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—Original—

Production of a mouse strain with impaired glucose tolerance by systemic heterozygous knockout of the glucokinase gene and its feasibility as a prediabetes model

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Abstract: Exon II of glucokinase (*Gk*) was deleted to produce a systemic heterozygous *Gk* knockout ($Gk^{+/-}$) mouse. The relative expression levels of *Gk* in the heart, lung, liver, stomach, and pancreas in $Gk^{+/-}$ mice ranged from 0.41–0.68 versus that in wild ($Gk^{+/+}$) mice. On the other hand, its expression levels in the brain, adipose tissue, and muscle ranged from 0.95–1.03, and its expression levels in the spleen and kidney were nearly zero. *Gk* knockout caused no remarkable off-target effect on the expression of 7 diabetes causing genes (*Shp*, *Hnf1a*, *Hnf1b*, *Irs1*, *Irs2*, *Kir6.2*, and *Pdx1*) in 10 organs. The glucose tolerance test was conducted to determine the blood glucose concentrations just after fasting for 24 h (FBG) and at 2 h after high-glucose application (GTT2h). The FBG-GTT2h plots obtained with the wild strain fed the control diet (CD), $Gk^{+/-}$ strain fed the CD, and $Gk^{+/-}$ strain fed the HFD were distributed in separate areas in the FBG-GTT2h diagram. The respective areas could be defined as the normal state, prediabetes state, and diabetes state, respectively. Based on the results, the criteria for prediabetes could be defined for the $Gk^{+/-}$ strain developed in this study.
Key words: glucokinase, glucose tolerance test, high-fat diet, prediabetes model mouse, systemic heterozygous knockout

Introduction

Recently, the number of persons considered to have or shortly have prediabetes [9] or to have borderline diabetes [17] is progressively increasing. Prediabetes represents an intermediate state between a normal blood glucose condition and diabetes and consequently increases the risk of diabetes as well as of other diseases such as vascular diseases [3, 14]. Therefore, prevention of the worsening of prediabetes is a critical issue today.

Except for the medicines and remedies already used in medical treatment, factors decreasing diabetes risk are, for instance, consumption of a well-balanced diet containing special amino acids, vitamins, and minerals [15, 16] and plant extracts [8]. There are also reports supporting the effects of prebiotics and probiotics [2, 4, 13]. Diabetes model animals were used for evaluation of the efficacy of most of these factors.

There are a number of factors that should be evaluated with a prediabetes model rather than a diabetes model. Cells and tissues for transplantation are typical examples. Their insulin-secreting activity might not be high enough

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to able remedy diabetes. Evaluation with a diabetes model should make a decision that those factors were not effective. If they were evaluated with a prediabetes model, however, their efficacy in improving prediabetes may be detected (unpublished data), and such a positive result would be of strategic importance from the viewpoint of development of better transplantation materials.

Various components in natural resources should also be evaluated with a prediabetes model from the following two points of view. They might contain a mixture of factors that both improve and worsen prediabetes/diabetes. A prediabetes model may detect both kinds of factors, while a diabetes model may not detect the factors that worsen prediabetes/diabetes. The sensitivity in detecting the factors that improve prediabetes/diabetes is also an issue. The concentrations of candidate factors in natural resources would be very low in most of cases. In screening of these factors, it is important to obtain a positive result even with crude samples in the first screening stage. The detection method should be as sensitive as possible. A prediabetes model is thought to be more sensitive improving effects than a diabetes model. Therefore, we decided to develop a useful prediabetes model. Moreover, we thought it important that its prediabetes level can be quantitatively defined.

Thus, we focused on a gene-modified mouse as a prediabetes model. Heterozygous knockout of a proper diabetes-causing gene was thought to be feasible. Considering the causing genes of type II diabetes, which is frequent in Japanese, we selected the glucokinase gene (*Gk*) as the target gene. Glucokinase is a rate-limiting enzyme in the glucose metabolic pathway predominantly in pancreatic β cells and hepatic cells [10, 12]. Heterozygous knockout of *Gk* was expected to cause impaired glucose-stimulated insulin secretion (GSIS) [5, 6, 11] and hyperglycemia but not to give rise to diabetes.

Previously, it was reported that a systemic heterozygous *Gk* knockout mouse could be produced by knocking out exon II [1]. However, no mouse of the developed strain or details of its production information was available. On the other hand, a pancreatic β-cell specific *Gk* knockout mouse was produced by disruption of exon Iβ [19]. From the viewpoint of future comparative study about systemic and pancreas-specific knockout strains, we decided to produce a systemic knockout strain for ourselves.

In order to confirm that the produced strain was really in a prediabetes state, we applied a high-fat diet (HFD) to induce diabetes. An HFD was reported to induce insulin resistance in a *Gk* knockout mouse and to cause diabetes [18, 21]. Such an effect of an HFD was speculated to be caused by the deficiency of *Irs2*. We intended to define the experimental criteria of the prediabetes state for the produced strain based on the blood glucose tolerance test. Moreover, we analyzed the expression levels of diabetes-causing genes to characterize the strain. In addition to *Gk*, we selected the following 7 genes: *Shp*, *Hnf1a*, *Hnf1b*, *Irs1*, *Irs2*, *Kir6.2*, and *Pdx1*. A decrease in the expression levels of these genes would imply the increase of risk of diabetes.

In this study, a *Gk* knockout mouse was developed, characterized, and qualified as a proper model of prediabetes.

Materials and Methods

Construction of a targeting vector

In order to knock out *Gk* systemically in every organ, a targeting vector designed to delete exon II of *Gk* was constructed. A long arm and short arm used for homologous recombination (Fig. 1) were obtained by TA cloning using primers listed in Supplementary Table 1. The long arm comprised 2 parts that were cloned separately and connected with an adaptor for the *Eco81I* terminal between them. A cassette of a diphtheria toxin A fragment (DT-A) was designed as a negative selection marker, while a cassette of a neomycin resistance gene (neo) was designed as a positive selection marker. Two parts of a long arm ((1) and (2)), a short arm, and 2 cassettes (DT-A and neo) were inserted into a multiple cloning site of a pBluescript® II SK (+) Phagemid Vector (Agilent Technologies, Santa Clara, CA, USA) according to the protocol illustrated in Supplementary Fig. 1.

ES cell culture

The ES cell line used in this study was EB3, which was derived from E14tg2a ES cells and kindly provided by Dr. H. Niwa (Riken CDB, Kobe, Japan). These cells were maintained in the absence of feeder cells in the medium for ES cells (ESM) on gelatin-coated dishes. ESM is Glasgow minimum essential medium (GMEM) supplemented with 10% fetal calf serum (FCS) (Biological Industries, Beit-Haemek, Israel), 1×nonessential amino acids (NEAA) (Invitrogen-GIBCO, Grand Island, NY, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 10^{-4} M 2-mercaptoethanol, and 1,000 U/ml leukemia

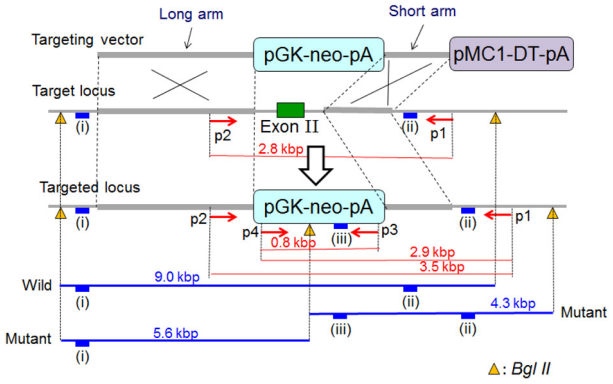

Fig. 1. A targeting vector for homologous recombination of exon II in *Gk*. Long arm, 4,849 bp; short arm, 914 bp; p1-p4, primers for genome PCR; (i), (ii), and (iii), probes for Southern blot analysis of DNA fragments prepared by *Bgl II* treatment. The lengths of the DNA fragments supposedly detected by PCR and Southern blot analysis are indicated in red and blue, respectively.

inhibitory factor (LIF) (ESG1107; Chemicon International, Temecula, CA, USA). Culture was conducted in a 5% CO_2-air mixture at 37°C.

Introduction of a targeting vector into ES cells

EB3 cells were suspended in a phosphate-buffered saline (PBS) at 1.0×10^7 cells/750 μl PBS and mixed with a 50 μl solution containing 50 μg linearized targeting vector. The mixture was placed on ice for 15 min and then transferred to a cooled cuvette for electroporation. An electric pulse was applied to the cuvette under the following conditions with an electroporation system (Gene Pulser II, Bio-Rad Laboratories, Tokyo, Japan): 0.8 kV, and 3.0 μF. The cuvette was placed for 3 min on ice. The cells were suspended in ESM at 2.0×10^5 cells/ml and cultured for 48 h at 37°C in a CO_2 incubator. Then the medium was replaced by a selection medium (ESM containing 200–300 μg/ml neomycin (G418, Sigma-Aldrich, St. Louis, MO, USA)) to select $Gk^{+/-}$ cells. The colonies obtained after culture for 7–10 days were transferred in a micro tube containing a 10 μl trypsin-EDTA solution. The cells were fully dispersed and distributed in a 48-well plate containing the selection medium. After 3–4 days culture, the cells in each well were dispersed again and distributed in each well of two 24-well plates containing the same selection medium. One plate was used for successive culture, and the other was used for the colony PCR described below.

Confirmation of $Gk^{+/-}$ ES cell clone

EB cell colonies obtained from the selection medium were analyzed by PCR with a primer set for neomycin (p4[Neo (F)]-p3[Neo (R)]) to confirm integration of the targeting vector (Fig. 1 and Supplementary Fig. 1). Thus confirmed colonies were analyzed by PCR with 2 other primers (p2[Long (F)]–p1[Cont (R)], p4[Neo (F)]-p1[Cont (R)]) to confirm homologous integration. Finally, the colonies were analyzed by Southern blotting using probes (i), (ii) and (iii). Probes (i) and (ii) were prepared by TA cloning of DNA isolated from EB3 cells using primers listed in Supplementary Table 1, while probe (iii) was part of a neomycin resistance gene from the targeting vector.

Production of $Gk^{+/-}$ mouse

$Gk^{+/-}$ ES cells were injected into blastocysts prepared from a C57BL/6J mouse. Thus produced chimera mice were crossbred with C57BL/6N mice. Then backcross with a mouse of the 129 strain was repeated to obtain subsequent generations (N_2-N_{10}). In order to characterize the strain thus developed, we investigated physical and functional properties using generations N_5-N_{10} of $Gk^{+/-}$ mice and their respective wild ($Gk^{+/+}$) littermates under the following conditions: (1) wild strain fed CD (control diet; W-CD), (2) wild strain fed HFD (W-HFD), (3) $Gk^{+/-}$ strain fed CD (G-CD), and (4) $Gk^{+/-}$ strain fed HFD (G-HFD). Eight mice of each strain were used per test. The test conditions were as follows unless otherwise indicated. At t=0 weeks, a glucose tolerance test was conducted (described below). Then 4 mice per group of each strain were bred on CD or HFD for 8 weeks. After another glucose tolerance test and an insulin test (described below), 3 out of the 4 mice per group were used for dissection and gene expression analysis. The same tests were repeated. To exclude variation due to gender, only male mice were used throughout.

Application of the high-fat diet

$Gk^{+/-}$ mice and wild mice were fed the control diet (solid diet MF, Oriental Yeast Co., Ltd., Tokyo, Japan) or high-fat diet (HFD 32, CLEA Japan, Inc., Tokyo, Japan). The HFD was composed of 32.0% fat, 25.5% protein, 2.9% fiber, 4.0% ash, 29.4% soluble nitrogen, and 6.2% water. The total calorie content was 507.6 kcal/100g, and fat accounted for 56.7% of the total (http://www.clea-japan.com/Feed/pdf/clea_hfd32.pdf).

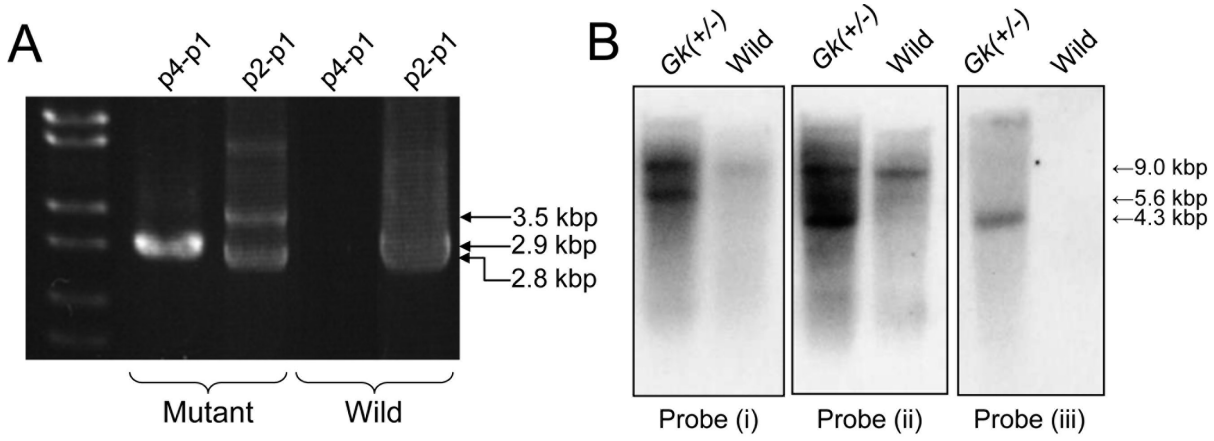

Fig. 2. Properties of $Gk^{+/-}$ ES cells and $Gk^{+/-}$ mice. (A) Results of genome PCR. (B) Result of Southern blot analysis.

Quantitative reverse transcription PCR (qRT-PCR) of diabetes-causing genes in various organs

Under anesthesia, various organs were removed from test mice. The organs were the brain, heart, lung, liver, stomach, pancreas, spleen, kidney, adipose tissue, and muscle. Total RNA was prepared from the respective organs using ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Oligo (dT)-primed cDNA was prepared from 1 μg of total RNA using SuperScript reverse transcriptase (Clontech Laboratories, Inc., Tokyo, Japan). The expression intensities of 8 genes were analyzed by qRT-PCR using primers listed in Supplementary Table 2. The expression of each target gene was normalized for the level of *Gapdh* or *Hprt* expression.

Glucose tolerance test and insulin test

After fasting for 12 h, 100 μl glucose solution (2 g glucose/kg mouse weight) was applied orally to test mice. An aliquot of blood was sampled from the tail vein at t=0, 15, 30, 60, 90, 120, and 180 min, respectively. The blood sample was assayed for glucose concentration directly with a glucose assay kit (G sensor, ARKRAY Inc., Kyoto, Japan). The data at t=0 min corresponded to the fasting blood glucose (FBG). The data at t=120 min corresponded to the blood glucose at 2 h in the glucose tolerance test (GTT2h). Based on the results of the glucose tolerance test, we proposed experimental criteria of diabetes, prediabetes, and normal states for the present $Gk^{+/-}$ strain. The plasma was separated from each blood sample and assayed for insulin concentration with an Ultrasensitive Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden).

Ethical guidelines

All experimental procedures involving animals were conducted according to the guidelines of the "Guide for the Care and Use of the Laboratory Animals" of Tokyo University of Agriculture and Technology, Japan.

Results

Establishment of a $Gk^{+/-}$ ES cell line

Genome PCR was conducted using primers p2-p1 and p4-p1 depicted in Fig. 1. and Supplementary Table 1. The expected length of the PCR product with the p2-p1 primer was 2.8 kbp for the wild ES cell line, while no product was obtained with the p4-p1 primer. On the other hand, in the $Gk^{+/-}$ ES cell line, primers p2-p1 and p4-p1 generated PCR products of 3.5 kbp and 2.9 kbp, respectively, plus those obtained with the wild ES cell line. As depicted in Fig. 2A, every product was detected at the position indicating its expected length.

Genomic DNA was then treated with a restriction enzyme, *Bgl II*, and applied to Southern blot analysis with probes (i), (ii), and (iii) depicted in Fig. 1 and Supplementary Table 1. In the case of a wild ES cell line, probe-bound signals are detected at 9.0 kbp with (i) and (ii), while no signal are detected with (iii). On the other hand, in the case of the $Gk^{+/-}$ ES cell line, they will appear at 5.6 kbp with (i), 4.3 kbp with (ii) and (iii), and at those positions observed in the wild ES cell line. Consequently, 1 colony showed these expected bands, as depicted in Fig. 2B. In total, we could establish 1 $Gk^{+/-}$

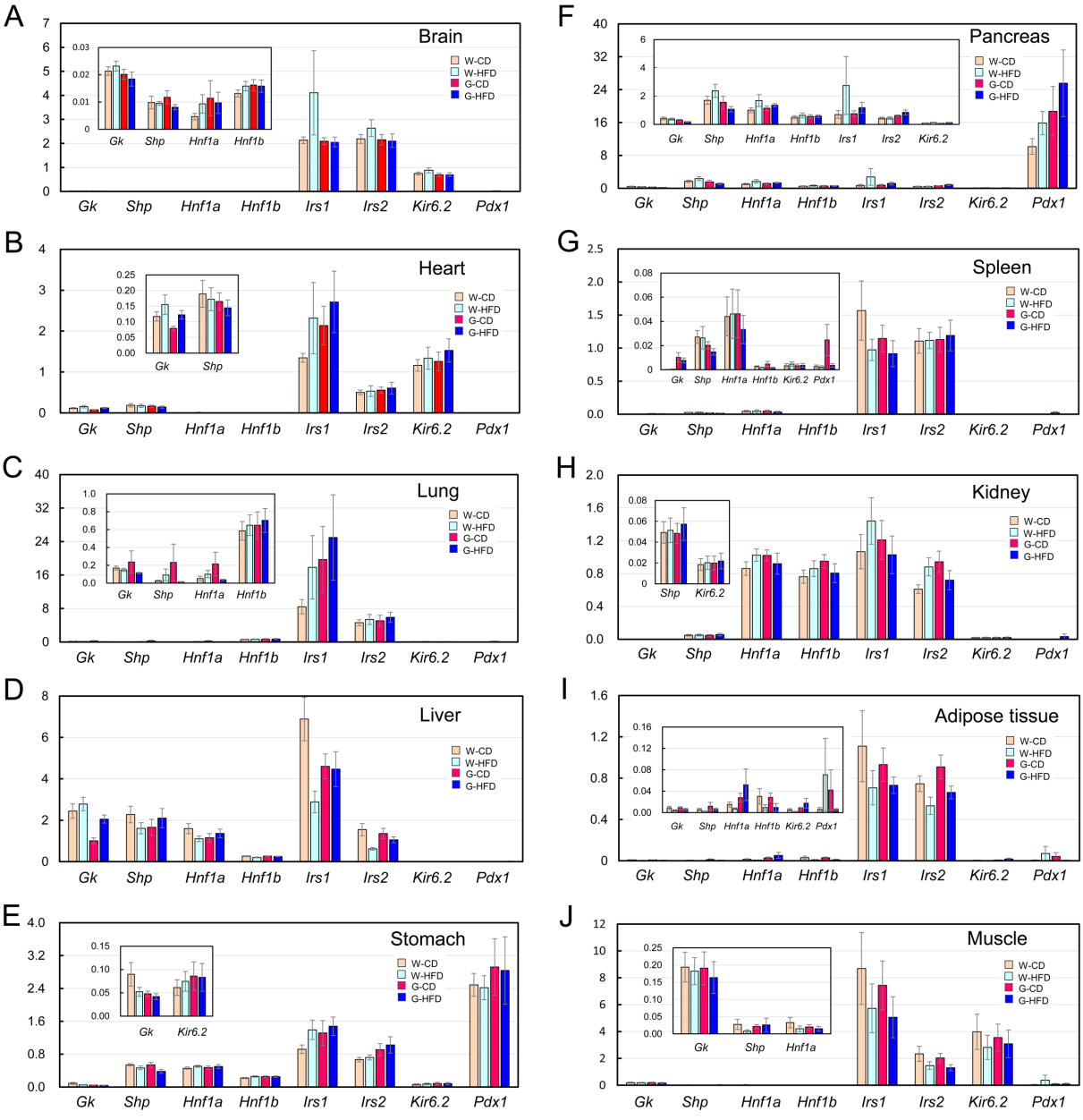

Fig. 3. Gene expression profiles of diabetes-causing genes in the $Gk^{+/-}$ strain and its wild mouse littermate fed the CD or HFD. Expression of each target gene was normalized for the level of *Gapdh* (brain, heart, lung, liver, stomach) or *Htpr* (pancreas, spleen, kidney, adipose tissue, muscle) expression. Error bars, mean ± SEM, n=9. Strains, N_5-N_8.

ES cell line out of 768 neo-positive colonies.

Gk expression in various organs

The gene expression profiles of *Gk* as well as of 7 other diabetes-causing genes in 10 organs were investigated under the following 4 conditions: W-CD, W-HFD, G-CD, and G-HFD (Fig. 3) The *Gk* expression levels under the W-CD and G-CD conditions are summarized in Table 1. Their relative values, G-CD versus W-CD, ranged from 0.41–0.68 in the heart, lung, liver, stomach, and pancreas, though the statistical significance levels in the stomach and pancreas were low. On the other hand, they were 0.95–1.03 in the brain, adipose tissue, and muscle. In the spleen and kidney, the *Gk* expression levels were very low. Considering the predominant expression properties of these organs, we concluded that

Table 1. *Gk* expression intensities at various organs. Data are mean values of *Gk* expression levels depicted in Fig. 3. t-value for DOF=16, $P<0.1$ (one-tailed) 1.34

	Brain	Heart	Lung	Liver	Stomach
W-CD (mean ± SEM, n=9)	0.021 ± 0.002	0.117 ± 0.015	0.168 ± 0.022	2.439 ± 0.348	0.090 ± 0.025
G-CD (mean ± SEM, n=9)	0.020 ± 0.002	0.079 ± 0.006	0.112 ± 0.013	1.006 ± 0.141	0.048 ± 0.006
t-value between W-CD and G-CD	0.51	2.32	2.15	3.82	1.62
G-CD/W-CD	0.95	0.68	0.67	0.41	0.53
	Pancreas	Spleen	Kidney	Adipose tissue	Muscle
W-CD (mean ± SEM, n=9)	0.473 ± 0.094	0.000 ± 0.000	0.000 ± 0.000	0.0089 ± 0.003	0.194 ± 0.043
G-CD (mean ± SEM, n=9)	0.295 ± 0.060	0.010 ± 0.004	0.001 ± 0.000	0.0086 ± 0.002	0.191 ± 0.048
t-value between W-CD and G-CD	1.63	—	—	0.33	0.04
G-CD/W-CD	0.62	—	—	1.03	0.98

systemic heterozygous knockout of *Gk* was successful and registered the developed strain as B6;129-*Gckt*^{m1Tms} (http://ilarlabcode.nas.edu/search_codes_full.php?labcode_id=9447&user_id=57616).

Effects of Gk knockout and/or HFD on the gene expression profiles

The effect of *Gk* knockout was evaluated by comparison of the results of W-CD versus G-CD depicted in Fig. 3A–J. The effect of the HFD was evaluated by comparison of the results of W-CD versus W-HFD. On the other hand, the *Gk* knockout+HFD effect was evaluated by comparison of the results of W-CD versus G-HFD. A noticeable event was the effect on the *Irs1* and/or *Irs2* expression. In fact, the *t*-test revealed that only the following cases were statistically significant at $P<0.05$: the effect of *Gk* knockout on *Irs2* in the kidney of W-CD vs G-CD and the effect of the HFD on *Irs1* and *Irs2* in the liver and *Irs2* in the kidney of W-CD vs W-HFD.

Another point to note is that the effects of *Gk* knockout and the HFD were sometimes opposite with respect to the same gene expression. A typical case was that observed in *Irs2* in adipose tissue. The gene expression level was lowered by the HFD (W-HFD) but raised by *Gk* knockout (G-CD) in comparison with W-CD. Consequently, W-HFD vs G-CD was statistically significant at $P<0.05$. In contrast, however, no statistical significance was observed between W-CD and G-HFD.

Effects of Gk knockout and/or HFD on body weight, blood glucose concentration, and insulin secretion

Effects of *Gk* knockout, the HFD, and both on body weight are depicted in Fig. 4A. Only the HFD was observed to have an effect. *Gk* knockout had no remarkable effect on body weight, indicating a prediabetic state without obesity.

The effects of the HFD on the FBG and GTT2h values were different between 2 strains. Initially, the values of wild mice were 82 and 120 mg/dl, while those of $Gk^{+/-}$ mice were 96–106 and 206–248 mg/dl, respectively (Fig. 4B). After breeding on the HFD for 8 weeks, they increased markedly (Fig. 4C), and the extent of their increase in the $Gk^{+/-}$ strain was much greater than that in the wild strain.

Glucose-responsive insulin secretion was lowered by *Gk* knockdown (Fig. 4D). In contrast, it was raised by the HFD, though the rate of increase in the $Gk^{+/-}$ mice was much lower than that in the wild strain.

Experimental criteria of the diabetes, prediabetes, and normal states

Glucose tolerance tests were applied to 3 groups of mice, W-CD, G-CD, and G-HFD for 8 weeks. The FBG-GTT2h plots of each group were distributed in separate areas (Fig. 5A). No generation dependence was observed with regard to generations N_5 and N_7-N_{10}. We defined these 3 areas as experimental criteria of diabetes (D), prediabetes (PD), and normal (N) states for this $Gk^{+/-}$ strain.

This diagram may be used for the evaluation of various factors that could induce or remedy diabetes. For instance, a $Gk^{+/-}$ mouse (G1 in Fig. 5B) was fed the HFD for 8 weeks, and its FBG and GTT2h values became 398 and 430 mg/dl, respectively, indicating a diabetes state. Then the mouse was fed the CD for 4 successive weeks. The FBG and GTT2h values changed to 153 and 356 mg/dl, respectively, indicating a prediabetes state. Therefore, CD feeding may be regarded as a remedy factor. On the other hand, the values for a $Gk^{+/-}$ mouse (G2) fed the HFD for 31 weeks were initially 299 and 394 mg/dl,

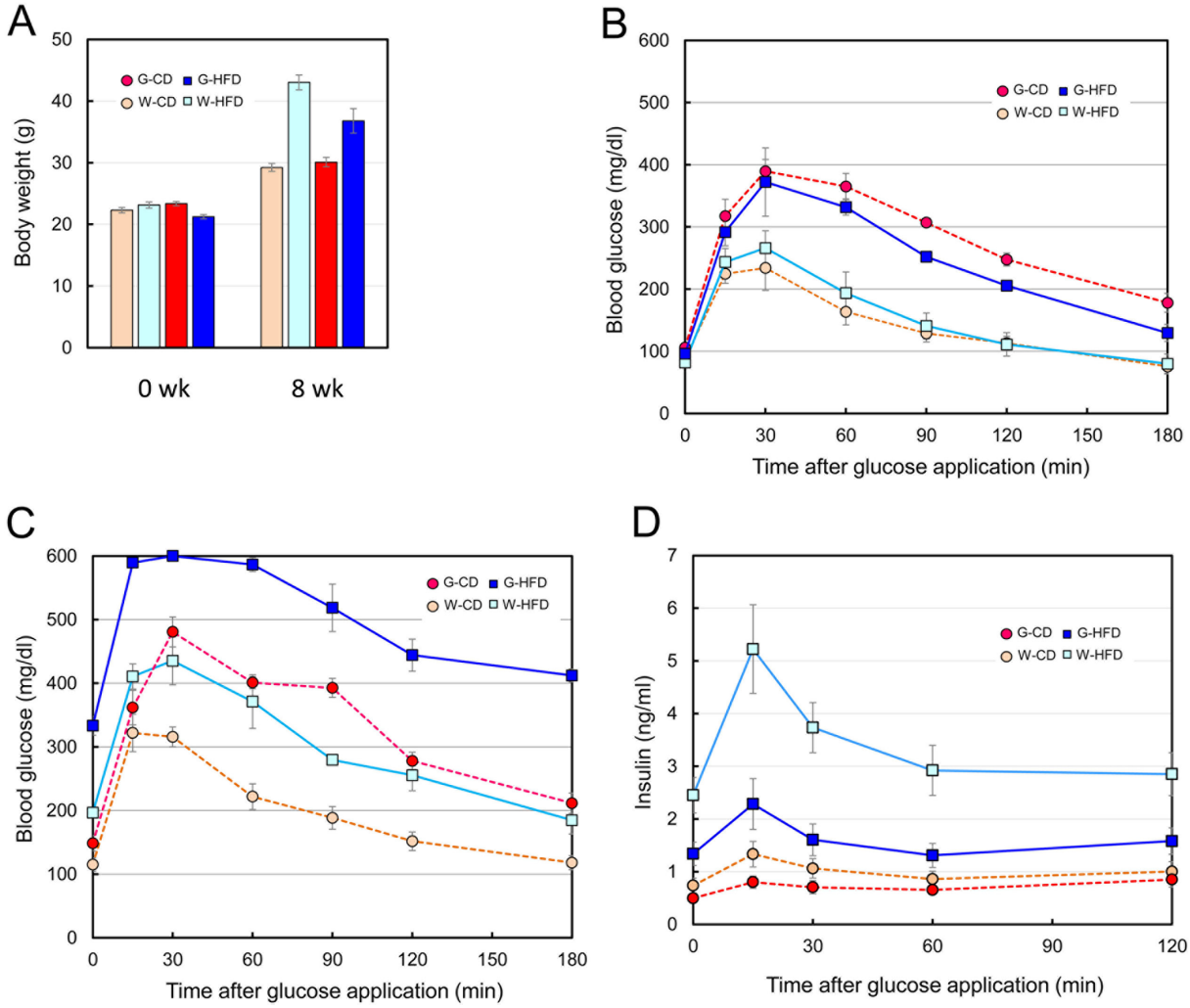

Fig. 4. Body weights and glucose tolerance test results of the $Gk^{+/-}$ strain and its wild strain littermate fed the CD or HFD. W-CD, wild strain fed CD; W-HFD, wild strain fed HDF; G-CD, $Gk^{+/-}$ strain fed CD; G-HFD, $Gk^{+/-}$ strain fed HFD. (A) Body weights of mice during breeding. Error bars, mean ± SEM, n=12 (W-CD), 11 (W-HFD), 10 (G-CD), 9 (G-HFD). Strains, N_8-N_{10}. (B) Result of glucose tolerance test before breeding (0 week). Error bars, mean ± SEM, n=4. Strain, N_7. (C) Results of the glucose tolerance test after breeding for 8 weeks. Error bars, mean±SEM, n=4. Strain, N_7. (D) Insulin secretion in response to the glucose tolerance test conducted after breeding for 8 weeks. Error bars, mean ± SEM, n=25 (W-CD), 26 (W-HFD), 23 (G-CD, G-HFD). Strains, N_5-N_{10}.

respectively, indicating also a diabetes state. However, those values remained at a high values of 227 and 431 mg/dl, respectively, even after successive breeding the CD for 8 weeks. This indicates a diabetes level that is difficult to remedy by only feeding the CD. Wild strain mice (W2), however, returned to the normal state after being fed the CD for 8 weeks even after being fed the HFD.

Discussion

Diabetes is thought to be caused by simultaneous impairment of multiple genes. Therefore, study efforts have been concentrated on the development of model animals deficient in multiple genes relevant to diabetes. Target genes actually knocked out and/or knocked down in experimental animals, or those proposed as an idea of double knockout and/or knockdown genes include *Pdx1-Irs1*, *Kir6.2-Irs1* [7], *Irs1-Gk* [20], *Irs2-Gk* [21], *Irs1-Irs2*, and *Gk-Shp*. On the other hand, we paid special

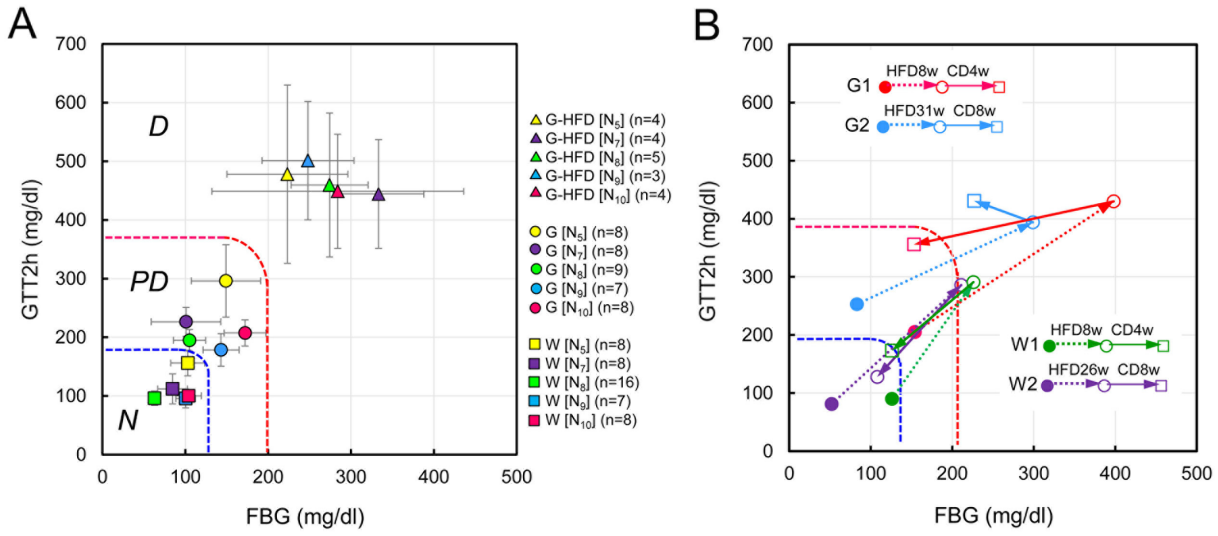

Fig. 5. Diagram of postulated criteria of diabetes, prediabetes, and normal conditions for mice based on the glucose tolerance test. (A) Definition of criteria. N, area surrounded by a broken blue line and the x- and y- axes, normal condition; PD, area between the blue and red broken lines, prediabetes condition; D, area beyond the red broken line, diabetes condition; G, $Gk^{+/-}$ strain; W, wild strain; N, mouse generation; n, number of mice. Error bars, mean ± 95% confident intervals.

attention to maturity onset of diabetes of the young (MODY). MODY is inherited, and the relatives of patients with a deficiency in genes causing MODY have a high risk of diabetes during their lifetimes. Therefore, correct diagnosis of MODY is important. To date, more than 10 genes have been classified as relevant genes. For instance, *Gk*, *Hnf1a*, *Pdx1*, and *Hnf1b* are classified as MODY2, MODY3, MODY4, and MODY5, respectively. Based on this information, we selected 7 genes described above and investigated the effects of *Gk* knockout on these genes. We thought it important to evaluate the off-target effects on these genes by *Gk* knockout alone before applying the $Gk^{+/-}$ strain to the evaluation of other factors. As demonstrated in this study, *Gk* knockout caused no remarkable off-target effect.

Another point to be considered in the prediabetes model was the effect of a HFD. As demonstrated in Fig. 5A, the FBG-GTT2h plots of G-CD and G-HFD were distributed in distinctively separate areas. Therefore, this diagram should be practically useful in evaluating the effects that induce diabetes from prediabetes and vice versa.

The involvement of *Gk* in the mechanism of glucose tolerance is well understood. However, in the case of other symptoms such as impaired function of vascular and alimentary functions, it is difficult to identify the involvement of *Gk* knockout. If a systemic *Gk* knockout mouse is used, it is possible to speculate that glucose tolerance should be caused by *Gk* knockout. Then any challenge towards its remedy may be focused on *Gk*-associated functions. In contrast, if a β cell-specific *Gk* knockout mouse is used, it would likely be difficult to determine the involvement of *Gk* knockout. Therefore, simultaneous use of both strains may provide us with clear evidence of the involvement of *Gk* in organs other than the pancreas. This would be of great importance from the viewpoint of use of the prediabetes model in studies of various factors inducing diabetes and other diseases.

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