*) and H3K27 trimethylation at the *Cdkn1c* promoter (Fig. 3*H*) were both attenuated in liver, brain, and adipose tissue of PH mice, *Cdkn1c* expression was increased significantly only in the brain of these animals (Fig. 3*I*). This difference may reflect the fact that the transcriptional repressor CCCTC-binding protein (CTCF) binds to *Kvdmr1* in a tissue-specific manner. The reduced expression of *Kcnq1ot1* in the brain has been found to result in an increase in the expression of *Cdkn1c* (18, 24), because the latter gene is unaffected by CTCF in this organ. Conversely, the reduced expression of *Kcnq1ot1* in the liver or kidney does not result in an increase in the expression of *Cdkn1c* after imprinting because of the backup role played by CTCF (18).

Mutation in the Paternal Allele Leads to Decreased Pancreatic β-Cell Mass and Impaired Glucose Tolerance in Mice with *Kcnq1ot1* Truncation. To examine whether the reduced expression of *Kcnq1ot1* in islets of PH mice was directly responsible for the reduced β-cell mass in these animals, we determined β-cell mass in mice in which *Kcnq1ot1* transcription was blocked as a result of the insertion of a poly(A) sequence downstream of the *Kcnq1ot1* promoter (*Kcnq1ot1* truncation mice) (18). Both the expression of *Kcnq1ot1* in islets at 6 wk of age (Fig. 4*A*) as well as β-cell mass at birth (Fig. 4*B*) and 24 wk of age (Fig. 4*C*) were significantly reduced in mice with *Kcnq1ot1* truncation on the paternal allele (TP mice) compared with WT mice or mice with *Kcnq1ot1* truncation on the maternal allele. *Cdkn1c* expression in islets at 24 wk of age was significantly increased in TP mice (Fig. 4*D*), likely accounting for the reduced β-cell mass. An OGTT also revealed increased blood glucose and decreased plasma insulin concentrations in TP mice at 24 wk of age compared with WT mice and mice with *Kcnq1ot1* truncation on the maternal allele (Fig. 4*E*).

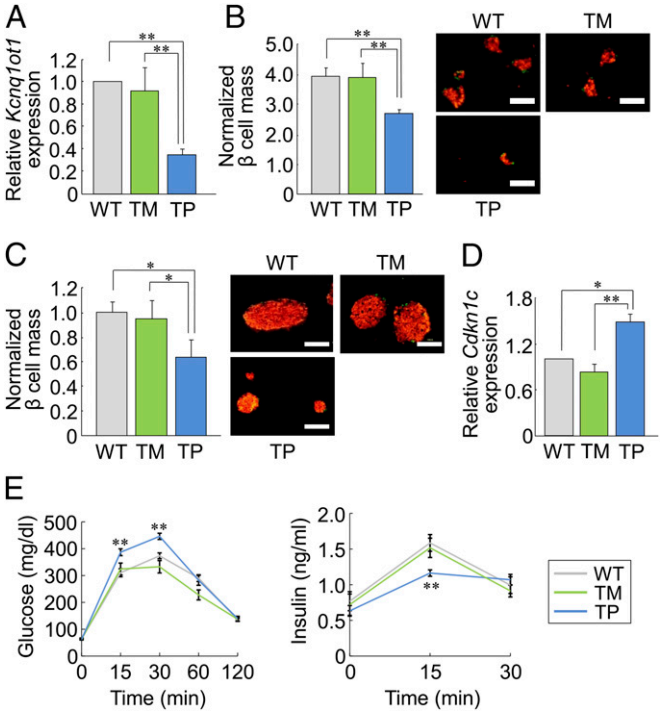

Fig. 4. Effects of allele-specific truncation of *Kcnq1ot1* on pancreatic β-cell mass, *Cdkn1c* expression in islets, and glucose tolerance. (*A*) Relative expression of *Kcnq1ot1* in islets isolated from 6-wk-old WT mice, mice with *Kcnq1ot1* truncation on the maternal allele (TM mice), or TP mice. (*B* and *C*) Pancreatic β-cell mass at (*B*) birth and (*C*) 24 wk of age in mice of three groups determined as in Fig. 1. (Scale bars: 50 μm.) (*D*) Relative expression of *Cdkn1c* in islets from 24-wk-old mice. (*E*) Blood glucose and plasma insulin levels during an OGTT in 24-wk-old mice. All quantitative data are means ± SEM for (*A*) 3–5, (*B*) 6–13, (*C*) 6–10, (*D*) 3–5, and (*E*) 6–10 mice. $*P < 0.05$; $**P < 0.01$ for the indicated comparisons or vs. the corresponding value for WT or TM mice.

Discussion

KCNQ1 is a well-known voltage-dependent potassium channel. Therefore, we expected that *Kcnq1* KO mice would show abnormal insulin secretion; however, a significant difference in insulin secretion was not observed, despite the loss of KCNQ1 function (Fig. 1*A* and Fig. S1), which suggests that the islets express additional Kv channels other than KCNQ1. Previous studies using KO mice reported that Kv2.1 played an important role in pancreatic β-cells (25, 26). Therefore, it was thought that the presence of KCNQ1 was not essential for insulin secretion in our experimental system. However, recent human in vitro and in vivo studies have reported increased insulin secretion as a result of loss of KCNQ1 function (27, 28). Insulin secretion was, thus, increased in human islets depleted of KCNQ1 by RNAi (27). In addition, consistent with this result, patients with long QT syndrome caused by loss of KCNQ1 function were found to manifest hyperinsulinemia and reactive hypoglycemia during an OGTT (28). These findings suggest that KCNQ1 plays a role in insulin secretion from human pancreatic β-cells and thus, warrants consideration as an effector transcript for type 2 diabetes risk at this locus.

Recent studies have shown that epigenetic regulation contributes to the onset of type 2 diabetes (29, 30). Epigenetic changes induced by starvation during fetal development have also been found to affect pancreatic β-cell mass (31). We have now shown that pancreatic β-cell mass is modulated by regulation of gene imprinting.

Temporal changes in the imprinting status of genes at the *KCNQ1* locus in human islets have been recently described, with imprinting of *CDKN1C* having been found to be maintained from the embryonic stage to adulthood (32). If the risk variants of this region are associated with imprinting control, *CDKN1C* should be considered an important candidate gene for type 2 diabetes.

We focused on CDKN1C ($p57^{KIP2}$), a cell cycle inhibitor. It has been reported that $p57^{KIP2}$ can be a therapeutic target in pancreatic β-cell failure (33, 34). Depletion of CDKN1C by RNAi was found to promote the replication of adult human β-cells (33), although the mechanism underlying the increased expression of $p57^{KIP2}$ in pancreatic β-cells in diabetes is not known. Our findings support the previous reports stating that $p57^{KIP2}$ could be a therapeutic target.

In a large-scale SNP analysis, we showed that some SNPs in intron 15 of *KCNQ1* were significantly associated with the development of type 2 diabetes. In addition, rs2237892 showed the strongest association with type 2 diabetes based on fine mapping of intron 15 of *KCNQ1* (3). Numerous additional SNPs in the *KCNQ1* region containing *KCNQ1OT1* have subsequently also been associated with this condition (1, 35). Transethnic fine mapping has recently improved the resolution of common variant

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association signals at several type 2 diabetes susceptibility loci (36). However, given the presence of multiple independent signals in the *KCNQ1* gene region, fine mapping of these independent association signals and elucidation of causal variants will require formal computation (36).

It remains to be determined whether SNPs in the *KCNQ1* region are associated with the expression of neighboring genes, including *KCNQ1OT1*, in human pancreatic islets. The relationship between SNPs and gene expression in human islets was examined recently by *cis*-expression quantitative trait loci analysis (37). However, current attempts to link type 2 diabetes risk variants to gene expression in the *KCNQ1* region have not found any significant associations with regional transcripts (32, 37), although this lack of association is likely the result of a lack of statistical power caused by the limited sample size. Our results provide an insight into a previously unidentified pathogenetic mechanism for type 2 diabetes and therefore, offer potential targets for the diagnosis and treatment of this disease.

Materials and Methods

Mice. *Kcnq1* KO mice were described previously (12). *Kcnq1* was inactivated by the insertion of a neomycin resistance gene cassette into exon 2, which is the first exon common to all known isoforms of KCNQ1. Genotyping of these mice was performed by PCR with genomic DNA, a forward primer (5′-CCAGGAGTGGGTGGTTCTAC-3′), a reverse primer (5′-GCCAGCACTAAAGATCTTGC-3′), and a *neo*-forward primer (5′-CGCTTCCTCGTGCTTTACG-3′). *Kcnq1ot1* truncation mice were described previously (18) and generated by the insertion of a poly(A) cassette 2.6 kb downstream of the *Kcnq1ot1* promoter by homologous recombination in ES cells. Genotyping of these mice was performed by PCR with genomic DNA, a forward primer (5′-CCATTTCTGCACCTGTTTTCC-3′), a reverse primer (5′-CATCTATGTTCACCAGGGAAG-3′), and a *mut*-forward primer (5′-CATATGCTGGCTGCCATGAAC-3′). All mice were fed normal laboratory chow unless indicated otherwise. The study was approved by the Animal Ethics Committee of Kobe University Graduate School of Medicine.

ChIP Analysis. ChIP analysis was performed with the use of a Magna ChIP G Kit (Millipore). In brief, islets were fixed with 1% formaldehyde for 30 min at room temperature and then, subjected to ultrasonic disruption in a solution containing a protease inhibitor mixture. The lysates were centrifuged to remove debris, diluted 1:10 with a solution containing 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl (pH 8.1), and 150 mM NaCl, and incubated for 2 h at 4 °C with protein G-Sepharose beads. The beads were removed by centrifugation; the supernatants were subjected to immunoprecipitation by incubation at 4 °C overnight with antibodies to trimethylated H3K27 (Millipore) or acetylated H3K9/K14 (Cell Signaling) or with normal mouse IgG (Santa Cruz Biotechnology), and then, they were incubated for 1 h with protein G-Sepharose. The precipitates were washed and then, subjected to extraction for 4 h at 65 °C with 1% SDS in 100 mM $NaHCO_3$. Proteins were digested with proteinase K, and the remaining DNA was purified with the use of a QIAquick PCR Purification Kit (QIAGEN) and subjected to PCR with *Cdkn1c*-specific primers (sense, 5′-CAGAGCTCTTTAACTCGAGC-3′; antisense, 5′-TTCAGTTTCAACAACACCGC-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 min at 4 °C in a microcentrifuge. Blood glucose concentration was determined with a glucometer (Glutest Pro; Sanwa Kagaku Kenkyusyo), and plasma insulin concentration was measured with an ELISA kit and the use of a mouse insulin standard (Shibayagi).

OGTT. Mice were deprived of food for 16 h. Blood was collected immediately before and at 15, 30, 60, and 120 min after oral administration of glucose (1.5 mg/g body weight).

Assay of Insulin Secretion from Isolated Islets. Islets were isolated from 12-wk-old mice as described previously (38). Five islets were selected manually, incubated in Krebs–Ringer solution, and stimulated at 37 °C for 30 min with glucose, KCl, or tolbutamide. The islets were then collected by centrifugation, and the supernatant was assayed for insulin with an ELISA kit as described above. The islets were solubilized in an acid–ethanol solution [74% (vol/vol) ethanol, 1.4% (vol/vol) HCL] overnight at 4 °C before analysis with the insulin ELISA kit.

RT-PCR and Real-Time PCR Analyses. Total RNA was extracted from islets pooled from three to five mice with the use of an RNeasy Kit (QIAGEN). The RNA was subjected to RT-PCR and real-time PCR analyses as described previously (39). The cDNA synthesized from the RNA was analyzed in a sequence detector (model 7500; Applied Biosystems) 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′-TGTCTTTGGAACTTTGTCTGCAA-3′; *Kcnq1ot1*, 5′-CTCAGTTCCACGATACCCTTCC-3′ and 5′-CTTACAGAAGCAGGGGTGGTCT-3′; *Cdkn1c*, 5′-CAGGACGGAATCAAGAGCAG-3′ and 5′-GAAGAAGTCGTTCGCATTGGC-3′; *Phlda2*, 5′-ACTGCGTGGAGCACACCTCT-3′ and 5′-ACACGGAATGGTGGGTTGGA-3′; and *Slc22a18*, 5′-TGTCTGCCTGGGATGTCTG-3′ and 5′-GGTAGGATGGAGAACTGCAT-3′.

Immunoblot Analysis. Lysates of isolated islets were prepared as described previously (40) and probed with antibodies to KCNQ1 (Santa Cruz Biotechnology), CDKN1C (Santa Cruz Biotechnology), proliferating cell nuclear antigen (Dako), and β-actin (Sigma-Aldrich).

Determination of β-Cell Mass. The pancreas was immersed in Bouin's solution, embedded in paraffin, and sectioned at a thickness of 4–5 μm. Sections were stained with antibodies to insulin and glucagon (Dako), and immune complexes were detected with secondary antibodies conjugated with Cy3 or FITC (Jackson ImmunoResearch Laboratories), respectively. Quantitation of β-cell mass was then performed as described previously (40). For Ki67 staining, pancreas sections fixed in 4% (vol/vol) paraformaldehyde were incubated with an anti-Ki67 antibody (Spring Bioscience), and the immune complexes were visualized using an EnVision/HRP Kit (Dako).

Islet Immunostaining. Islets were isolated as described previously (38) and cultured overnight in RPMI 1640 supplemented with (vol/vol) 10% FBS and antibiotics. They were then fixed in Bouin's solution for 5 min and processed for immunostaining with antibodies to CDKN1C (Santa Cruz Biotechnology) as described previously (41).

Infection with Retroviruses. PLAT-E retroviral packaging cells were transfected with the retroviral vector pBABE containing mouse *Kcnq1*, *Phlda2*, *Slc22a18*, or *Cdkn1c* cDNA (or with the empty vector) with the use of the Lipofectamine 2000 Reagent (Invitrogen). Recombinant retroviruses released into the culture medium were harvested at 48 h after transfection and added to INS-1 cells cultured in RPMI 1640 supplemented with 10% (vol/vol) FBS. After culture for 8 h, the infected cells were subjected to selection in medium containing puromycin.

Perfusion Analysis. Perfusion of the mouse pancreas was performed as described previously (42, 43). Male mice at 8–10 wk of age were deprived of food overnight (16 h) before analysis. The perfusion protocol began with a 10-min equilibration period with Krebs–Ringer bicarbonate Hepes buffer. The flow rate was 1 mL/min.

Statistical Analysis. Data are presented as means ± SEM and were compared by ANOVA followed by two-tailed Student's *t* tests. A P value of <0.05 was considered statistically significant.

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