千葉大学学位申請論文

Roles of insulin signaling on survival and function of pancreatic β cells

（膵β細胞の生存と機能におけるインスリンシグナルの重要性）

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ABSTRACT

Insulin signaling is essential for the maintenance of pancreatic β-cell mass and function. However, it remains unsettled whether such maintenance is mediated by direct action on β-cells. We generated pseudo-islets from pancreatic β-cell line MIN6 cells and transplanted them into the sub-renal capsule of wild-type mice (SRT mice), which subsequently developed severe, progressive, and eventually lethal hypoglycemia. Histological analysis of these mice revealed that the mass of insulin-positive (Ins^+) cells was markedly reduced and the number of apoptotic Ins^+ cells was increased. As insulin signaling blockade potently induced cell death of MIN6 cells *in vitro*, we hypothesized that autocrine insulin action is required for β-cell survival. In fact, intra-pancreas transplantation of pseudo-islets to mice (IPT mice) resulted in fewer apoptotic Ins^+ cells than in SRT mice. On the other hand, β-cell mass was decreased in proportion to the decreased blood glucose levels in both SRT and IPT mice, suggesting a contribution of systemic insulin action and/or its consequential hypoglycemia. Thus, insulin plays distinct roles in pancreatic β-cell survival and regulation of its mass through both systemic and local actions on β-cells.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by chronic hyperglycemia, and is caused by defective insulin secretion from pancreatic β-cells and/or defective insulin action (1; 2) and usually occurs in middle age. However, impairment of insulin secretion and/or action arises prior to onset of hyperglycemia and decline in β-cell function has been shown to be progressive as in a clinical study in a Pima Indian population, in whom the transition from impaired glucose tolerance to T2DM was monitored in a cohort study (3). In addition, several clinical trials have indicated that none of oral anti-diabetic drugs in current clinical use are effective in attaining durable glycemic control in T2DM patients and that their protective effects on progressive β-cell devastation are questionable (4; 5). Insulin resistance has long been considered to play a central role in the development of T2DM, as typically seen in obese Caucasians (6; 7). In these subjects, β-cell failure has been considered to occur secondarily to the precedent compensatory β-cell hyperplasia. However, in 2003, Butler *et al.* found that pancreatic β-cell mass was significantly decreased in patients with impaired fasting glucose as well as T2DM (8), suggesting that the β-cell loss is a primary event in progression of the disease.

The regulatory mechanism of β-cell mass has been examined in several animal models, in which β-cell mass is acutely decreased by experimental procedures, such as partial pancreatectomy (9; 10) and β-cell ablation by toxins (11-13). After these procedures, β-cells have been shown to proliferate and to recover their mass. In order to clarify the mechanism of pancreatic β-cell mass regulation, we conferred an excess amount of β-cells to mice. To accomplish this, pseudo-islets generated from the β-cell line MIN6 were transplanted into the sub-renal capsule of wild-type mice (SRT mice). Approximately 2 weeks after transplantation, SRT mice developed severe, progressive, and eventually lethal hypoglycemia following growth of the transplants. Histological analysis of these severely hypoglycemic mice revealed that endogenous pancreatic β-cell mass was markedly reduced and that the number of apoptotic β-cells was increased. Using this mouse model in the present study, we examined the regulatory mechanism of β-cell mass and its function in detail.

RESEARCH DESIGN AND METHODS

Reagents

Hydroxyl-2-naphthalenylmethylphosphonic acid triscetoxymethyl ester (HNMPA) was purchased from Millipore (Billerica, MA). Insulin and diazoxide were

purchased from Sigma Aldrich (St. Louis, MO). Matrigel was purchased from Corning Inc. (Corning, NY).

Animals

We generated *Rip-Cre; Rosa26*$^{tdTomato/+}$ mice by crossbreeding promoter-driven Cre transgenic mice (*Rip-Cre*) (14) with fluorescent protein (Tomato)-reporter mice (*Rosa26*$^{tdTomato/+}$) for a cell lineage tracing experiment. Eight-week-old C57BL/6J mice and *Rip-Cre:Rosa26-tdTomato* mice were subjected to transplantation experiments. The mice were housed in a climate controlled room with a temperature of 23±3 °C, humidity of 55±15%, and a 12 h light/12 h dark cycle, and were fed standard laboratory chow (CE-2) (Clea Japan Inc., Tokyo, Japan) *ad libitum*. Blood glucose was measured at indicated time points using Glutestmint (Sanwa Kagaku Kenkyusho Co. Ltd., Nagoya, Japan). All animal experiments were approved by the Animal Care Committee of Chiba University.

Generating pseudo-islets and MIN6 cells overexpressing GFP

MIN6 (K20) cells, a pancreatic β-cell line (15), were used for generating pseudo-islets. MIN6 cells were cultured as previously described (16). To generate pseudo-islets, 20 μl MIN6 suspension (1.5×10^5 cells/ml) in 25mM glucose (HG) -DMEM was cultured in a hanging drop for 3 days, and the cell spheres were then

transferred to a gelatin (1%)-coated dish and cultured for another 3 days before being used for transplantation.

Pseudo-islets transplantation

During surgery, mice were anesthetized by inhalation of 1.7-1.9% isoflurane (DS Pharma Animal Health Co. Ltd, Osaka, Japan) under sterile conditions. For transplantation into the sub-renal capsule, the capsules of the kidneys were incised, and 150 pseudo-islets were implanted around the upper pole of the left kidney in eight-week-old female mice. For transplantation into the pancreas, MIN6 cells ($1x10^5$) were mixed with Growth Factor Reduced Matrigel (Corning Inc.) and injected directly into the pancreas.

Histological analysis

Extracted pancreata were fixed and embedded in paraffin, and 4 μm sections were cut. For quantitative analysis of cell mass, we examined all parts of the whole pancreas in sections spaced 160 μm apart. All sections were stained with hematoxylin-eosin (HE) or immunostained as previously described (16). Primary antibodies used were: anti-insulin (1:100; Abcam, Cambridge, UK), anti-RFP (1:500; MBL, Nagoya, Japan or 1:1000; Chromotek, Planegg, Germany), anti-glucagon (1:200; Cell signaling technology, Boston, MA or 1:1000; Sigma),

anti-MafA (1:100; Bethyl laboratories, Montgomery, AL), anti-Pdx1 (1:5000; Abcam) and anti-GFP (1:5000; Abcam). Anti-RFP antibody was used to stain Tomato. Secondary antibodies used were anti-IgG antibodies conjugated with Alexa Fluor 488 and 555 for each of the species (anti-guinea pig, anti-rabbit, anti-rat, or anti-chicken) (1:1000; Life Technologies). MafA and Pdx1 immunofluorescence was visualized by the TSA fluorescence system (PerkinElmer, Waltham, MA) according to the manufacture's instructions. A TUNEL assay kit (ApopTag; Millipore) was used to detect apoptosis. We quantified TUNEL-positive cells as the ratio of positive cells per total cell number, by counting at least thirty islets randomly selected (3000-7000 cells in total) per mouse. The nuclei were counterstained with Hoechst 33342 (Sigma). The slides were analyzed by FV10i confocal microscope (Olympus, Tokyo, Japan).

Cell viability assay

Mouse aortic endothelial cells (MAE) (17) were cultured in Ham's F-12 medium (Wako) supplemented with 10% FBS and COS-1 cells (18) were cultured in HG-DMEM containing 10% FBS. MIN6 cells were seeded in 96 well plates (1.5 x 10^4 cells/well) 2 days before the experiment. For cell viability assay, MIN6 cells were cultured for 12 hrs in DMEM containing 1 mM glucose, sodium pyruvate (1

mM), and L-glutamine (4 mM) or in HG-DMEM supplemented with diazoxide (200 μM; Sigma) or Insulin (1 μM; Sigma). HNMPA (100 μM) was added 2 hrs before sampling. Cell viability was determined using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) and the viability was expressed as arbitrary units [A.U.(%)]. Experiments using the same protocol were repeated three times to ascertain reproducibility.

Quantitative real-time PCR

Quantitative real-time PCR was performed under standardized protocol as previously described (19). The primers used were *Ddit3*: Fw 5'GAGCTGGAAGCCTGGTATGA3': Rev 5'ACGCAGGGTCAAGAGTAGTG3' and *Hprt*: Fw 5'-GCGTCGTGATTAGCGATGA-3': Rev 5'-ATGGCCTCCCATCTCCTT-3'.

Statistical analysis

Values are represented as means ± SEM, and tests were performed using SAS version 9.3 (SAS Institute). Comparisons between two groups were assessed using unpaired Student's *t*-test for normally distributed variables. Analysis of multiple comparisons was made using one-way ANOVA followed by Bonferroni *post-hoc*

test. To investigate the relationship between two variables, Peason's correlation coefficient was used. *P* values were considered significant at $P < 0.05$.

RESULTS

Transplantation of pseudo-islets into sub-renal capsule evokes marked reduction in pancreatic β-cell mass

To induce a state of excessive β-cell mass, we transplanted 150 pseudo-islets into the sub-renal capsule of a wild-type mouse. About 2-4 weeks after transplantation, the mice with sub-renal transplantation (SRT mice) developed severe hypoglycemia (Fig. 1A). Pancreata of SRT mice were sampled at differential time points with various degrees of hypoglycemia; the endogenous β-cell mass, as assessed by insulin immunoreactivity, was found to be decreased in the mice exposed to sustained, severe hypoglycemia. The pancreatic islets of SRT mice with severe hypoglycemia were small in size and irregular in shape, and contained fewer insulin-positive (Ins^+) cells along with reduced immunoreactivity (Fig. 1B). The β-cell mass of SRT mice was found to be proportional to the sum (area under the curve) of glycemia for 7 days before sampling (AUC_{Glc}) (Fig. 1C). To consider the cause of the reduction of β-cell mass, we quantified cellular size and apoptosis of

Ins^+ cells (Fig. 1D, E) in SRT mice. Histological analysis revealed that Ins^+ cell size, as well as its mass, was decreased proportionally to AUC_{Glc} (Fig. 1D). By contrast, the frequency of apoptotic Ins^+ cells, as assessed by TUNEL staining, was drastically increased only under severely hypoglycemic (< 300 AUC_{Glc}) conditions (Fig.1E).

Quantitative analyses of SRT mice having < 300 AUC_{Glc} revealed that the Ins^+ cell mass was decreased to 18.3 % (Fig. 1F), Ins^+ cell size was decreased to 54.8% (Fig. 1G), and the frequency of TUNEL-positive ($TUNEL^+$) cells was increased by 15 folds (Fig. 1H) compared with those of the controls.

Glucose and insulin signals are essential for β-cell survival; hyperpolarization does not trigger cell death

We then examined the mechanism of β-cell apoptosis in SRT mice with < 300 AUC_{Glc}. The pancreatic β-cell is electrically excitable, and is reported to require extracellular glucose (20), β-cell firing (21), and insulin signaling (22; 23) for its survival and/or its proliferation. To evaluate the effects of these factors on β-cell survival, we treated MIN6 cells with low glucose, the ATP-sensitive K^+ channel opener diazoxide, high dose of exogenous insulin, or the insulin receptor specific

tyrosine kinase inhibitor HNMPA for 12 hrs, 12 hrs, 12 hrs, and 2 hrs, respectively. Morphological observation by detecting dead cells with trypan blue suggested that cell death was induced mildly by low glucose and fiercely by HNMPA (data not shown). Cell death was then assessed by quantifying the surviving cells after exposure to each stimulus (Fig. 2A). The surviving cell number was mildly but significantly decreased by low glucose treatment. By contrast, treatment with diazoxide or a high concentration of insulin failed to reduce the cell number. Importantly, cell number was markedly reduced by HNMPA treatment.

Imbalance between demand and supply of ATP, including hypoxia, has been reported to induce ER stress and oxidative stress, resulting in cellular apoptosis (24). Considering that intracellular ATP concentrations in pancreatic β-cells should be decreased in SRT mice with severe hypoglycemia, we examined whether low glucose treatment of MIN6 cells induces the expression of CHOP (*Ddit3*), the central player of ER stress-induced apoptotic cell death (25). As expected, low glucose significantly increased gene expression of CHOP (Fig. 2B). In addition, removal of glutamine, another important ATP substrate, from the medium significantly potentiated low glucose induced cell death (Fig. 2C) and CHOP expression (Fig. 2D). By contrast, diazoxide suppressed CHOP expression, while a

high concentration of insulin did not affect CHOP expression. HNMPA mildly induced CHOP expression regardless of its lethal effect (Fig. 2B).

We also examined whether HNMPA-induced cell death is an event that occurs specifically in insulin-secreting β-cells as a consequence of the blockade of autocrine insulin action. For this purpose, we repeated the same experiment in other cell lines: a simian kidney fibroblast-like cell line COS-1 cells and a mouse artery endothelial MAE cells. Interestingly, HNMPA treatment did not induce cell death in either COS-1 cells or MAE cells (Fig. 2E), suggesting that insulin signaling has a role in cell survival specifically in pancreatic β-cells.

Auto/paracrine insulin signal input from neighboring β-cells is required for their survival

Our results in MIN6 cells reveal that attenuation of insulin signaling in pancreatic β-cells triggers their cellular death. In SRT mice having < 300 AUC_{Glc}, the extracellular insulin concentrations in the vicinity of endogenous β-cells may be diminished due to cessation of insulin secretion. Accordingly, apoptotic cell death in β-cells of SRT mice could be attributable to attenuated insulin signaling. To test this, we transplanted the MIN6 cells directly into the pancreas (rather than the sub-renal

capsule) to elevate the extracellular insulin concentrations around the β-cells, and evaluated the frequency of β-cell apoptosis.

By using MIN6 cells stably expressing green fluorescent protein (GFP), we confirmed successful intra-pancreas transplantation (IPT) of MIN6 cells to pancreas, as demonstrated by formation of GFP-positive MIN6 tumors (Fig. 3A). As the time course of development of hypoglycemia in the mice receiving IPT (IPT mice) was comparable to that of SRT mice, we repeated the same analyses in IPT mice.

Similarly to SRT mice, IPT mice showed a decrease in cell mass (Fig. 3B), and cellular size (Fig. 3C) of Ins^+ cells, and an increase in apoptotic β-cells ($TUNEL^+$/ Ins^+ cells) under hypoglycemia (Fig. 3D). As expected, when compared with those in SRT mice, $TUNEL^+$ cells were detected less frequently in hypoglycemic (<300 AUC_{Glc}) IPT mice, the relative abundance of $TUNEL^+$ cells (in Ins^+ cells) being significantly ($p<0.001$) lower in IPT mice (1.22±0.16 %) than that in SRT mice (2.83±0.29 %) (Fig. 3G).

MIN6 cells transplantation to pancreas (rather than kidney) protects endogenous β-cells from cell death, but deprives them of insulin immunoreactivity

Surprisingly, in spite of the lesser apoptosis in Ins^+ cells of hypoglycemic (< 300 AUC_{Glc}) IPT mice (Fig. 3F), their Ins^+ cell mass was much less than that of SRT mice (Fig. 3D). This apparent contradiction might be explained by insulin depletion in β-cells of IPT mice rather than by increase in β-cell death. To assess this possibility, we employed a lineage tracing technique to specifically mark β-cells using *RipCre:Rosa26-tdTomato*, a mouse permanently expressing a red fluorescent protein Tomato in most (~80%) β-cells (Fig. 4A).

Quantitative analyses in *RipCre:Rosa26-tdTomato* mice revealed that the decrease in $Tomato^+$ cell mass was significantly milder after IPT than that after SRT (Fig. 4B). We then examined insulin immunoreactivity in $Tomato^+$ cells (i.e., cells that once had expressed the insulin gene) of IPT-treated *RipCre:Rosa26-tdTomato* mice. Interestingly, insulin-negative but Tomato-positive ($Ins^-/Tomato^+$) cells were frequently found after IPT, but only occasionally after SRT (Fig. 4C).

Expression of MafA and subcellular-localization of Pdx1 was altered similarly in SRT and IPT mice with severe hypoglycemia

The appearance of $Ins^-/Tomato^+$ cells in IPT-treated *RipCre:Rosa26-tdTomato* mice (Fig. 4C) prompted us to evaluate protein expression of the critical genes in

β-cells: MafA and Pdx1. Immunostaining of pancreata of IPT-treated *RipCre:Rosa26-tdTomato* mice revealed a marked decrease in MafA expression and cytoplasmic distribution of Pdx1 in Tomato^{+} cells (Fig. 5A, B). Similar change was also observed in SRT mice (Fig. 5A, B)

Transplant removal induced functional and morphological recovery of the residual β-cells in SRT mice

We next examined whether the deterioration of β-cells in hypoglycemic SRT mice is reversible. Hypoglycemic (< 300 AUC_{Glc}) SRT mice were subjected to transplant removal by nephrectomy, which induced a rapid and drastic shift in glycemic levels from hypoglycemia to a transient (< 4 days) hyperglycemia (peaking at 431.4 ± 75.0 mg/dl, n = 5), followed by sustained normoglycemia (Fig. 5A). Since the time-course of glycemia strongly suggests functional recovery of endogenous β-cells, we next examined morphological changes of islets in nephrectomized-SRT mice on day 4. We found that the attenuated immunoreactivity of insulin and MafA and cytoplasmic expression of Pdx1 in SRT mice was normalized 4 days after nephrectomy (Fig. 5B, C). Morphological analyses of the islets revealed a

significant (2.2 fold) increase in Ins^+ cell mass (Fig. 5D) and enlargement of Ins^+ cell size (Fig. 5E), which accords with normalization of glycemic control.

DISCUSSION

In healthy individuals, β-cell mass is steadily maintained throughout life with slow turnover (26). In addition, in contrast to the β-cell mass decrease in T2DM, their marked hypertrophy, so-called nesidioblastosis, has recently been shown to occur after bariatric surgery, which leads to hyperinsulinemic hypoglycemia (27). Accordingly, β-cell mass is likely to be regulated strictly in response to both its surplus and shortage through an intricate mechanism but yet to be elucidated. In the present study, using mice transplanted with pseudo-islets of MIN6 cells, we found that the cell mass and function of endogenous β-cells is substantially jeopardized by the existence of excessive β-cells. Interestingly, although SRT mice exhibited both increased β-cell apoptosis and decreased β-cell mass, the two phenotypes may well be induced by distinct mechanisms, as the former occurs in proportion to the decrease of AUC_{Glc} while the latter occurs only under severely hypoglycemic conditions.

Notably, the attenuated apoptotic β-cell death in IPT mice (compared to SRT mice) strongly suggests that this difference can be attributed to the humoral factors released from the transplanted β-cells, including insulin, Zn^{2+} (28), islet amyloid polypeptide (IAPP) (29), and GABA (30). Among these, insulin signaling in β-cell could play a critical role, as the blockade of insulin signaling by the insulin receptor antagonist HNMPA markedly induced apoptotic cell death in MIN6 cells (Fig. 2A).

The importance of insulin signaling on β-cell function and mass has been demonstrated in many studies. Especially, analyses of genetically engineered mice deficient in any insulin signaling molecule revealed that the signaling is essential for both β-cell survival and its function (23; 31). Mice lacking the insulin receptor in β-cells exhibited loss of glucose-responsive insulin secretion, progressive impairment of glucose tolerance (32), and decreased β-cell mass (22). IRS2 deficient mice showed developed late onset hyperglycemia associated with progressive decrease in β-cell mass (33). In addition, insulin receptor and IRS2 are required for the actions of glucose on β-cell survival and proliferation (34). Moreover, other downstream signaling molecules, such as Akt (35), PDK1 (36), and mTOR (37), are known to play a role in β-cell survival and its proliferation.

On the other hand, the physiological relevance of insulin signaling in β-cells has long been questioned by many researchers. Rhodes *et al.* question the concept of a physiological role of autocrine action of insulin on β-cells (38): 1) chronic exposure to insulin secreted from β-cells themselves should downregulate insulin receptor expression of β-cells and desensitize the IRS signaling pathway; 2) secreted insulin from β-cells should be rapidly cleared from the islet, in which microcirculation directs blood flow away from β-cells via the other pancreatic endocrine islet cell types; 3) there are many untested growth factors other than insulin that might act on the IRS signaling pathway in β-cells. These issues remain difficult to be answered convincingly. While definitive evidence for the importance of local insulin in β-cell survival is still lacking, our present findings of attenuated β-cell apoptosis in IPT mice and induction of MIN6 cell death by the insulin receptor antagonist strongly suggest that insulin secreted from neighboring β-cells may play an important role in β-cell survival.

We observed a drastic decrease in Ins^+ cell number in both SRT and IPT mice as well as a decrease in immunoreactivity in the pancreas. As such decrease could be due to either a decrease in β-cell number and/or loss of insulin protein expression in β-cells, we performed cell lineage tracing experiments using

RipCre:Rosa26-tdTomato mice. Analyses with Tomato^{+} cells revealed milder β-cell loss (associated with decreased β-cell apoptosis) in IPT mice than that in SRT mice, suggesting that β-cell death could be alleviated through a direct action of insulin released from the intra-pancreatic transplants.

To our surprise, Ins^{-}/Tomato^{+} cells (β-cells without insulin protein) were frequently detected in IPT mice, but only sparsely in SRT mice. These results suggest that local humoral factors, most likely insulin, protect endogenous β-cells from cell death at the expense of insulin secretory capacity. Interestingly, Goginashvili *et al.* recently reported that pancreatic β-cells evoke insulin granule destruction under metabolic deprivation through a p38δ/PDK1 signaling pathway (39). Local insulin signaling could be involved in insulin degranulation in Tomato^{+} cells after IPT through this mechanism.

By subjecting MIN6 cells to low glucose and/or glutamine-deficient conditions, we demonstrated that deficiency of ATP substrate induces CHOP expression, which is a central player in apoptotic β-cell death induced by ER stress (25). This suggests a mechanism of the contribution of severe hypoglycemia to apoptotic cell death in SRT and IPT mice.

ER and oxidative stress can be induced by insufficient supply of nutrients and oxygen leading to apoptotic cell death of pancreatic β-cells in the situation of hyper-function and compensatory hyperplasia under hyperglycemic condition (40). As glutamine removal failed to induce cell death under high (25mM) glucose condition, the shortage of ATP substrates (either glucose or amino acids) is likely to trigger ER stress, as demonstrated by CHOP induction, contributing to the cell death in this experimental condition. Diazoxide did not affect surviving cell number and significantly suppressed CHOP gene expression. Taken together with the previous report that diazoxide increased the ATP/ADP ratio in rat islets (41), membrane hyperpolarization is unlikely to participate in the β-cell death seen in SRT mice.

In the present study with MIN6 cells, cell death was induced markedly by HNMPA but only mildly by low glucose, while CHOP expression was increased markedly by low glucose but only mildly by HNMPA. Accordingly, the mechanism of cell death of MIN6 cells by HNMPA is likely to be distinct from that by low glucose. Moreover, considering that HNMPA-induced cell death occurred only in MIN6 cells, insulin signaling might well fulfill a role in cell survival specifically in pancreatic β-cells.

Immunostaining with MafA and Pdx1 helped us to understand the functional state of β-cells in IPT-treated *RipCre:Rosa26-tdTomato* mice. We found a marked decrease in MafA expression and cytoplasmic distribution of Pdx1 in β-cells of IPT-treated *RipCre:Rosa26-tdTomato* mice, in which $Ins^-/Tomato^+$ cells were frequently detected. However, these findings on MafA and Pdx1 did not support dedifferentiation of β-cells (42), as similar findings were also observed in SRT mice in which $Ins^-/Tomato^+$ cells were barely detected.

Alternatively, considering the significant reduction in MafA expression by low glucose treatment in MIN6 cells (data not shown), our finding suggests that hypoglycemia directly or indirectly inhibits functions of MafA and Pdx1 in β-cells of SRT mice and IPT mice, which should contribute to the suppression of insulin secretion in addition to its hyperpolarizing action. Both MafA and Pdx1 participate synergistically in the transcriptional regulation of the insulin gene (43; 44). MafA is a key regulator of various genes implicated in the maintenance of β-cell function (43), while Pdx1 is critical in subsequent β-cell regeneration and maintenance of β-cell mass (42).

The level of MafA is known to be regulated tightly in response to a change in glucose levels through transcriptional and/or post-transcriptional mechanisms. By

contrast, Pdx1 function has been reported to be regulated through its nucleo-cytoplasmic translocation as well as its transcription (45; 46). Thus, changes in MafA and Pdx1 in β-cells of SRT and IPT mice may contribute to their dysfunction under severe hypoglycemia.

Normalization of hypoglycemia within 4 days after transplant removal might suggest functional recovery of β-cells, although the recovery of Ins^+ cell mass and Ins^+ cell size remained marginal (~ 2 folds). Previously, Miyaura *et al.*conducted a similar experiment, in which the changes in pancreatic islets were precisely examine in the rats subjected to transplantation and removal of an insulinoma tumor (47). Their findings were quite similar to ours except for the better recovery in β-cell mass in their experiment. Although the reason for this discrepancy is unknown at present, the difference in severity and/or duration of the hypoglycemia might be involved.

By contrast, rapid restoration of MafA and Pdx1 expressions in β-cells occurred in parallel to normalization of glycemia. Thus, insulin replenishment and not β-cell proliferation played the substantial role in the recovery of β-cell function. Hyperglycemia induced by transplant removal in SRT mice restored the insulin

secretory capacity of once-flawed β-cells, possibly through normalizing MafA and Pdx1, the two major transcription factors critical for normal β-cell function.

In conclusion, our present study demonstrates that local insulin action on the β-cell is critical for β-cell survival. Although the importance of insulin signaling in β-cells has been shown in various genetically engineered mouse models with defective insulin signaling, our study differs in that marked β-cell death can be induced in wild-type mice by severe sustained hypoglycemia. Our study also shows that blood glucose levels that are maintained through systemic insulin action may play a critical role in the regulation of β-cell mass. Accordingly, insulin plays distinct roles in pancreatic β-cell survival and regulation of β-cell mass through direct and indirect action on β-cells.

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Figure. 1

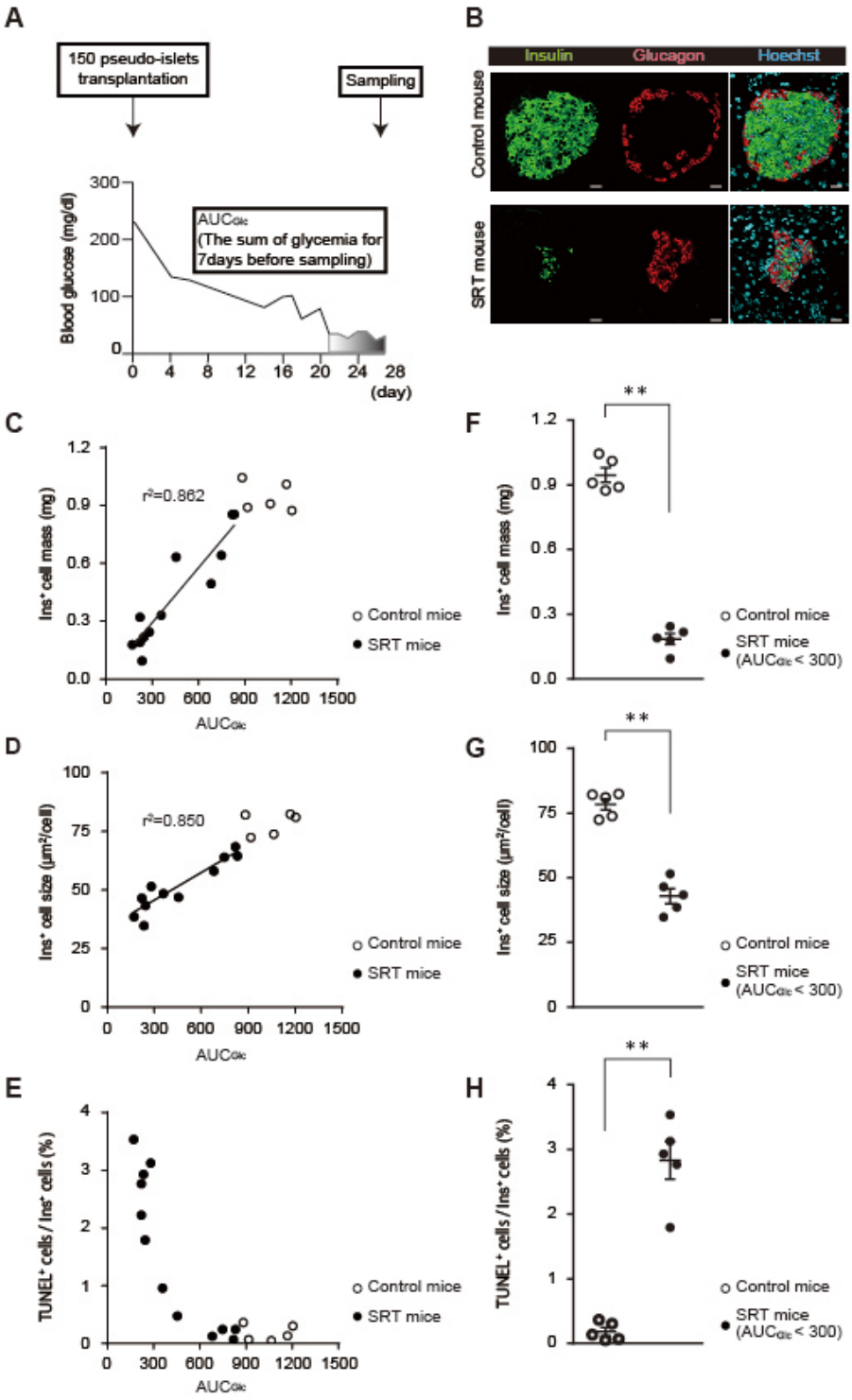
A
150 pseudo-islets transplantation
Sampling
Blood glucose (mg/dl)
AUCGlc
(The sum of glycemia for 7days before sampling)
(day)
B
Insulin
Glucagon
Hoechst
Control mouse
SRT mouse
C
Ins+ cell mass (mg)
r²=0.862
AUCGlc
Control mice
SRT mice
D
Ins+ cell size (μm²/cell)
r²=0.850
AUCGlc
Control mice
SRT mice
E
TUNEL+ cells / Ins+ cells (%)
AUCGlc
Control mice
SRT mice
F
Ins+ cell mass (mg)
**
Control mice
SRT mice (AUCGlc < 300)
G
Ins+ cell size (μm²/cell)
**
Control mice
SRT mice (AUCGlc < 300)
H
TUNEL+ cells / Ins+ cells (%)
**
Control mice
SRT mice (AUCGlc < 300)

Figure 1 - Transplantation of pseudo-islets into subrenal capsule influences pancreatic β-cell mass and survival.

(A) Representative transition of blood glucose levels in an eight-week-old control mouse after pseudo-islets transplantation. Blood glucose levels of the mice were measured in the same time (16:00-18:00) every other day and AUC_{Glc} was calculated. (B) Comparison of representative immunofluorescence images between control mouse and SRT mouse having < 300 AUC_{Glc}. (C, D) Relationship between control mice without transplantation (white circles; $n = 5$) and SRT mice (black circles; $n = 11$). Correlation coefficient is calculated with only SRT mice. (E) Relationship between the frequency of $TUNEL^+$ cells in Ins^+ cells and AUC_{Glc}. (F-H) Quantification of Ins^+ cell mass, size and the frequency of $TUNEL^+$ cells (control mice vs. SRT mice with < 300 AUC_{Glc}). Data are mean ± SEM. r = Pearson's correlation coefficient. $^{**}P < 0.01$ vs. control mice, Scale bars = 20 μm

Figure. 2

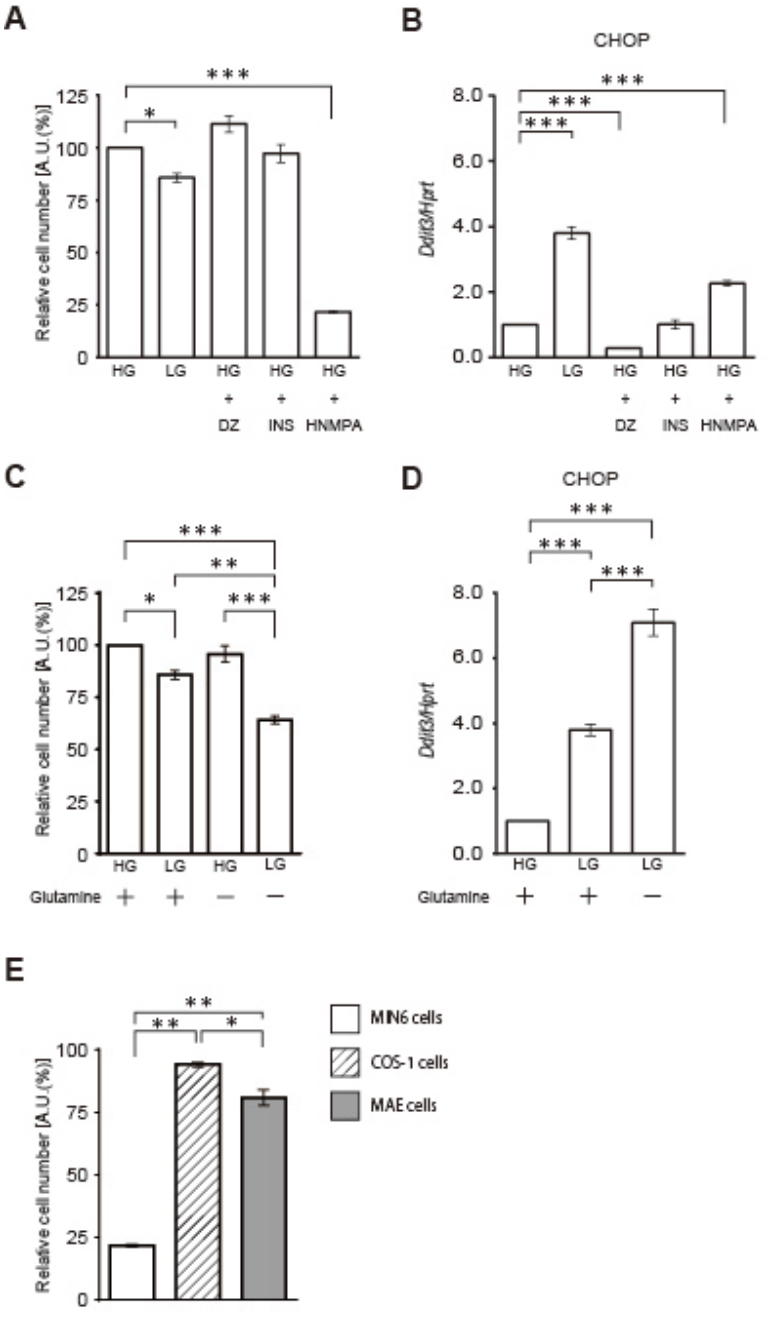
A
Relative cell number [A.U.(%)]
HG
LG
HG + DZ
HG + INS
HG + HNMPA
B
CHOP
Ddit3/Hprt
C
Glutamine
D
E
MIN6 cells
COS-1 cells
MAE cells

Figure 2 - Insulin signaling is essential for cell survival specifically in pancreatic β-cells.

(A) Viability of control (MIN6 cells cultured in HG-DMEM (HG)) and MIN6 cells with low (1 mM) glucose (LG), diazoxide (DZ), 1 μM insulin (INS), and HNMPA treatment. (B) mRNA expression levels of *Ddit3* in MIN6 cells with each treatment. (C-D) Cell viability assay and mRNA expression levels of *Ddit3* for MIN6 cells cultured in HG or LG with/without glutamine. (E) Cell viability assay for MIN6, COS-1, and MAE cells with HNMAP treatment. All values represent means ± SEM obtained from 3 independent experiments. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Figure. 3

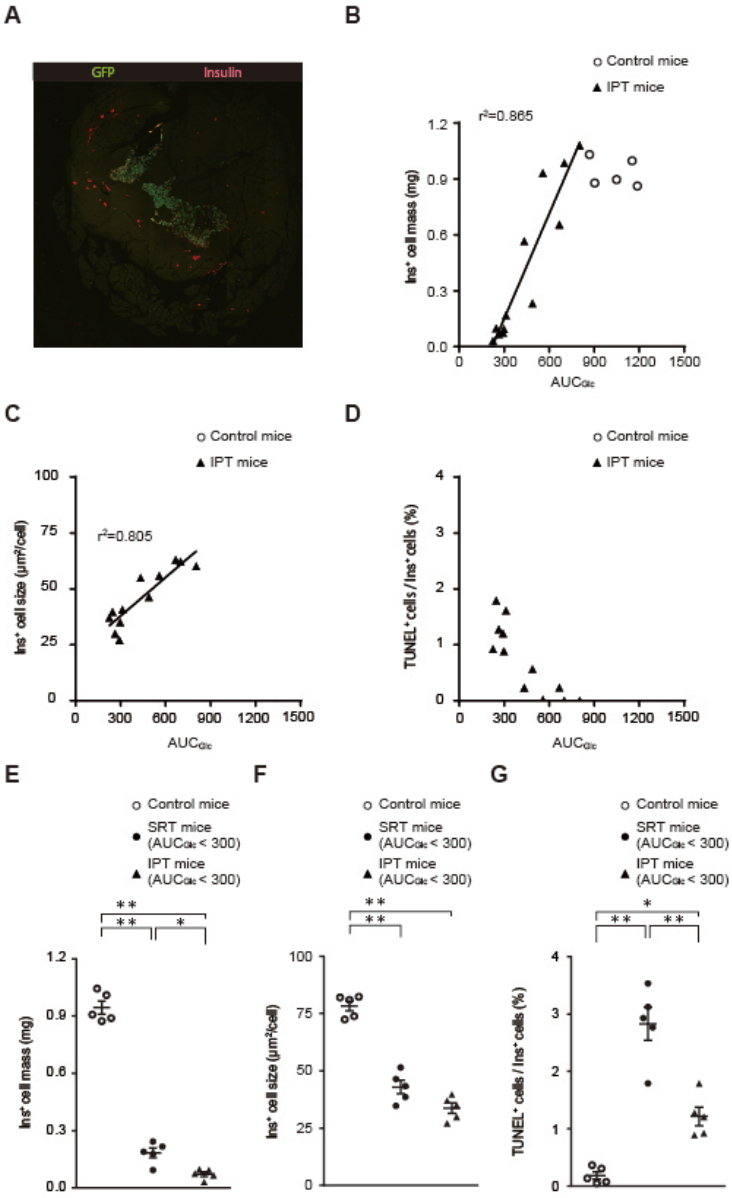
A
GFP
Insulin
B
Control mice
IPT mice
r²=0.865
Ins⁺ cell mass (mg)
AUCGlc
C
Control mice
IPT mice
r²=0.805
Ins⁺ cell size (μm²/cell)
AUCGlc
D
Control mice
IPT mice
TUNEL⁺ cells / Ins⁺ cells (%)
AUCGlc
E
Control mice
SRT mice (AUCGlc < 300)
IPT mice (AUCGlc < 300)
Ins⁺ cell mass (mg)
F
Control mice
SRT mice (AUCGlc < 300)
IPT mice (AUCGlc < 300)
Ins⁺ cell size (μm²/cell)
G
Control mice
SRT mice (AUCGlc < 300)
IPT mice (AUCGlc < 300)
TUNEL⁺ cells / Ins⁺ cells (%)

Figure 3 - β-cell death is suppressed by MIN6 cells transplantation to pancreas. (A) Discrimination by double immunofluorescence staining of GFP (green) and Ins (red) could discriminate MIN6 cells from endogenous β-cells. (B, C) Relationship between control mice without transplantation (white circles; $n = 5$) and IPT mice (black triangles; $n = 12$). Correlation coefficient is calculated with only IPT mice. (E) Relationship between the frequency of $TUNEL^+$ cells in Ins^+ cells and AUC_{Glc}. (E-G) Quantification of Ins^+ cell mass, size and frequency of $TUNEL^+$ cells (control mice, SRT mice having < 300 AUC_{Glc}, and IPT mice having < 300 AUC_{Glc}). Data are mean ± SEM. r = Pearson's correlation coefficient. $^*P < 0.05$, $^{**}P < 0.01$

Figure. 4

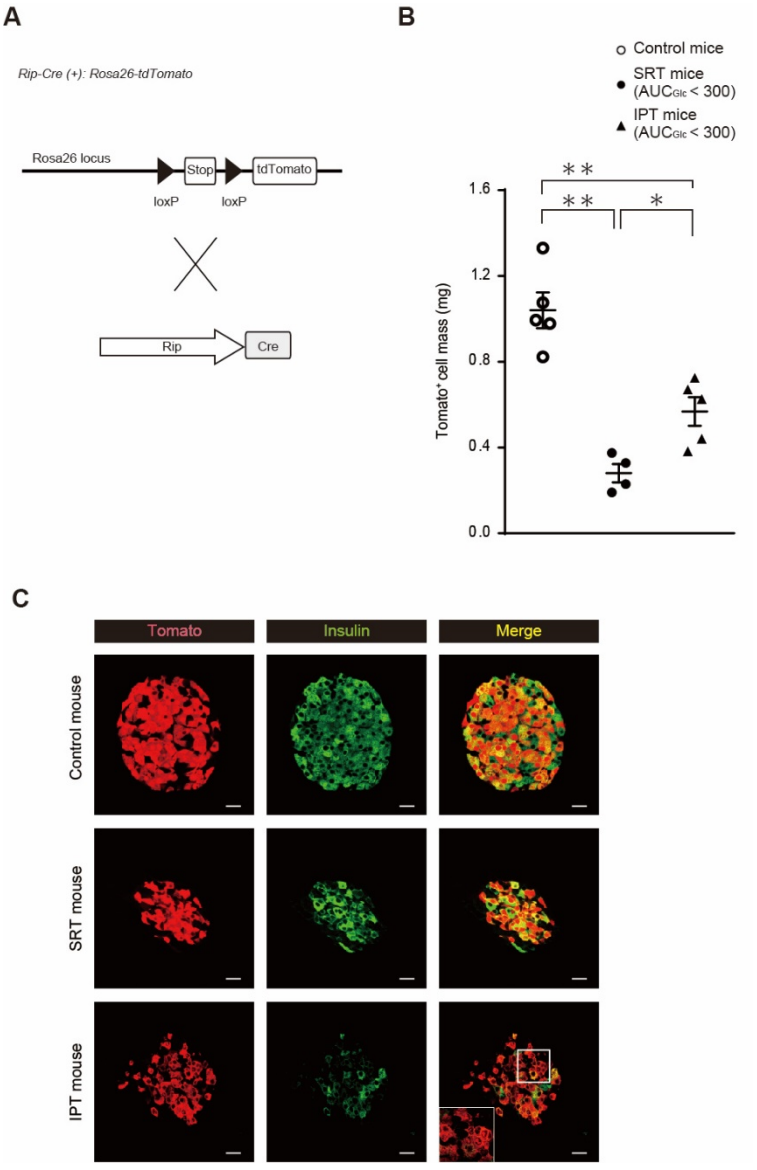
A
Rip-Cre (+): Rosa26-tdTomato
Rosa26 locus
Stop
tdTomato
loxP
loxP
Rip
Cre
B
Control mice
SRT mice
(AUCGlc < 300)
IPT mice
(AUCGlc < 300)
**
**
*
Tomato+ cell mass (mg)
1.6
1.2
0.8
0.4
0.0
C
Tomato
Insulin
Merge
Control mouse
SRT mouse
IPT mouse

Figure 4 - β-cells in IPT mice are protected from cell death in comparison with those in SRT mice; insulin immunoreactivity is suppressed.

(A) Scheme of *RipCre: Rosa26-tdTomato* mice. (B) Quantitative analysis of RFP^+ cell mass of control, SRT, and IPT mice (n = 4-5 each group). (C) Immunofluorescence of RFP (red) and insulin (green) in *RipCre: Rosa26-tdTomato* mice with SRT or IPT. $Ins^-/Tomato^+$ cells are detected frequently after IPT. Data are mean ± SEM. Scale bars = 20 μm $^*P < 0.05$, $^{**}P < 0.01$

Figure. 5

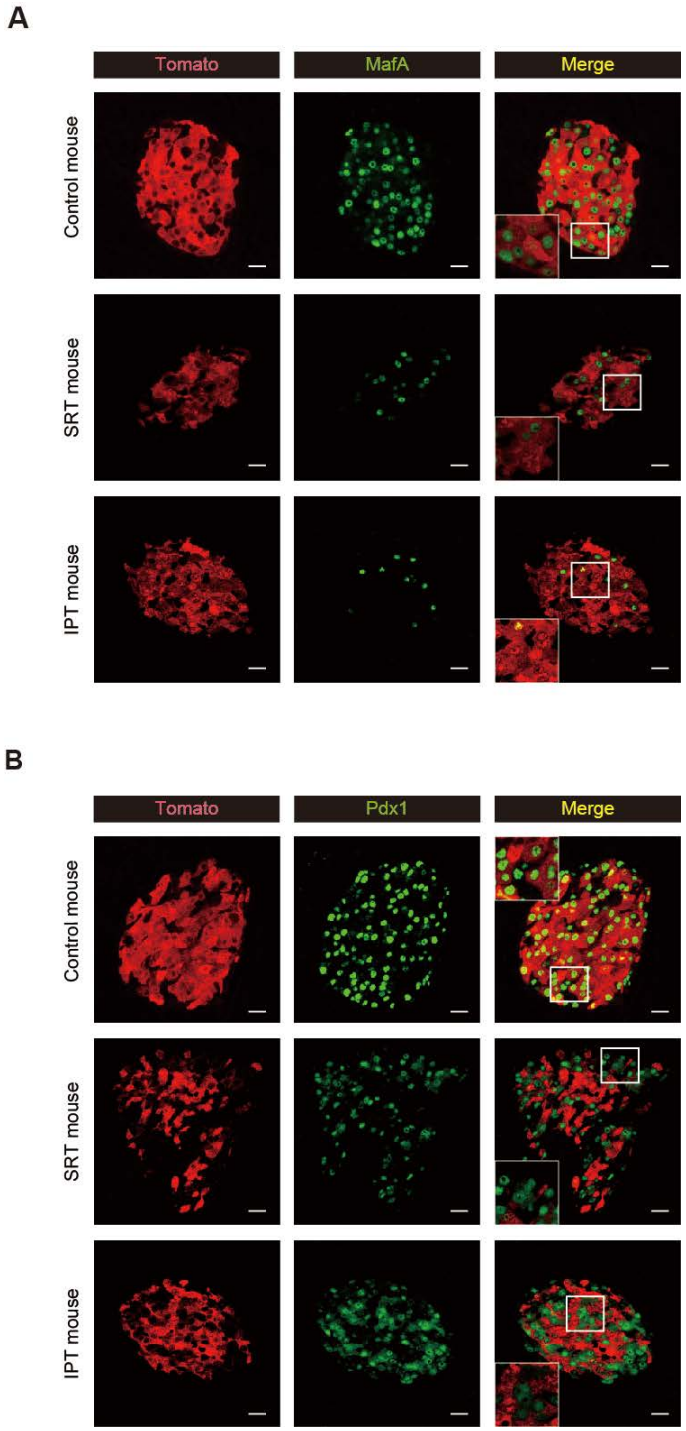
A
Tomato
MafA
Merge
Control mouse
SRT mouse
IPT mouse
B
Tomato
Pdx1
Merge
Control mouse
SRT mouse
IPT mouse

Figure 5 - Both SRT and IPT show decreased expression level of MafA and altered cytoplasmic distribution of Pdx1.

(A and B) Representative images showing co-staining for Tomato (red) and MafA (green, A) or Tomato and Pdx1 (green, B) in control, SRT mice, and IPT mice. Scale bars = 20 μm

Figure. 6

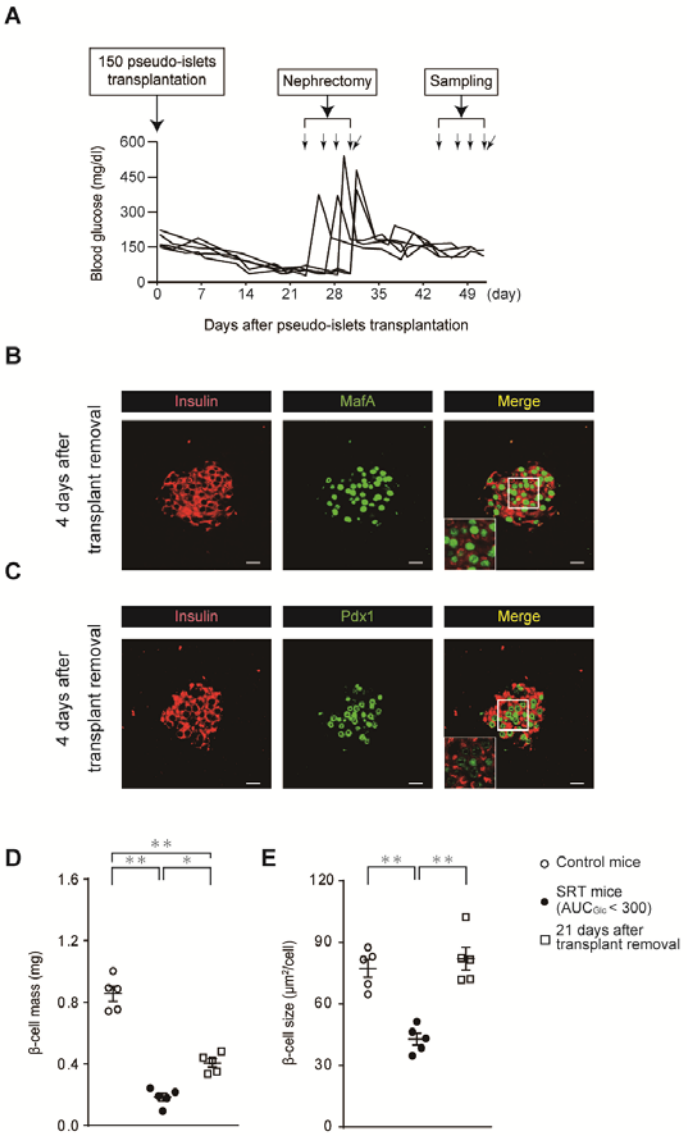
A
150 pseudo-islets transplantation
Nephrectomy
Sampling
Blood glucose (mg/dl)
600
450
300
150
0
0 7 14 21 28 35 42 49 (day)
Days after pseudo-islets transplantation
B
Insulin
MafA
Merge
4 days after transplant removal
C
Insulin
Pdx1
Merge
4 days after transplant removal
D
β-cell mass (mg)
1.6
1.2
0.8
0.4
0.0
E
β-cell size (μm²/cell)
120
90
60
30
0
Control mice
SRT mice (AUCGlc < 300)
21 days after transplant removal

Figure 6 - β-cell function and Ins^+ cell mass and size are recovered after transplant removal.

(A) Blood glucose levels before and after transplant removal. An arrow indicates the time point of nephrectomy of each mouse. (B and C) Representative images showing staining for Tomato (red) and MafA (green, B) or Tomato and Pdx1 (green, C) 4 days after transplant removal. (D) Comparison of β-cell mass and size among control (white circles), SRT mice having < 300 AUC_{Glc} (black circles), and SRT mice after transplantation removal. Data are mean ± SEM. Scale bars = 20 μm. $^{*}P < 0.05$, $^{**}P < 0.01$

Diabetes

平成 28 年 12 月 14 日　　投稿中