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Ablation of *Rnf213* retards progression of diabetes in the Akita mouse

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ABSTRACT

Moyamoya disease (MMD) and moyamoya syndrome are vasculopathies characterized by progressive stenosis in the circle of Willis and its branches. The *RNF213* gene, which encodes a novel class of proteins, characterized by both E3 ligase and AAA + ATPase activities, has been identified as the susceptibility gene for MMD. However, its physiological functions remain unknown. MMD and moyamoya syndrome are often accompanied by diabetes mellitus. In this study, we generated *Rnf213* knockout (KO) C57BL/6 mice (*Rnf213*$^{-/-}$; *Ins2*$^{+/+}$), which were mated with Akita (C57BL/6 *Rnf213*$^{+/+}$; *Ins2*$^{+/C96Y}$) mice, a strain that develops diabetes spontaneously by 5 weeks of age, to obtain mice lacking *Rnf213* and carrying the Akita mutation (KO/Akita, *Rnf213*$^{-/-}$; *Ins2*$^{+/C96Y}$). Body weight and blood glucose concentration were measured from 6 to 20 weeks. Glucose tolerance, insulin resistance, plasma insulin and leptin concentrations, food consumption, pancreatic insulin content and histopathology were evaluated at 18 weeks of age. We found that glucose tolerance, as indicated by AUC, was 20% lower ($p < 0.05$) and insulin contents in pancreas were 150% higher ($p < 0.05$), in KO/Akita than in Akita mice. The number of CHOP positive β-cells assayed by histopathological examination was 30% lower and food consumption was 34% lower in KO/Akita than in Akita mice ($p < 0.05$ each). These findings indicated that the disruption of *Rnf213* improved glucose tolerance by protecting islet β cells.

1. Introduction

Moyamoya disease (MMD) and moyamoya syndrome are vasculopathies characterized by occlusion at the internal carotid arteries in the circle of Willis and the compensatory formation of an abnormal vascular network, resembling "puffs of smoke", that are called moyamoya vessels [1]. Patients with moyamoya syndrome have a predisposing disease [2], including Down's syndrome [3], neurofibromatosis 1 [4], or microcephalic osteodysplastic primordial dwarfism type Majewski II (MOPDII) [5], whereas patients with MMD have no such predisposing conditions.

Conditions predisposing to moyamoya syndrome are frequently accompanied by diabetes [2,5–7]. Moreover, the prevalence of type 1 diabetes mellitus was shown to be much higher in patients with MMD than in the general population [8], suggesting a pathological link between MMD and diabetes. We recently demonstrated that *RNF213* was the susceptibility gene for MMD, and that the p.R4810K polymorphism (ss179362673: G>A) is a founder variant commonly found in East Asian patients [9]. Although knockdown of *RNF213* in zebrafish caused abnormal vascular development [9], the physiological function of *RNF213* remains largely unknown.

RNF213 encodes a unique, 591-kDa protein with both a ring finger domain and Walker motifs, and *RNF213* mRNA is expressed in various tissues [9]. The E3 ligase activity of the ring finger domain was confirmed by self-ubiquitination, and ATPase in the Walker motifs was confirmed biochemically [9]. Ring-base E3 ligases have been linked to the control of many cellular processes, including proteasome-dependent proteolysis, DNA repair, signal transduction, apoptosis, immunological processes and transcription [10]. *RNF213* is also an AAA + ATPase because it has Walker A and Walker B motifs. AAA + ATPases usually exist and function as oligomers; their cellular functions include vesicular transport, quality control, cargo trafficking and microtubule homeostasis [11].

In this study, we tested whether ablation of *Rnf213* can modify diabetes mellitus in Akita mice (C57BL/6 *Rnf213*$^{+/+}$; *Ins2*$^{+/C96Y}$), a model for type 1 diabetes [12], in which β-cell destruction results from endoplasmic reticulum (ER) stress. We found that ablation of *Rnf213* unexpectedly alleviates diabetes by preserving β-cell function through moderating the vicious cycle of hyperphagia and hypoinsulinemia.

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2. Materials and methods

2.1. Generation of Rnf213 knockout mice

An *Rnf213*-targeting construct was produced using a Multisite Gateway Three-Fragment Vector Construction Kit (Invitrogen). Briefly, a loxP site was cloned into the 5′ site of exon 20, and a fragment containing a loxP site and a neomycin-resistance gene (Neo) was cloned into the 3′ site of exon 20 (Fig. 1A, Supplemental material). The construct was linearized and electroporated into RENKA C57BL/6 ES cells and selected with G418. Integration of the targeting vector into the mouse genome by homologous recombination was verified in targeted ES clones by Southern blotting (data not shown). Correctly targeted clones were injected into C57BL/6 blastocysts to generate chimeric mice with the targeted allele incorporated into the germ lines. The resulting chimeric male mice were mated with female C57BL/6 mice, and germ line transmission of the targeted allele was examined in the offspring. Offspring carrying the target allele were bred with Cre-transgenic C57BL/6 mice to generate mice heterozygous for the *Rnf213* deficiency ($Rnf213^{-/+}$). Heterozygous male and female mice were bred to produce homozygous offspring (KO, $Rnf213^{-/-}$).

2.2. Experimental animals

Akita ($Ins2^{+/C96Y}$) mice on a C57BL/6 background and C57BL/6 (WT) mice were purchased from Japan SLC. To generate mice lacking *Rnf213* and carrying the Akita mutation (KO/Akita, $Rnf213^{-/-}$; $Ins2^{+/C96Y}$), male double-heterozygous ($Rnf213^{+/-}$; $Ins2^{+/C96Y}$) mice were generated and mated with female *Rnf213* KO mice. Experiments were performed on four groups of male mice: (1) KO/Akita ($Rnf213^{-/-}$; $Ins2^{+/C96Y}$), (2) Akita ($Rnf213^{+/+}$; $Ins2^{+/C96Y}$), (3) KO ($Rnf213^{-/-}$; $Ins2^{+/+}$), and (4) WT ($Rnf213^{+/+}$; $Ins2^{+/+}$). Progeny of (1–3), aged 4 weeks, were selected by PCR genotyping for *Rnf213* (Supplemental material) and the *Ins2* locus, as described [13]. Mice were allowed free access to a standard diet (CLEA, Rodent Diet CE-7, 3.4 kcal/g) and tap water. The care of the animals and all experimental procedures were in accordance with the Animal Welfare Guidelines of Kyoto University.

2.3. Culture of Akita and min-6 cell lines and real-time PCR (RT-PCR)

To test *Rnf213* expression in β cells, we used Akita cells and the min-6 cell line [14,15]. Quantitative RT-PCR for *Rnf213* was performed using the specific primers, Rnf213cex29–31F (5′-TAA GGA TGT CCG CTC CTG GTT-3′) and Rnf213cex29–31R (5′-TTG ATG GCA GTA TAC TTG GCA-3′).

2.4. Western blotting

Protein samples from mice pancreas or cultured cells were subjected to immunoblotting using the rabbit polyclonal anti-RNF213 antibody and anti-GAPDH antibody (Santa Cruz Biotechnology). The rabbit polyclonal antibody was produced by inoculation of rabbits with cloned human full-length RNF213 as an antigen. The polyclonal IgG was purified from rabbit serum.

2.5. Measurement of glucose, insulin, proinsulin and leptin

Blood glucose was measured by Glutest Neo Super (Sanwa). All values above 600 mg/dl were treated as 600 mg/dl. Glucose tolerance testing (GTT) was performed by fasting 18-week-old mice for 16 h, followed by an intraperitoneal injection of 1.5 g/kg glucose. Insulin tolerance testing (ITT) was performed by fasting 18-week-old mice for 6 h, followed by an intraperitoneal injection of 1.5 U/kg insulin (Eli Lilly and Company). To measure leptin concentrations, blood was collected from the tail veins of 18-week-old mice after a 16 h fast. Plasma concentrations of insulin, leptin and proinsulin were measured by ELISA (Shibayagi).

2.6. Measurement of pancreatic insulin and proinsulin contents

Mice were sacrificed at 18 weeks of age in the morning after a 6 h fast. Each pancreas was homogenized in acid ethanol (75% ethanol, 1.5% HCl) and extracted at 4 °C overnight. The extracts were centrifuged, and the insulin and proinsulin concentrations of the supernatants were measured.

2.7. Pathological investigations

Mice were sacrificed under sevoflurane at 18 weeks of age after a 6 h fast. Each pancreas was fixed in 10% formaldehyde, embedded in paraffin, and sectioned. The sections were immunostained with guinea pig anti-insulin antibody (Dako) or rabbit anti-C/EBP homologous protein (CHOP)/GADD153 antibody (Santa Cruz Biotechnology). To estimate β-cell mass, consecutive paraffin sections 75 μm apart and spanning the entire pancreas (5–8 sections per pancreas) were prepared, and islet areas and relative abundance of insulin- and CHOP-positive cells were quantified on more than 20 islets per pancreas in three or four mice per genotype using Image-J software (National Institutes of Health). For electron microscopy, pancreases were fixed in 2% glutaraldehyde and post-fixed in 1% osmium tetroxide.

2.8. Statistical analysis

Results are presented as the mean ± standard deviation (SD) or standard error (SE). Differences were analyzed by *t*-test or ANOVA followed by Tukey's honestly significant difference test using STATISTICA software (StatSoft). $p < 0.05$ was considered statistically significant.

3. Results

3.1. General characterization of Rnf213 KO mice

To determine the physiological function of *Rnf213*, we generated mice with targeted deletion of *Rnf213* exon 20. This targeting strategy, in which a frame shift mutation was introduced into this exon, resulted in the disruption of the Walker motifs and the ring finger domain (Fig. 1A). Complete removal of *Rnf213* exon 20 from genomic DNA (Fig. 1B) and the absence of Rnf213 protein expression (Fig. 1C), were confirmed in KO mice. KO mice were born in the predicted Mendelian ratio and did not show any apparent health problems, including a cerebrovascular phenotype similar to MMD, even at around 80 weeks of age. Both males and females were fertile and produced normal-sized litters (mean, 6–8 pups). The body weight of KO mice was about 13% less than that of WT mice after 25 weeks of age ($p < 0.05$), and GTT results in KO and WT mice did not differ at 50 weeks of age (Supplemental Fig. 1).

3.2. Expression of Rnf213 in Akita and min-6 cells

Rnf213 protein was expressed in the pancreas (Fig. 1C). To assess the expression of *Rnf213* in β cells, we investigated the expression of *Rnf213* mRNA and protein in Akita and min-6 cell lines by quantitative RT-PCR and western blotting, respectively. We found that *Rnf213* mRNA and protein were expressed in these cells, with no differences between Akita and min-6 cell lines (Fig. 1D and E).