Normal pancreatic β-cell function in mice with RIP-Cre-mediated inactivation of p62/SQSTM1

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Abstract. Recent studies have suggested that decreased pancreatic β-cell function and mass are common features of patients with type 2 diabetes mellitus. Pancreatic β-cell homeostasis is regulated by various types of signaling molecules and stress responses. Sequestosome 1/p62 (SQSTM1, hereafter referred to as p62) is a ubiquitin-binding adaptor protein involved in cell signaling, oxidative stress, and autophagy. Because p62 appears to play an important role in maintaining mitochondrial quality control, it is possible that the loss of p62 in pancreatic β cells contributes to mitochondrial dysfunction, and thus leading to impaired glucose tolerance. In this study we investigated the physiological roles of p62 by inactivating p62 in a β-cell specific manner. We found that firstly, rat insulin-2 promoter-Cre (RIP-Cre)-mediated p62 inactivation did not cause body weight gain, although ubiquitous inactivation of p62 was previously shown to result in severe obesity. Secondly, we found no gross structural disorganization of the islets of p62-deficient mice. Consistent with normal islet morphology, no impairment in glucose tolerance was observed in mice with RIP-Cre-mediated p62 deletion. These results suggest that p62 is dispensable for normal islet organization and β-cell function.

Key words: Islets, β cell, Diabetes, p62, Sequestosome 1

TYPE 2 DIABETES MELLITUS is a metabolic disorder characterized by hyperglycemia resulting from the complex interplay of multiple genetic and environmental factors, resulting in both decreased insulin action on target tissues and defective pancreatic β-cell insulin secretion in response to glucose [1]. The natural history of diabetes is strongly associated with the disability of pancreatic β cells to adapt to meet the increased demand for insulin secretion caused by increased insulin resistance [2]. Therefore, elucidating the molecular mechanisms underlying pancreatic β-cell dysfunction is a key to understanding the pathology of diabetes [3, 4].

Sequestosome 1 (SQSTM1, referred to hereafter as p62) is a multifunctional scaffold protein that can interact with several signaling pathways through its functional subdomains, including the Phox and Bem1 (PB1) domain, zinc-finger domain, TNF receptor-associated factor 6 (TRAF6)-binding domain, and Kelch-like ECH-associated protein 1 (Keap1) interacting region (KIR) [5-7]. p62/SQSTM1 can activate the antioxidant response by interacting with the Keap1-Nrf2 pathway [8-10]. Therefore, elucidating the molecular mechanisms underlying pancreatic β-cell dysfunction is a key to understanding the pathology of diabetes [3, 4].

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and is thus selectively degraded by autophagy together with ubiquitinated proteins that are recruited to autophagosomes via p62 [13, 14]. Based on the assumption that the degradation of p62 largely depends on autophagy, the accumulation of cellular p62 has been widely used as a diagnostic marker for autophagic failure [15]. In fact, p62 accumulates in tissues such as liver, brain, and heart under autophagy-deficient conditions [16-18].

p62 has been reported to play a crucial role in the regulation of metabolism in white adipose tissue and liver. Mice with global inactivation of p62 (p62–/–) were found to develop mature-onset obesity and several features of metabolic syndrome, including excess fat accumulation in white adipose tissue and liver, impaired glucose tolerance, and insulin sensitivity [19]. A subsequent study on brain-specific p62 knockout mice demonstrated that a lack of p62 in the brain causes leptin resistance, thereby leading to hyperphagia and obesity [20]. The p62 protein is also involved in several ageing-related pathologies. p62–/– mice have a reduced lifespan and show premature aging phenotypes with increased mitochondrial damage and dysfunction [21]. Given the importance of p62 in maintaining mitochondrial homeostasis through the autophagic degradation of damaged mitochondria, or so called mitophagy, it is possible that loss of p62 in pancreatic β cells contributes to mitochondrial dysfunction and thereby reduces insulin release, resulting in impaired glucose tolerance [5]. On the other hand, a recent study reported that in contrast to what has been reported for ubiquitin-induced pexophagy and xenophagy, p62 appears to be dispensable for mitophagy [22]. The same study also reported that mitochondrial-anchored ubiquitin is sufficient to recruit p62 to mitochondria and promote mitochondrial clustering, but does not promote mitophagy, further demonstrating the controversy regarding the role of p62 in the mitochondrial homeostasis of β cells. Therefore, in this study, as the first step towards elucidating the role of p62 itself in β cells, we investigated the effects of β-cell-specific p62 ablation on β-cell function.

Materials and Methods

Animal experiments

All mice were housed in specific pathogen-free barrier facilities, maintained under a 12-hour light/12-hour dark cycle, and provided standard rodent food (Oriental Yeast, Tokyo, Japan) and water ad libitum. Rat insulin 2 promoter-driven Cre recombinase [23] was used to delete p62 in a pancreatic β-cell-specific manner. The generation of p62flox/+/Cre mice was performed as described previously [20]. RIP-Cre mice were crossed with p62flox–/– mice to generate p62flox–/–:RIP-Cre mice. Next, p62flox–/– mice were crossed with p62flox–/–:RIP-Cre mice to generate p62flox–/–:RIP-Cre mice.

Measurement of blood glucose and insulin levels

To measure non-fasting glucose levels, glucose levels were measured in the morning. For the intraperitoneal glucose tolerance test (IPGTT), after an overnight fast (16 h), age-matched 22-week-old male mice were injected intraperitoneally with glucose (0.5 g/kg body weight). A few microliters of blood were taken from the tail vein of awake mice, and glucose levels were measured using whole blood with a compact glucose analyzer (ACCU-CHEK® Compact Plus, Roche Diagnostics, Basel, Switzerland) at the indicated time points. To measure insulin levels, whole blood samples were collected from the orbital sinus in awake mice and centrifuged. After centrifugation, plasma was stored at –80°C until analysis. Insulin levels were measured using an enzyme-linked immunosorbent assay kit (Morinaga Co., Kanagawa, Japan).

Isolation of mouse islets

After anesthetization of mice and euthanasia by cutting of their carotid arteries, the distal ends of the common bile duct were clamped adjacent to the duodenum. Subsequently, common bile ducts were cannulated with a 30-G needle near the liver. Acinar tissue was disrupted by injecting 1.5 mL of a 0.15% collagenase solution. Then, pancreata were removed, and incubated in 1 mL of 0.15% collagenase solution for 40 min at 37°C. For isolating islets from the digested acinar tissue, pancreata were shaken for 1 min in conical tubes with 40 mL Hank’s balanced salt solution (HBSS). Next, solutions were incubated for 90 sec until the islets sunk to the bottom of the tube. Then, supernatants were aspirated and 50 mL of HBSS was added. This cycle of incubating, aspirating, and adding was continued until the solution became clear. The islets were transferred to dishes and collected using a micropipette under a dissecting microscope.

RT-PCR analysis

Total RNA was extracted from isolated islets using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized using Ovation RNA Amplifica-
tion System V2 (Nugen, San Carlos, CA, USA) according to the manufacturer’s protocols.

Real-time PCR was performed using TaqMan Custom Arrays (Applied Biosystems, Foster City, CA, USA). The expression levels of Sqstm1/p62 (Mm00448091_m1) mRNAs were quantified by TaqMan Real-Time PCR Assays (Applied Biosystems, Foster City, CA, USA), and normalized to glucuronidase beta (Gusb, Mm01197698_m1). Data are expressed as the mean ± SE.

Immunohistochemistry and morphometric analysis

After anesthetization, 22-week-old mice were thoroughly perfused with saline followed by 4% paraformaldehyde. Pancreata were removed and fixed with 4% paraformaldehyde for at least 2 days at 4°C until embedding. Fixed tissues were embedded in paraffin and then cut into 4-μm-thick sections and mounted onto slides. The sections were blocked with 2% bovine serum albumin for 30 min at room temperature, and then incubated with each primary antibody overnight at 4°C. The primary antibody for guinea pig anti-human insulin (Dako, Glostrup, Denmark) was diluted to 1:1,000 in 2% bovine serum albumin. The streptavidin-biotin complex method was used for detection, and hence the sections were incubated with biotinylated goat anti-guinea pig IgG (1:1,000) secondary antibody for 60 min at room temperature. β-cell areas were determined using five insulin-stained sections from each mouse, and each section was separated by at least 200 μm to avoid double scoring of the same islet. Images of pancreatic tissue were captured using a microscope (BZ-9000; Keyence, Osaka, Japan). Areas of insulin-stained pancreatic islets were determined automatically using image analysis software (WinROOF; Mitani Corp., Fukui, Japan).

Statistical analyses

All quantitative data are reported as the mean ± SEM. Statistical analyses were performed using the unpaired two-tailed Student t-test or nonrepeated ANOVA. A p-value of less than 0.05 was considered to indicate a significant difference between groups.

Ethical approval

The animal experiment protocol was approved by the Ethics Review Committee of Animal Experimentation of Juntendo University.

Results

To analyze the physiological roles of p62 in pancreatic β cells, p62ff:RIP-Cre mice were generated by crossing p62ff mice with p62ff:RIP-Cre mice. Quantitative RT-PCR analysis indicated that p62 mRNA levels of p62ff:RIP-Cre (referred to hereafter as p62ff:Cre islets) were reduced by more than 70% of that of p62ff+/-:Cre islets (Fig. 1). Given that β cells constitute 70% of normal islet cells [24] and the recombination efficiency of RIP-Cre is reported to be 80%–90% [25], p62 expression was efficiently eliminated from β cells in p62ff:Cre islets. No gross abnormalities were observed in p62ff:Cre mice. In contrast to the body weight gain observed in ubiquitous p62-knockout mice [19] and brain-specific p62-knockout mice [20], p62ff:Cre mice were indistinguishable in body weight from age-matched control p62ff+/-:Cre littermates between the ages of 6 and 22 weeks (Fig. 2A). There was no difference in non-fasting blood glucose levels between the two groups (Fig. 2B). The glucose tolerance test (GTT) indicated no deterioration of glucose tolerance in p62ff:Cre mice (Fig. 3A). During the treatment period, glucose levels were not increased in p62ff:Cre mice compared to p62ff+/-:Cre mice (Fig. 3B). These data suggest that p62 plays an important role in β-cell function.
GTT, a normal insulin secretion profile was observed, which was comparable to that of $p62^{+/+}$:Cre mice (Fig. 3B). Moreover, histological analysis demonstrated that there were no apparent morphological abnormalities or degenerative changes in the islets of $p62$-deficient mice (Fig. 4A). There was no significant change in islet cell mass, assessed by insulin immunostaining, in $p62^{tet}$:Cre mice compared with $p62^{+/-}$:Cre mice (Fig. 4B, C). The normal β-cell mass observed in β-cell-specific $p62$-deficient mice is consistent with the normal β-cell proliferation in response to increased insulin resistance observed in global $p62$-deficient mice [19]. Taken together, we conclude that there are no differences between β-cell-specific $p62$-deficient mice and control mice in terms of body weight, non-fasting blood glucose levels, glucose tolerance, islet cell mass, and β-cell morphology. These results indicate that $p62$/SQSTM1 in pancreatic β cells is dispensable for normal islet architecture, normal glucose tolerance, and β-cell function.

**Discussion**

Ubiquitous inactivation of $p62$ ($p62^{-/-}$) has been reported to result in early onset glucose intolerance and maturity-onset obesity [19]. Our results further confirmed that impaired glucose tolerance in $p62^{-/-}$ mice is primarily due to peripheral insulin resistance, excluding the possible involvement of $p62$ function in pancreatic β
Fig. 4. Ablation of p62 in islets does not result in morphological abnormalities of islets
(A) Representative images of hematoxylin and eosin staining of islets of 22-week-old p62<sup>+/+</sup>:Cre, p62<sup>ff</sup>:Cre, and p62<sup>ff</sup>:Cre mice. Scale bars represent 100 μm.
(B) Representative images of insulin staining of the islets of 22-week-old p62<sup>+/+</sup>:Cre, p62<sup>ff</sup>:Cre, and p62<sup>ff</sup>:Cre mice. Scale bars represent 100 μm.
(C) Percent islet area of p62<sup>+/+</sup>:Cre (n = 4), p62<sup>ff</sup>:Cre (n = 5), and p62<sup>ff</sup>:Cre mice (n = 6). Data represent the mean ± SEM.
cells. p62−/− mice develop insulin resistance before their obesity becomes apparent [19]. p62−/− mice have increased fat content due to the hyperactivation of ERK, which causes adipogenesis and obesity. At the molecular level, it has been demonstrated that p62 controls adipogenesis by inhibiting ERK activation via a direct interaction [19]. In contrast to such a cell-autonomous role of p62 in adipocyte differentiation, our study highlights the dispensable role of p62 in normal β-cell function and homeostasis, at least when mice are fed a normal diet.

As p62 appears to play an important role in maintaining mitochondrial quality control through mitophagy, it is possible that the loss of p62 in pancreatic β cells may cause mitochondrial dysfunction and thus result in impaired glucose tolerance [5]. Normal β-cell function and mass in β-cell-specific p62-deficient mice suggested redundant autophagy receptors; for instance, NBR1, which shares similar functional domains with p62, may have a compensatory role in mitophagy when p62 is deficient [26]. However, a recent study reported that NBR1 is dispensable for PARK2-mediated mitophagy regardless of the presence or absence of p62 [27]. Thus, other mitophagy receptors, such as BCL2L13 and FKBP8, may be involved in the autophagic degradation of damaged mitochondria [28, 29]. Further studies will hence be needed to determine the molecules that may compensate for p62 in p62ΔΔ.RIP-Cre mice.

Harada et al. reported that brain-specific p62 disruption by nestin-Cre results in significant body weight gain compared with control mice at 20 weeks of age [20]. Importantly, pair feeding completely abolished the obese phenotype of brain-specific p62 KO mice, indicating that p62 in the brain inhibits appetite and thus controls body weight homeostasis [20]. It has been demonstrated that RIP-Cre is expressed in a subset of neurons in the hypothalamus, and that deletion of Stat3 results in progressive obesity [30]. We found that p62ΔΔ.RIP-Cre mice show no noticeable obesity, suggesting that p62 function in a brain area other than the Rip-Cre-expressing area is important for appetite regulation.

We previously reported that autophagy deficiency in β cells is associated with β-cell dysfunction [31]. Mice with β-cell specific autophagy deficiency (Atg7f/f.RIP-Cre) showed impaired glucose tolerance with abnormal β-cell morphology. In that study, a large amount of p62 accumulation was observed. It has been reported that the liver injury occurring in liver-specific autophagy-deficient mice is largely suppressed by the concomitant loss of p62, indicating that liver injury is caused by a p62-dependent mechanism [14]. In contrast, the molecular mechanisms as to how β cells are damaged under autophagy deficiency is largely unknown. Here we show that the loss of p62 in pancreatic β cells does not affect their morphology or function. Therefore, future studies should focus on investigating whether the accumulation of p62/SQSTM1 contributes to β-cell failure under autophagy-deficient conditions, by generating β-cell specific Atg7/p62 double KO mice.

**Conflicts of Interest**

All authors report no conflicts of interest.

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