Disruption of insulin receptor substrate 2 (Irs2) causes non-obese type 2 diabetes with β-cell dysfunction in the golden (Syrian) hamster

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Abstract

Because of the advent of genome-editing technology, gene knockout (KO) hamsters have become attractive research models for diverse diseases in humans. This study established a new KO model of diabetes by disrupting the insulin receptor substrate-2 (Irs2) gene in the golden (Syrian) hamster. Homozygous KO animals were born alive but delayed postnatal growth until adulthood. They showed hyperglycemia, high HbA1c, and impaired glucose tolerance. However, they normally responded to insulin stimulation, unlike Irs2 KO mice, an obese type 2 diabetes (T2D) model. Consistent with this, Irs2 KO hamsters did not increase serum insulin level upon glucose administration and showed β-cell hypoplasia in their pancreas. Thus, our Irs2 KO hamsters provide a unique T2D animal model that is distinct from the obese T2D models. This model may contribute to a better understanding of the pathophysiology of human non-obese T2D with β-cell dysfunction, the most common type of T2D in East Asian countries, including Japan.

Main text

Animal models have played major roles in the etiological analysis and the therapeutic development of human diseases. In particular, laboratory mice and rats have provided many model strains carrying spontaneous mutations or artificially modified genes. However, because of the differences between humans and mice/rats in various metabolic systems, they cannot appropriately reproduce some human diseases, such as lipid metabolic disorders [1-3]. Although monkeys and domestic animals can compensate for the shortcomings of mice and rats, these nonrodent animals are not widely used because of the time and cost required to create genetically modified animals.

The golden hamster (*Mesocricetus auratus*) is a rodent widely used in biomedical research, including oncology, immunology, physiology, and reproductive biology. Hamsters belong to the family Cricetidae, which is taxonomically distinct from that of mice and rats, the Muridae rodents. Hamsters have unique reproductive characteristics, such as a stable 4-day estrous cycle, high responsiveness to conventional superovulation methods, and the shortest gestation period (16 days) among eutherian mammals [4]. Like mice and rats, hamsters can be easily kept and bred in standard laboratories [5](5).

Gene knockout (KO) technology plays a major role in the generation of model animals. In mice, generating KO lines became practical as early as the 1980s with the availability of embryonic stem cells that can be gene-targeted and contribute to the germ line [6]. However, for many animals, including hamsters, the inability to establish high-quality embryonic stem cells has made generating KO animals difficult. In 2013, genome-editing technology using the CRISPR/Cas9 system was introduced into mice, making it possible to create KO animals using fertilized eggs in mammals [7,8]. In hamsters, the first gene-edited knockout strain (*Stat2* KO) was produced in 2014 [9]. The golden hamster has cardiovascular and lipid metabolism systems similar to humans, so it may provide unique and useful animal models for research on human metabolic diseases [10]. Based on this advantage, genome-edited KO hamsters have

been generated for models of aortic atherosclerosis with the lecithin-cholesterol acyltransferase mutation [11] and coronary atherosclerosis with the LDL receptor mutation[12].

Although human lipid metabolism abnormalities caused by metabolic syndrome have been well reproduced in a hamster model, there is no hamster model for diabetes, the other major disease related to metabolic syndrome. Therefore, we attempted to generate a diabetic hamster model by targeting the *Irs2* gene, a causative factor of diabetes in mice and humans [13,14]. As expected, *Irs2* KO hamsters developed congenital diabetes with typical hyperglycemia and glucose intolerance. However, unlike *Irs2* KO mice, *Irs*2 KO hamsters showed a non-obese (lean) phenotype, insulin responsiveness, and heavy β-cell hypoplasia. These animals are expected to provide a unique animal model for human type 2 diabetes (T2D) with β-cell dysfunction, the most prevailing diabetes in East Asian countries, including Japan [15,16].

Results

Generation of Irs2 KO hamsters. We designed seven single-guide RNAs (sgRNAs) that targeted the sequences of either the 5' or 3' side of the CDS domain (exon 1) of the hamster *Irs2* gene (Fig. 1A). In mice and humans, the 5' half of the *Irs2* gene contains the highly conserved PH- and PTB-encoding sequences that target IRS-2 to the cell membrane and insulin receptor (IR), respectively [17,18]. As expected, hamster *Irs2* also contains these sequences, indicating that the same functions of IRS2 may be conserved in hamsters (**Supplementary Fig. S1**). We injected the seven sgRNAs, together with the Cas9 protein, into the oviducts of 47 females on day 0.5 (the day after mating) and immediately applied electric pulses to the oviducts. Sixteen females became pregnant and gave birth to 101 pups on day 15.5. Of them, 12 (8 females and 4 males) carried mutant alleles. We mated these 12 mutant founders (F0) with wild-type (WT) hamsters to generate heterozygous KO hamsters of F1. As F0 #35 first gave birth to 25 pups, including one heterozygous KO female, we used its mutant allele to generate an *Irs2* KO hamster line (Table 1). The *Irs2* mutation consisted of a 2653-bp deletion at exon 1 (Fig. 1B).

Table 1
Distribution of genotypes in the offspring of each generation

Generation of parents	No. of offspring		Genotype of offspring (%)		
	Male	Female	+/+	+/-	-/-
F1	7	6	7	6	0
F2	13	11	9	15	0
F3	14	20	16	13	5
F4	11	6	11	16	0
F5	16	23	14	20	4
F6	34	30	16	45	5
F7	34	29	26	29	8
Total	129	125	101 (37.8)	144 (53.9)	22* (8.2)

*$P < 0.0001$ (vs. expected number, χ^2-test)

By intercrossing heterozygous KO hamsters, we found that homozygous KO hamsters were born alive but showed retarded growth postnatally and maintained low body weight in adults in both sexes (Fig. 1C **and D**). However, they showed no deteriorated systemic condition until at least 14 months of age. Heterozygous KO hamsters were indistinguishable from WT hamsters in their appearance. We observed that homozygous KO hamsters of both sexes were infertile according to natural mating tests with heterozygous or WT hamsters of the opposite sex. Therefore, homozygous KO hamsters were produced by mating heterozygous males and females in this study. The results of the mating of heterozygous parents in F1 to F7 are shown in Table 1. The proportion of homozygous KO pups was 8.2%, far less than the expected Mendelian ratio ($P < 0.0001$). This indicates that homozygous KO pups died before or around birth or were cannibalized by mothers before genotyping (2 to 3 weeks of age) because of their weakness.

Gene expression analysis. The *Irs2* gene was expressed in all tissues analyzed in heterozygous KO and WT hamsters, but no expression was observed in homozygous KO hamsters (Fig. 1E). The *Irs1* gene, from the same gene family of *Irs2*, was expressed in all these tissues, irrespective of the genotype, but its expression level in the pancreas was significantly decreased in homozygous KO hamsters. This is likely due to the decrease of β-cell masses in homozygous KO hamsters (see below; Fig. 1E). There seemed to be no compensatory mechanism of *Irs1* for the *Irs2* deficiency, except for the muscle that showed a significant increase of the *Irs1* expression in homozygous *Irs2* KO hamsters (Fig. 1E).

Serum and blood tests. All serum biochemical and blood values were analyzed by two-way ANOVA (genotype × sex). The genotype had significant effects on glucose, urea nitrogen (UN), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and hemoglobin A1c (HbA1c), but not in other parameters, including insulin (Fig. 2A). Of these, glucose, UN, TG, and HbA1c had no significant interactions between two factors, indicating that the genotype affected these parameters independently of sex. Statistical analysis revealed that the glucose level was significantly higher in homozygous KO hamsters than in heterozygous KO or WT hamsters, indicating that they suffered from hyperglycemia (Fig. 2B). Consistently with this, HbA1c, which reflects the long-term glycemic status, was significantly higher in homozygous KO hamsters than in heterozygous KO or WT hamsters (Fig. 2B). The UN level was slightly but significantly higher in homozygous KO hamsters (Fig. 2B). The results of other parameters are shown in **Supplementary Fig. S2**.

In vivo glucose homeostasis. Upon glucose administration, the increase in blood glucose levels was significantly greater and stayed higher until 120 min in homozygous KO hamsters than in heterozygous KO and WT hamsters (Fig. 2C). There were no significant differences in the glucose level between heterozygous KO hamsters and WT hamsters (Fig. 2C). This indicated that glucose tolerance ability was decreased in homozygous KO hamsters but not in heterozygous KO hamsters. The insulin tolerance test revealed that homozygous and heterozygous KO hamsters reacted normally to insulin administration (Fig. 2D) and reached the same serum glucose level (**Supplementary Fig. S3**). We also confirmed that the insulin level in homozygous KO hamsters did not increase upon glucose treatment (Fig. 2E).

Histological observations and immunostaining. *In vivo* glucose homeostasis assays (above) indicated insufficient β-cell function in *Irs2* KO hamsters. We then examined the population of insulin-positive β-cells in their pancreas by immunohistochemistry. The relative areas of insulin-positive cells in homozygous KO hamsters were significantly decreased and many of the islets were smaller than those of heterozygous KO hamsters (Fig. 3). Although the serum UN value was elevated in homozygous KO hamsters, there were no histological changes in their kidney tissue (**Fig. S4**).

Discussion

Animal models have long played an important role in understanding the etiology and clinical complications of human diabetic diseases. Historically, most diabetes models in mice and rats have been established by selective breeding of natural mutants based on their phenotypes [19,20]. Therefore, only a few genetically modified animals with clear diabetic phenotypes are available. Genetically modified models are advantageous in the preciseness of the causative gene involved in pathophysiology, especially when the models have a defined genetic background, i.e., inbred mice. In mice, *Irs2* KO strains are such typical models that they have been generated by mixed breeding of two inbred strains, such as C57BL/6 × CBA [21,22]. Syrian hamsters are considered genetically homogeneous because all hamsters in laboratories are descendants of two females and one male captured from their native habitat [5]. This study demonstrated that *Irs2* KO hamsters consistently develop diabetes with typical hyperglycemia and

glucose intolerance. Given their homogeneous genetic background, these phenotypes will be reproducible in any laboratory.

Mouse and hamster models with *Irs2* deficiency share common diabetic phenotypes (hyperglycemia and glucose intolerance), but their pathophysiology seems to be largely different. *Irs2* KO hamsters showed a lean phenotype throughout their lifespan, while *Irs2* KO mice developed an obese phenotype due to heavy adiposity [23,24]. Interestingly, unlike *Irs2* KO mice [13], *Irs2* KO hamsters normally responded to insulin treatment, indicating that they did not develop insulin resistance even under diabetic conditions. In relation to this, *Irs2* KO hamsters lost the ability to increase serum insulin after glucose load, most likely because of the β-cell dysfunction indicated by immunohistochemistry. In *Irs2* KO mice, it was reported that insulin secretion ability per β-cell increased to maintain the total insulin secretion level [13]. Although we did not perform an *in vitro* assay on the insulin secretion ability of β-cells in *Irs2* KO hamsters, it was very likely that they had little, if any, compensatory potential because KO hamsters did not increase serum insulin after glucose load (Fig. 2E).

Human T2D can be roughly classified into obese and non-obese cases. *Irs2* KO mice have greatly contributed to understanding the pathophysiology of obese T2D, such as the underlying mechanisms of insulin resistance, the roles of *Irs2* and related factors in different tissues, and the insulin signaling pathway under diabetic conditions [24–26]. Therefore, much information has been accumulated on the pathophysiology of obese T2D by this mouse model. By contrast, the pathophysiology of non-obese T2D remains poorly understood, while its cases are rapidly increasing, especially in East Asian countries [15,16]. T2D in East Asians is characterized by β-cell dysfunction and less obesity than in Caucasians [27–29]. Thus, there may be some pathophysiological difference between the T2D of East Asians and Caucasians, which may reflect the long-term dietary difference between them; East Asians have been primarily agriculturalists since the domestication of rice in China about 10,000 years ago [30]. The phenotypes of *Irs2* KO hamsters we identified resemble those of human non-obese T2D regarding their non-obese feature and β-cell dysfunction tendency. Interestingly, it is known that hamsters prefer plant diets, such as grains, while mice have more omnivorous tendencies [5,31]. Perhaps such differences in diet between mice and hamsters for over tens of thousands of years may have caused the differences in the β-cell robustness and the metabolic responsiveness to diabetic disorders.

A major clinical issue associated with human diabetes is that it is highly prone to various complications. Major complications include chronic kidney disease, visual impairment, nerve damage, and heart disease [32]. However, *Irs2* KO hamsters did not show such severe complications under our standard breeding condition. Although the serum UN level increased significantly in *Irs2* KO hamsters, they showed no signs of renal pathological changes, at least at the microscopic level. In hamsters, the APA strain is known to be predisposed to glomerulopathy [33] and can develop persistent hyperglycemia and diabetic nephropathy after a single low dose of streptozotocin injection. *Irs2* KO hamsters may also develop diabetic nephropathy if a strain prone to nephropathy can be used as the genetic background. Additionally, it

would be interesting to see whether a high-fat diet may further aggravate hyperglycemia and compromise its systemic condition in *Irs2* KO hamsters.

Another important characteristic of the golden hamster as a laboratory species is its susceptibility to many experimentally induced infectious diseases, especially coronavirus. Since the outbreak of severe acute respiratory syndrome (SARS) in 2003, a high virus titer has been known to develop in the respiratory tract of hamsters following intranasal inoculation of SARS-CoV, the agent of SARS [34]. In 2019, a new coronavirus outbreak (COVID-19) by SARS-CoV-2 occurred, and several waves of pandemics have followed to date. It is known that people with diabetes are more likely to experience severe outcomes [35]. Hamsters are also infected with SARS-CoV-2 but with milder symptoms than humans [36,37]. It is interesting to see if our diabetic hamsters would show severer symptoms than wild type hamsters upon infection with SARS-CoV-2.

In conclusion, we generated a T2D hamster model with a non-obese phenotype that may become a unique experimental model for human non-obese T2D with β-cell dysfunction. Further experiments, including high-fat diet treatment or detailed analysis at an early age, would elucidate their advantages as T2D models in the future. They would also provide important information on the mechanisms underlying the severe clinical symptoms of SARS-Co-2 infection in patients with diabetes. Our *Irs2* KO hamsters are freely available from the RIKEN BioResource Research Center (AO) on request.

Methods

Animals. Seven- to ten-week-old golden (Syrian) hamsters were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were used for experiments at 15 to 40 weeks of age. All hamsters were kept under specific pathogen-free conditions, provided with commercial laboratory mouse food and water *ad libitum*, and housed under controlled lighting conditions (daily light period, 04:00 to 18:00 hrs.) at a temperature of 24°C and humidity of 50%. The care and use of animals in this study were performed according to the guidelines for the use and maintenance of experimental animals from the Japanese Ministry of Environment. All animal experiments included in this study were approved by the Institutional Animal Care and Use Committee of RIKEN Tsukuba Branch. This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org). All the operations during *i*-GONAD experiments were performed on animals under ketamine anesthesia. When samples were collected from animals, they were euthanized with high concentrations of isoflurane.

Genome editing by the CRISPR/Cas9 system. The sgRNAs were designed using CRISPOR (http://crispor.tefor.net) and produced using a GeneArt Precision gRNA Synthesis Kit (#A29377; Thermo Fisher Scientific). In this study, we employed an *in vivo* genome-editing method (improved genome editing via oviductal nucleic acid delivery, i-GONAD [38,39] to avoid *in vitro* developmental arrest of hamster embryos that are highly sensitive to an *in vitro* milieu [4]. Briefly, a solution containing seven sgRNAs and the Cas9 protein (#1081059; IDT) was prepared (Fig. 1A). Approximately 3.5 to 4.0 μL of the solution was injected from the upper segment of the ampulla to the lower segments using a fine glass micropipette.

After injection, the oviduct was pinched by a forceps-type electrode (#CUY650P5; NEPA GENE). Electroporation was performed using NEPA21 (NEPA GENE). Electroporation conditions consisted of three sequential poring pulses (40–50 V, 5-ms duration, 50-ms intervals) followed by three transfer pulses (10 V, 50-ms duration, 50-ms intervals). On day 15.5 of pregnancy, the fetuses were delivered naturally, and live pups were examined for CRISPR-Cas9-induced mutations at the target sites.

Gene expression analysis. Total RNAs from fresh tissues were purified using the RNeasy Mini Kit (#74104; Qiagen, Nordrhein-Westfalen, Germany). DNaseI treatment was performed on columns with the RNase-Free DNase Set (Qiagen). cDNAs were synthesized from purified RNA using the SuperScript IV First-Strand Synthesis System (#18091050; Thermo Fisher Scientific). RT-PCR was performed using PowerUp SYBR Green Master Mix (#A25742; Thermo Fisher Scientific) with the QuantStudio 7 system (Thermo Fisher Scientific). Ct values were normalized to those of *beta-actin*, and relative gene expression levels were calculated using the ΔΔCt method. Each experiment was performed in duplicate. Primer sets used are as follows: *Irs1* (CTGACATTGGAGGTGGGTCC and TTTGGAATGAGGCAGGGCAT), *Irs2* (GACAGCGACCAGTATGTGCT and GAGTGGTGAGGCTGGGTATG), and *beta-actin* (CAAGAGATGGCCACTGCCG and GTGGATGCCACAGGATTCCATA).

Serum and blood tests. Blood samples were taken from WT and homozygous and heterozygous KO hamsters by cardiac puncture. Serum was centrifuged at 5000 × *g* for 10 min at 8°C. Biochemical parameters were measured using an automated biochemical analyzer, BioMajesty JCA-BM6070 (JEOL Ltd.,). Serum insulin levels were measured using a Bio-Plex mouse diabetes insulin set (Bio-Rad Laboratories, Inc.) and a Bio-Plex200 system (Bio-Rad Laboratories, Inc.).

In vivo glucose homeostasis. For the glucose tolerance test, the hamsters were fasted for 20 h and peritoneally injected with 1 mg/kg glucose solution. Blood samples were taken from the tail or orbital sinus at different time points and glucose was measured using a GLUCOCARD G + meter (ARKRAY, Inc.) or insulin as described above. For the insulin tolerance test, the hamsters were fed freely and fasted during the experiment. They were peritoneally injected with 0.75 U / kg of human insulin (Eli Lilly, Japan), and blood sample collection and glucose measurement were performed as described above.

Histology and immunostaining. Hamster tissue samples with different genotypes were collected from the brain, lung, heart, kidney, liver, pancreas, and muscle (hind leg). They were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (4–5-μm thickness) were stained with hematoxylin and eosin. Pancreatic sections were stained with mouse anti-insulin monoclonal antibody (INS monoclonal antibody, cat no. 66198-1-Ig, 1:5000 dilution) followed by biotinylated antimouse IgG antibody (1:400 dilution). The stained biotin signals were visualized using the VECTASTAIN Elite ABC Kit (VECTOR, cat no. PK-6100) and diaminobenzidine (DAB) as a brown color. Immunized sections were counterstained with hematoxylin. The areas of DAB-positive insulin-producing cells (β-cells) were traced, and ImageJ was used to calculate their proportions with the entire pancreas.

Statistical analysis. Body weights at each week of age were analyzed by Scheffe's F multiple comparison test. The proportion of β-cell areas in the pancreas and the expression levels of the expression levels of

the *Irs1* and *Irs2* genes were analyzed using unpaired two-tailed Student t test. The two-way ANOVA analyzed clinical blood parameters. Parameters with significant interactions between two factors (genotype and sex) were further analyzed using Tukey multiple comparisons. Other statistical analyzes are indicated as appropriate. A *P* value less than 0.05 was considered to indicate a statistically significant difference.

Declarations

Competing interests

The authors declare no competing interests.

Author Contribution

M.H., N.I. and A.O. designed the study. M.H., K.I., S.M., T.Tatebe, S.T., Y.D., T.Tomishima, A.Honda., M.O., A.S., R.Y. and A.O. performed the experiments. M.H., M.T., A.Hasegawa and A.O. analyzed the data. M.H. and A.O. wrote the article. All authors have reviewed the manuscript.

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Data availability

All data are contained within the article and supporting information.

Additional information

Supplementary Information The online version contains supplementary materials available at ...

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Figures

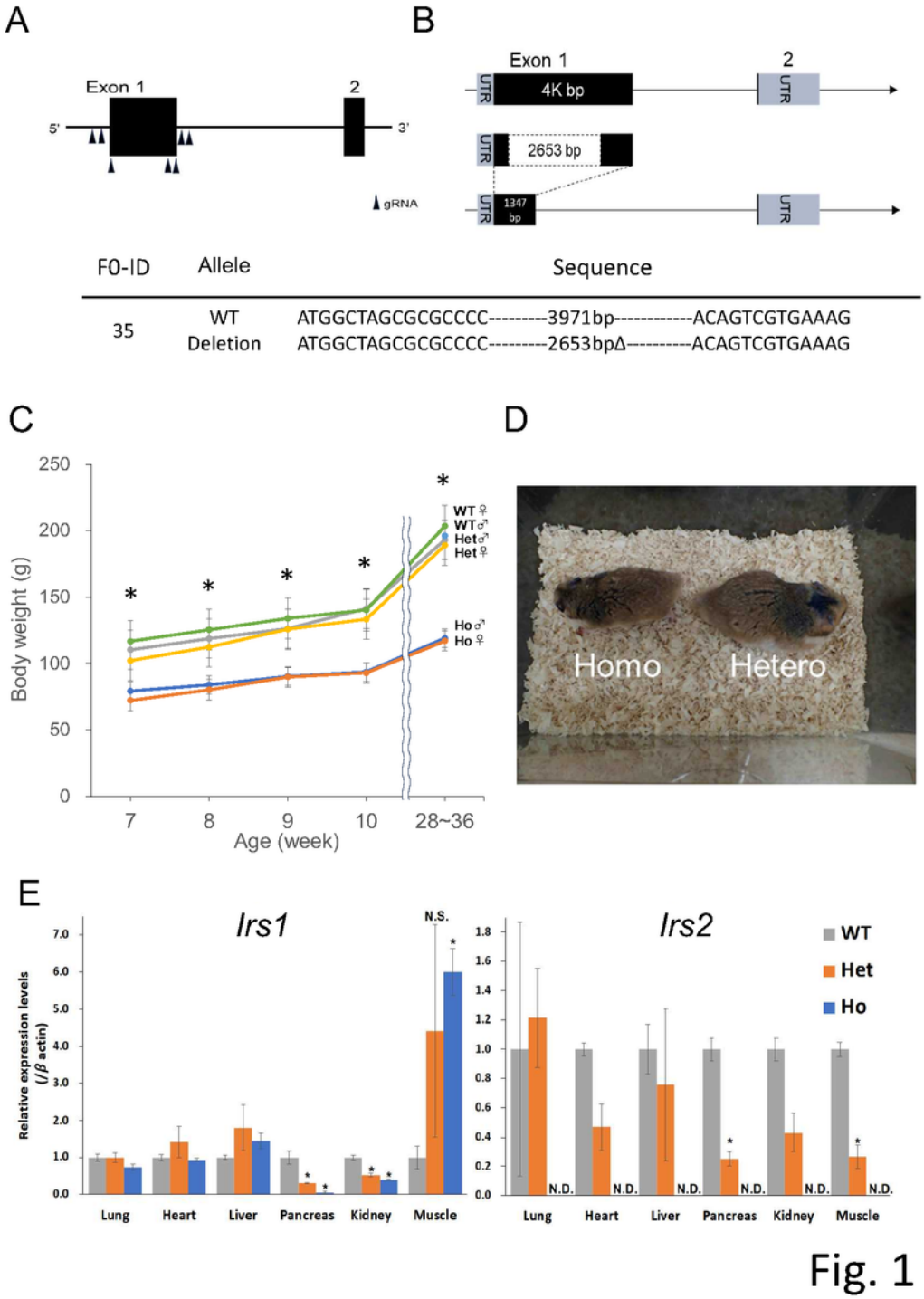

Fig. 1

Figure 1

Generation of *Irs2*KO hamsters. (A) The exon composition of thehamster *Irs2* gene. Specific gRNAs were designed to target sequences on the 5¢or 3¢side of the CDS domain (exon 1) of the hamster *Irs2* gene. (B) The mutant allele ofa founder F0#35 was used to establish the *Irs2* KO strain. There is a 2653-bp

deletion at the first exon. (C) Body weight change of *Irs2* KO hamsters. Homozygous (Ho) KO hamsters of both sexes had lower body weight than heterozygous (Het) KO or wild-type (WT) hamsters (*P< 0.05, Scheffe's F multiple comparison test). There were no significant differences between homozygous KO groups and between heterozygous KO/wild-type groups. Each group consisted of 2 to 6 animals. (D) Appearance of homozygous (homo)and heterozygous (hetero) KO hamsters, which can be distinguished by their size. (E) Expressionlevels of *Irs1* and *Irs2* in *Irs2* KO hamsters. *P < 0.05 (vs. wild type). N.D., not detected.

A

	CPK	AMY	TP	Glu	UN	T-Bil	HDL-C	LDL-C	T-CHO	TG	Insulin	HbA1c
Genotype	0.27560	0.35803	0.36289	0.02095	0.01495	0.06033	0.00536	0.87431	0.28018	0.00486	0.186649	0.00000
Sex	0.52634	0.17894	0.74666	0.46989	0.93939	0.07759	0.00003	0.20811	0.00083	0.00622	0.174746	0.41296
Interaction	0.75421	0.40381	0.53374	0.78486	0.10121	0.04470	0.00436	0.03308	0.76882	0.53895	0.360715	0.88384

CPK, Creatine phosphokinase; AMY, Amylase; TP, Total protein; Glu: Glucose, UN, Urea nitrogen; T-Bil, Total bilirubin; HDL-C, High density lipoprotein cholesterol; LDL-C, Low density lipoprotein cholesterol; T-CHO, Total cholesterol; TG, Triglyceride, HbA1c: Hemoglobin A1c

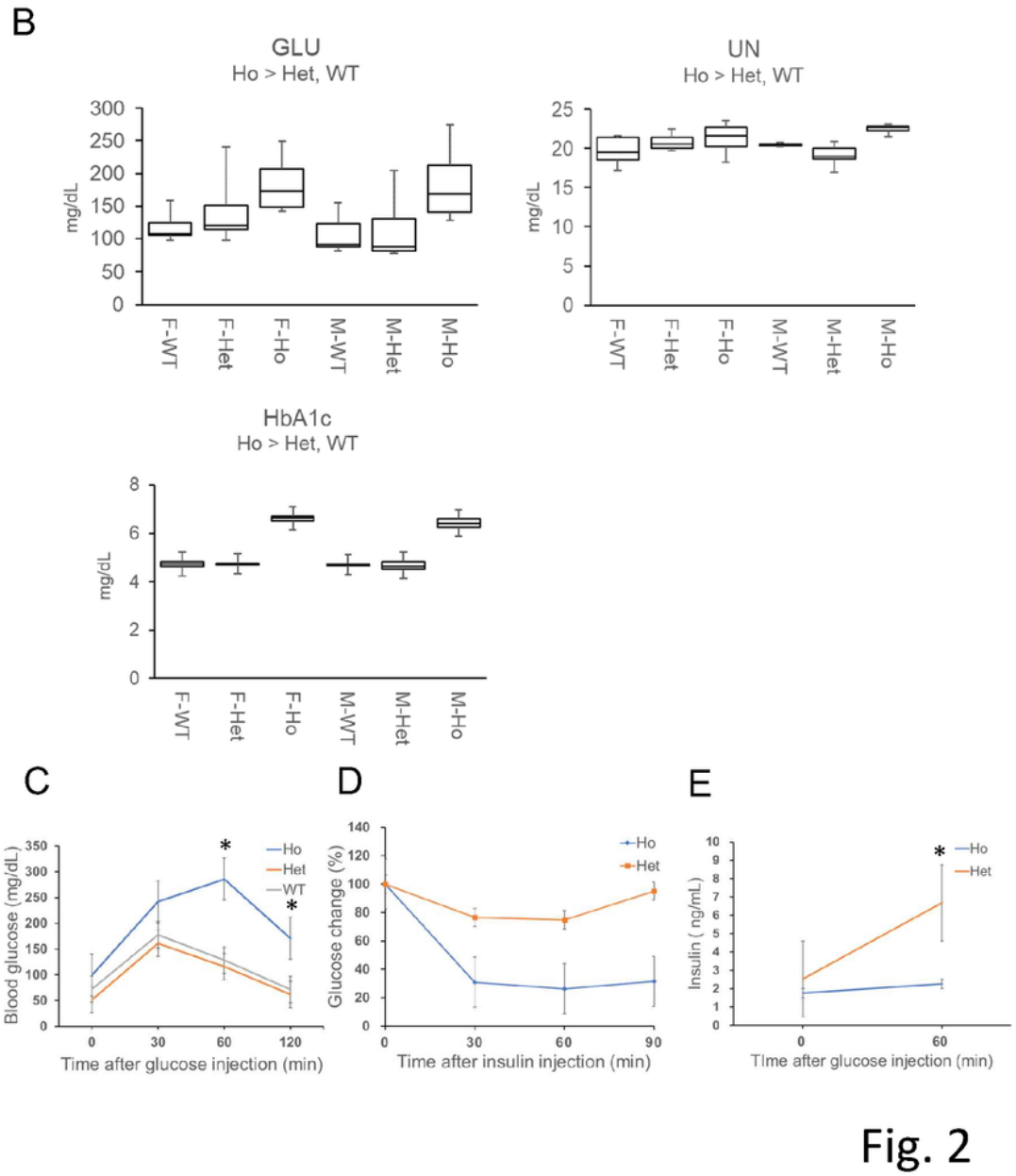

Fig. 2

Figure 2

Clinical blood parameters and glucose metabolic tests in Irs2 KO hamsters. (A) Results of the two-way ANOVA analysis of clinical blood/serum parameters. Each group consistedof 3 to 7 animals. The underlined numbers indicate significant differences ($P < 0.05$). (B) Four parameters with significant differences in genotype. (C) Glucose tolerance test. (D) Insulin tolerance test. (E) The serum insulin level after glucose load. Asterisks in C and E indicate significant differences between homozygous KO and heterozygous KO/wild-type hamsters. Scheffe's F multiple comparison test for the glucose tolerant test and Student's t-test for the other two tests. Ho, homozygous KO (n = 3–6); Het, heterozygous KO (n = 3–5); WT, wild-type (n = 2).

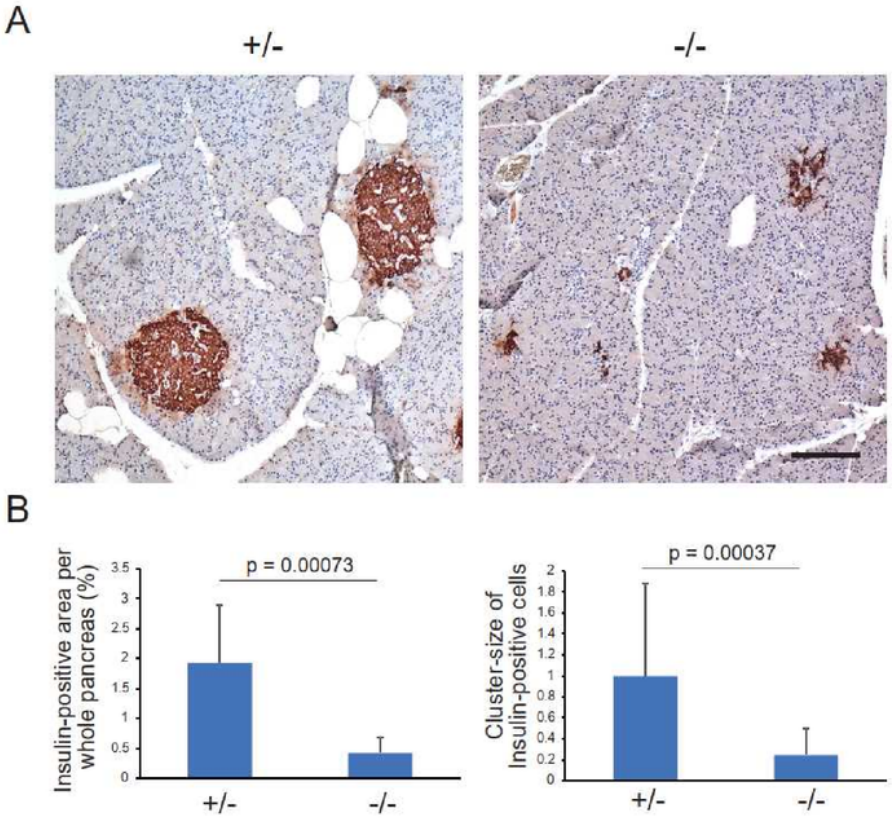

Fig. 3

Figure 3

Histological observation of b-cells in Irs2 KO hamsters. (A) Insulin-positive β-cells in heterozygous (+/−) and homozygous (−/−) KO hamsters. (B) Relative insulin-positiveareas of the pancreases in heterozygous (+/−) and homozygous (−/−) KO hamsters. Results from 6 and 3 animals, respectively. Bar: 100 μm.

Supplementary Files

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- Hiroseetal.Supplchecked2.pdf