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Roles of FoxM1-driven basal β-cell proliferation in maintenance of β-cell mass and glucose tolerance during adulthood

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ABSTRACT

Aims/Introduction: Whether basal β-cell proliferation during adulthood is involved in maintaining sufficient β-cell mass, and if so, the molecular mechanism(s) underlying basal β-cell proliferation remain unclear. FoxM1 is a critical transcription factor which is known to play roles in 'adaptive' β-cell proliferation, which facilitates rapid increases in β-cell mass in response to increased insulin demands. Therefore, herein we focused on the roles of β-cell FoxM1 in 'basal' β-cell proliferation under normal conditions and in the maintenance of sufficient β-cell mass as well as glucose homeostasis during adulthood.

Materials and Methods: FoxM1 deficiency was induced specifically in β-cells of 8-week-old mice, followed by analyzing its short- (2 weeks) and long- (10 months) term effects on β-cell proliferation, β-cell mass, and glucose tolerance.

Results: FoxM1 deficiency suppressed β-cell proliferation at both ages, indicating critical roles of FoxM1 in basal β-cell proliferation throughout adulthood. While short-term FoxM1 deficiency affected neither β-cell mass nor glucose tolerance, long-term FoxM1 deficiency suppressed β-cell mass increases with impaired insulin secretion, thereby worsening glucose tolerance. In contrast, the insulin secretory function was not impaired in islets isolated from mice subjected to long-term β-cell FoxM1 deficiency. Therefore, β-cell mass reduction is the primary cause of impaired insulin secretion and deterioration of glucose tolerance due to long-term β-cell FoxM1 deficiency.

Conclusions: Basal low-level proliferation of β-cells during adulthood is important for maintaining sufficient β-cell mass and good glucose tolerance and β-cell FoxM1 underlies this mechanism. Preserving β-cell FoxM1 activity may prevent the impairment of glucose tolerance with advancing age.

INTRODUCTION

Pancreatic β-cells secrete insulin in response to systemic demand, thereby preventing blood glucose elevation. Therefore, sustaining appropriate β-cell mass is critical for the maintenance of glucose homeostasis. Indeed, in not only type 1 but also in type 2 diabetes, a reduction of β-cell mass and the resultant impairment of insulin secretion play a critical role in the pathogenesis of diabetes.[1]

Several lines of evidence indicate that even terminally differentiated β-cells retain significant proliferative capacity *in vivo*.[2-5] Under various physiological and pathological conditions, such as pregnancy[6] and obesity,[7,8] β-cells proliferate and increase their mass. In contrast to such adaptive proliferation, basal β-cell proliferation rates under normal conditions are very low during adulthood,[5,9] compared with those in other intra-abdominal organs, such as the liver, gastrointestinal tract, and the exocrine pancreas, which show ongoing active cell proliferation.[10-13] The extent of involvement of basal and low-level proliferation in maintaining sufficient β-cells during adulthood

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remains unclear. In addition, if basal β-cell proliferation is important, identifying the elusive molecules that play important roles in the underlying mechanism is essential.

FoxM1 is a critical transcription factor in cell cycle progression. This mitogenic transcription factor modulates several aspects of the cell cycle,[14] such as promotion of G1/S transition by triggering the transcription of several cyclins, including cyclin A (Ccna),[15-17] and the control of the proper progression of mitosis by increasing the expression of several mitotic genes, such as polo-like kinase 1 (Plk1).[18] In addition, FoxM1 is known to function during cellular proliferation in various cell types.[19-21] As for β-cells as well, expression of an activated form of FoxM1 in middle-aged mice, i.e., those 4–12 months old, promotes β-cell proliferation.[22] In addition, FoxM1 reportedly plays roles in 'adaptive' β-cell proliferation, which facilitates rapid increases in β-cell mass in response to alterations of insulin demands, such as the situation after partial pancreatectomy,[23] during pregnancy,[24] and in obesity settings.[8] On the other hand, the roles of FoxM1 in 'basal' β-cell proliferation remain unclear. Therefore, we herein focused on the roles of β-cell FoxM1 in the basal β-cell proliferation during adulthood.

The necessity of FoxM1 for the maintenance of adequate β-cell mass during growth periods (until 9 weeks of age) was reported previously using congenital pancreas-specific FoxM1 knockout mice, which had been generated by crossing pancreatic and duodenal homeobox 1 (pdx1)-Cre mice and FoxM1-floxed mice.[25] However, in this mouse model, FoxM1 deficiency from the embryonic period in the whole pancreas had great impacts on islet structure and growth until 9 weeks of age. Therefore, the effects of FoxM1 deficiency, exclusively in β-cells after the development of islet structure, on the maintenance of β-cell mass during adulthood, merit intensive study. For this purpose, we generated inducible β-cell-specific FoxM1 knockout mice, and induced FoxM1 gene deficiency at 8 weeks of age to evaluate the effects of FoxM1 deficiency on β-cells, followed by analyzing β-cells and glucose metabolism in these knockout mice at approximately 1 year of age, to explore the long-term effects of β-cell FoxM1 on the maintenance of β-cell mass during adulthood.

MATERIALS AND METHODS

Animals

The rat insulin 2 promoter-CreER (RIP-CreER)[2] mice with a mixed genetic background were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). These mice, which had been crossed more than 15 times with C57BL/6N mice were purchased from SLC Japan (Shizuoka, Japan). To obtain tamoxifen-inducible β-cell-specific FoxM1 knockout (iβ-FoxM1KO) mice, we crossed RIP-CreER mice and FoxM1flox/flox mice.[26] At 8 weeks of age, RIP-CreER +/–; FoxM1flox/flox mice and RIP-CreER –/–; FoxM1flox/flox mice (as controls) were injected intraperitoneally with tamoxifen (Combi-Blocks, San Diego, CA, USA) dissolved in corn oil (Sigma, St Louis, MO, USA), at 80 μg/g body weight every 24 h for 5 consecutive days. All experiments used male mice that were either 10 weeks or approximately 1 year (48–53 weeks) of age, and all were housed in a controlled environment (room temperature 25°C) with a 12 h light–dark cycle. The animal studies were conducted in accordance with the Tohoku University institutional guidelines. Ethics approval was obtained from the Institutional Animal Care and Use Committee of the Tohoku University Environmental & Safety Committee.

Islet isolation and insulin secretion study

Islets were isolated from control- and iβ-FoxM1KO mice at 10 weeks of age and at 1 year of age by retrograde injection of cold Hanks' balanced salt solution containing 1.0 mg/mL collagenase V (Sigma) into the pancreatic duct. The pancreases were digested for 8.5 min at 37°C, and the islets were then collected by hand-picking using a microscope as described previously.[27]

For insulin secretion studies, isolated islets from 1-year-old control and iβ-FoxM1KO mice were maintained overnight in RPMI1640 medium containing 10% fetal calf serum, 25 mM glucose, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamycin at 37°C with 5% CO_2 and 95% air. The following day, batches of 10 islets were pre-incubated at 37°C for 30 min with Krebs-Ringer bicarbonate HEPES buffer (KRBH; 135 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, and 0.1% BSA) containing 1.67 mM glucose, then incubated for 60 min in KRBH with 1.67 or 16.7 mM glucose. Insulin contents were measured after acid-ethanol treatment (77.5 mM HCl in 75% ethanol) extraction.

Quantitative RT-PCR analysis

Total RNA was extracted from 50 isolated mouse islets using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Using a 100 ng quantity of RNA, cDNA synthesis was performed with a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Real-time PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-rad Laboratories, Hercules, CA, USA). The relative amount of mRNA was calculated with *Actb* as the invariant control using the Ct method. Primer sequences were as follows: *Actb*, forward, 5'-GATGCCC TGAGGCTCTT-3′; *Actb*, reverse, 5'-TGTGTTGGCATAGAGG TCTTTAC-3′; *Foxm1*, forward, 5'-GCTCCATAGAAATGTGA CCATC-3′; *Foxm1*, reverse, 5'-AACCTTCACTGAGGGCTGT AAC-3′; *Ccna2*, forward, 5'-CCTTAAGTACCTGCCTTCA CTC-3′; *Ccna2*, reverse, 5'-ACAAGGCTTAAGACTCTCCA-3′; *Plk1*, forward, 5'-ACGGCACCGTGCAGATTA-3′; *Plk1*, reverse, 5'-AGGCGGTACGTTTGGAAGTC-3′; *Mafa*, forward, 5'-TGC AGCAGCGGCACATTCT-3′; *Mafa*, reverse, 5'-CTTGTACAG GTCCCGCTCCTTG-3′; *Pdx1*, forward, 5'-GAAATCCACC AAAGCTCACG-3′; *Pdx1*, reverse, 5'-CGGGTTCCGCTGTG TAAG-3′; *Ins2*, forward, 5'-CCACCACCTTCCAGCTCA-3′; *Ins2*, reverse, 5'-GCAGTACGGGTCCTCTTGTT-3′; *Slc2a2*, forward, 5′- AGCCAGCCTGTGTATGCAAC-3′; and *Slc2a2*, reverse, 5′- CGTAACTCATCCAGGCGAAT-3′.

Glucose and insulin tolerance tests

Glucose tolerance tests were performed on mice fasted for 16 h. The mice were injected with glucose (2 g/kg of body weight) intraperitoneally, followed by blood glucose measurement employing Glutestmint (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Insulin tolerance tests were performed on *ad libitum* fed mice. Human regular insulin (0.25 or 0.5 U/kg of body weight) was injected into the intraperitoneal space. Plasma insulin levels were measured using a mouse/rat insulin ELISA kit (Morinaga, Tokyo, Japan) as described previously.[28]

β-Cell mass measurement

Excised whole pancreatic tissues were fixed with 10% formalin at 4°C overnight and embedded in paraffin. Pancreatic sections of 3 μm thickness were made at an interval of 100 μm, and then immunostained with insulin antibody (I2018; Sigma). Five sections per sample were analyzed by observing sections, selected at random, of the entire pancreas from head to tail. Immunoreactivity was then visualized by incubation with a substrate solution containing 3,3'diaminobenzidine tetrahydrochloride. After immunostaining, total pancreatic and insulin-positive areas in each section were measured using BIOREVO BZ-X710 and BZ-II Analyzers (Keyence, Osaka, Japan). We then determined the β-cell mass, calculating the average ratio of the total insulin-positive area to the total pancreatic area and multiplied this ratio by total pancreatic weight.

Immunohistochemistry

Pancreatic tissues were excised and fixed in 10% formalin at 4°C overnight and embedded in paraffin. Pancreatic sections of 3 μm thickness were made at an interval of 100 μm. The specimens were stained with insulin (IR002; Dako, Carpinteria, CA, USA), glucagon (MAB1249; R&D Systems, Minneapolis, MN, USA), and somatostatin (MAB354; Millipore, Temecula, CA, USA) using the respective primary antibodies. Alexa Fluor 647 donkey anti-guinea pig IgG (706–605-148; Jackson ImmunoResearch, West Grove, PA, USA), Alexa Fluor 488 donkey anti-mouse IgG (715–545-151; Jackson ImmunoResearch), and Alexa Fluor 594 donkey anti-rat IgG (712–585-153; Jackson ImmunoResearch) were used as secondary antibodies. DAPI (D9542; Sigma) was used for nuclear staining.

For Ki67 *in situ* detection, the specimens were stained with Ki67 (#12202; Cell Signaling Technology, Danvers, MA, USA) and insulin (I2018, Sigma) using the respective primary antibodies. Alexa Fluor 488 goat anti-mouse IgG (ab150117; Abcam, Cambridge, UK) and Alexa Fluor 594 conjugate (#8889; Cell Signaling Technology) were used as secondary antibodies. For β-cell analysis, at least 25 islets and over 1,000 nuclei per sample were counted.

For terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) *in situ* detection, the specimens were processed using a DeadEnd™ Colorimetric TUNEL System (G7360; Promega Corporation, Madison, WI, USA) to detect DNA fragmentation associated with apoptosis. One percent methyl green stain solution (Muto Pure Chemicals, Tokyo, Japan) was used for nuclear staining. For analysis, at least 20 islets and over 1,000 nuclei per sample were counted. Insulin-deficient diabetes and TUNEL positive model mice were created by intraperitoneal infusion of 50 mg/kg per body weight of streptozotocin (Sigma) for 5 consecutive days. Streptozotocin was dissolved in 0.05 M citrate sodium buffer (pH 4.5) and injected into 8-week-old C57BL/6N mice.

Immunoblotting

Islet samples were boiled in Laemmli buffer containing 10 mM dithiothreitol at 100°C for 5 min, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, then blocked in Tris-buffered saline with 3% fetal bovine serum. Nitrocellulose membranes were incubated with primary antibodies to FoxM1 (ab207298, Abcam) at a 1:2,000 dilution and Actin (A2066, Sigma) at a 1:5,000 dilution, and then incubated with a secondary horseradish peroxidase-conjugated antibody (NA9340; GE Healthcare, Tokyo, Japan) at a 1:10,000 dilution. These antibodies were dissolved in Can Get Signal (Toyobo). As blottings of FoxM1 and actin were performed using the same membrane. Quantitative data were obtained by employing the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and the ChemiDoc Touch Imaging System (Bio-rad Laboratories). Relative intensities were standardized employing actin intensity as the invariant control using ImageJ Fiji. Uncropped images are presented in Figure S1.

Statistical analyses

All data are expressed as mean ± SEM. The statistical significance of differences between two groups was assessed using the two-tailed unpaired *t*-test. Analyses were performed with BellCurve for Excel (Social Survey Research Information Co. Ltd Tokyo, Japan). For experiments yielding data requiring multiple comparisons, one-way ANOVA was followed by Holm's *post hoc* test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Long-term β-cell FoxM1 deficiency during adulthood impairs basal β-cell proliferation

To investigate the role of the FoxM1 pathway in maintaining β-cell mass in adult animals, we generated tamoxifen-inducible β-cell-specific FoxM1 knockout (iβ-FoxM1KO) mice by crossing FoxM1-floxed mice[26] and RIP-CreER mice.[2] We administered tamoxifen at 8 weeks of age in order to exclude the effects of FoxM1 deficiency during the developmental stage, and maintained these mice up to approximately 1 year of age (48–53 weeks of age). *Foxm1* gene expressions in isolated islet cells 2 weeks after tamoxifen administration were decreased by around 70% in iβ-FoxM1KO mice (hereafter referred to '10-week-old iβ-FoxM1KO mice') (Figure 1a). Since rodent islets reportedly consist of 80% β-cells and 20% other endocrine cells,[29] *Foxm1* gene expression was estimated to be absent in

80–90% of β-cells in 10-week-old iβ-FoxM1KO mouse islets. According to the decreased expression of FoxM1 in 1-year-old control mice, the difference in expression of the *Foxm1* gene was not statistically significant in the islet cells of 1-year-old iβ-FoxM1KO mice and control mice, although there was a tendency for *Foxm1* expression decrements in iβ-FoxM1KO mice (Figure 1a). Importantly, expressions of FoxM1 protein were decreased significantly in islets of iβ-FoxM1KO mice compared with the controls at both 10 weeks and 1 year of age (Figure 1b). These results suggest that down-regulation of FoxM1 expression is maintained throughout the experimental period in β-cells of iβ-FoxM1KO mice.

Next, we explored the effects of $\beta_{\text{-cell FoxM1}}$ deficiency in basal β-cell proliferation. First, we histologically estimated β-cell proliferation by examining Ki67-positive β-cells in both 10 week- and 1 year-old mice. Ki67-positive β-cells were decreased significantly in 1-year-old compared with 10-week-old control mice, indicating that the basal rates of β-cell proliferation diminish over time during adulthood. In addition, Ki67-positive β-cells were decreased significantly in 10-week-old, and tended to be decreased in 1-year-old iβ-FoxM1KO mice, compared with those in control mice of the same ages (Figure 1c,d). Thus, β-cell FoxM1 plays a critical role in basal β-cell proliferation throughout adult periods from youth to middle age.

Long-term β-cell FoxM1 deficiency leads to reduction of β-cell mass

Subsequently, we examined the β-cell masses of 10-week-old and 1-year-old iβ-FoxM1KO mice. Histological measurements of β-cell mass revealed that there was no difference between 10-week-old iβ-FoxM1KO and control mice (Figure 1e,f), suggesting that the levels of basal β-cell proliferation were too low to affect β-cell mass in the short term. The β-cell mass rose significantly in control mice, increasing by 3.6-fold, from 10 weeks of age to 1 year of age (Figure 1e). In contrast, increases in the β-cell mass of iβ-FoxM1KO mice were markedly suppressed, and the difference in β-cell mass values between 10-week-old and 1-year-old iβ-FoxM1KO mice was not statistically significant (Figure 1e). As a result, 1-year-old iβ-FoxM1KO mice had a significantly smaller β-cell mass than control mice (Figure 1e). We also histologically estimated β-cell apoptosis employing the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining method. TUNEL-positive islet cells were scarce in the islets of the control and iβ-FoxM1KO mice at 10 weeks of age, and the ratios of TUNEL-positive islet cells were similar in control and iβ-FoxM1KO mice at 10 weeks of age, suggesting that β-cell apoptosis has a minimal impact on the suppression of β-cell mass expansion in iβ-FoxM1KO mice (Figure 1g). These results indicate that long-term FoxM1 deficiency in β-cells and the resultant impairment of basal, low-level β-cell proliferation, for more than 40 weeks, markedly suppress β-cell mass increases. Thus, FoxM1-driven basal β-cell proliferation is essential for β-cell mass expansion with advancing age.

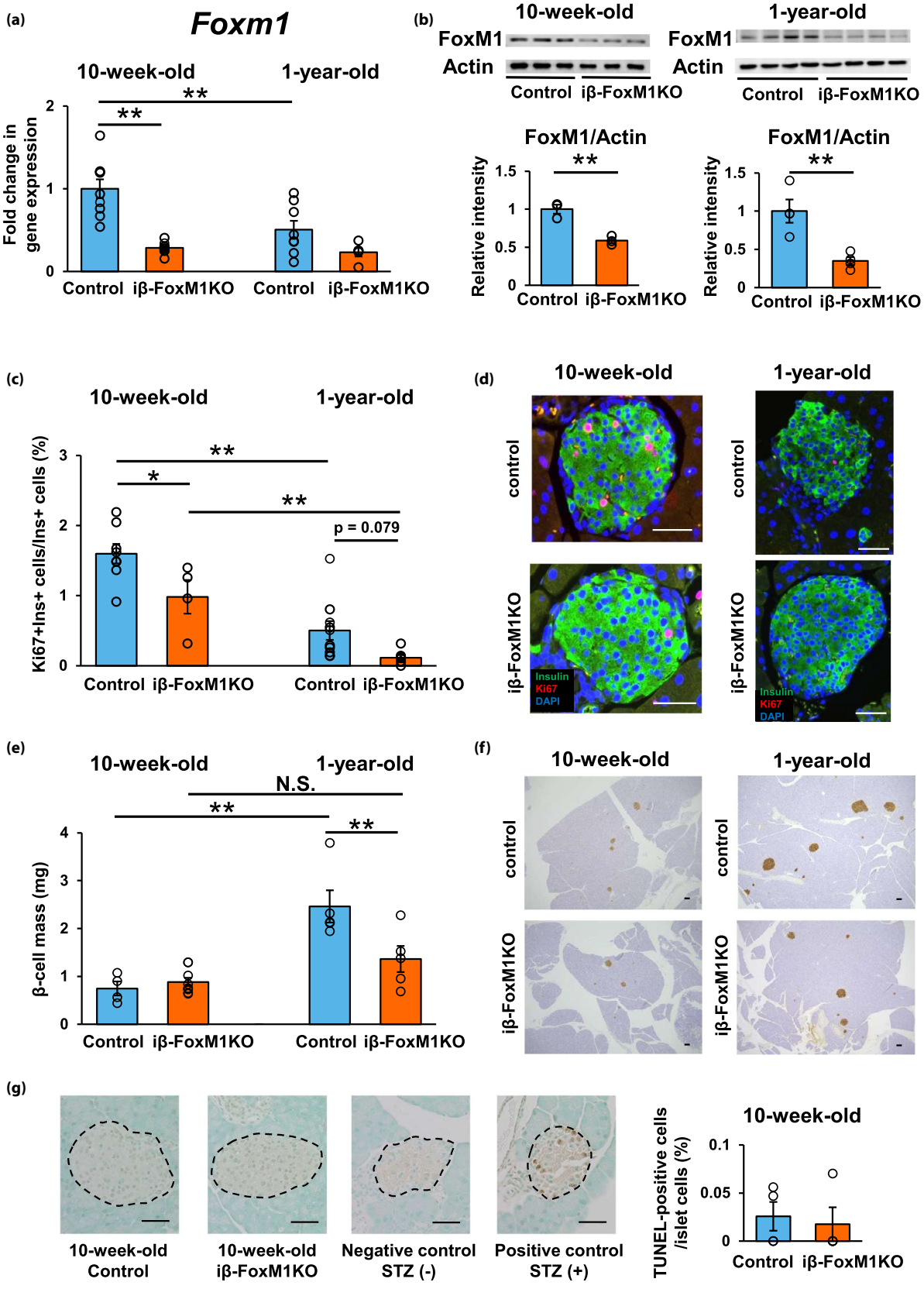
Inducible β-cell-specific FoxM1 deficiency disturbs cell cycle progressions in β-cells without inducing islet structural abnormalities

A previous report showed that 9-week-old congenital pancreas-specific FoxM1 knockout mice exhibited islet structure collapse.[25] To explore whether alterations of islet structure occur in 1-year-old iβ-FoxM1KO mice, immunostaining was performed for multiple islet hormones, including somatostatin and glucagon, using pancreatic tissues. These analyses showed that the structure of the islets as well as the composition of each endocrine cell type in islets of 1-year-old iβ-FoxM1KO mice were similar to those of the control mice (Figure 2b). Thus, FoxM1 deficiency specifically in β-cells after development and growth stages does not induce structural abnormalities in the islets.

Long-term, but not short-term, β-cell FoxM1 deficiency during adulthood worsens glucose tolerance due to impaired insulin secretion

We evaluated the glucose tolerance of iβ-FoxM1KO mice at 10 weeks and 1 year of age. There was no difference in the body weights between iβ-FoxM1KO and control mice at either

Figure 1 | (a) Relative expression levels of *Foxm1* gene in islets isolated from control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 9$, KO $n = 10$. 1-year-old; control $n = 8$, KO $n = 5$). (b) (Upper panels) Immunoblotting images of islets from control and iβ-FoxM1KO mice at 10 weeks and 1 year of age treated with anti-FoxM1 and anti-Actin antibodies. (Lower panels) Relative intensity of FoxM1, which was normalized by Actin intensities, in isolated islets from control and iβ-FoxM1KO mice at 10 weeks and 1 year of age (10-week-old; $n = 3$ per group. 1-year-old; $n = 4$ per group). (c) Ki67 and insulin co-positive cell ratios in insulin positive cells of control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 8$, KO $n = 4$. 1-year-old; control $n = 10$, KO $n = 7$). (d) Representative histological images of pancreases from control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age, immunostaining with DAPI (blue), anti-Ki67 (red), and anti-insulin (green) antibody. Scale bars indicate 50 μm. (e) β-cell mass of control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 4$, KO $n = 9$. 1-year-old; control $n = 5$, KO $n = 5$). (f) Representative histological images of pancreases from control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age, immunostaining with insulin antibody. Scale bars indicate 100 μm. (g) (Left panels) TUNEL staining images of pancreatic tissues from control and iβ-FoxM1KO mice at 10 weeks of age. Images of pancreatic tissues from negative and positive control mice are also shown. Positive control mice were treated by streptozotocin (STZ). Scale bars indicate 50 μm. (Right panel) TUNEL-positive cell ratios in islet cells of control and iβ-FoxM1KO mice at 10 weeks of age ($n = 4$ per group). Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; N.S., not significant, as assessed by one-way ANOVA followed by Holm's *post hoc* test (a, c, e) or the unpaired t test (b, g).



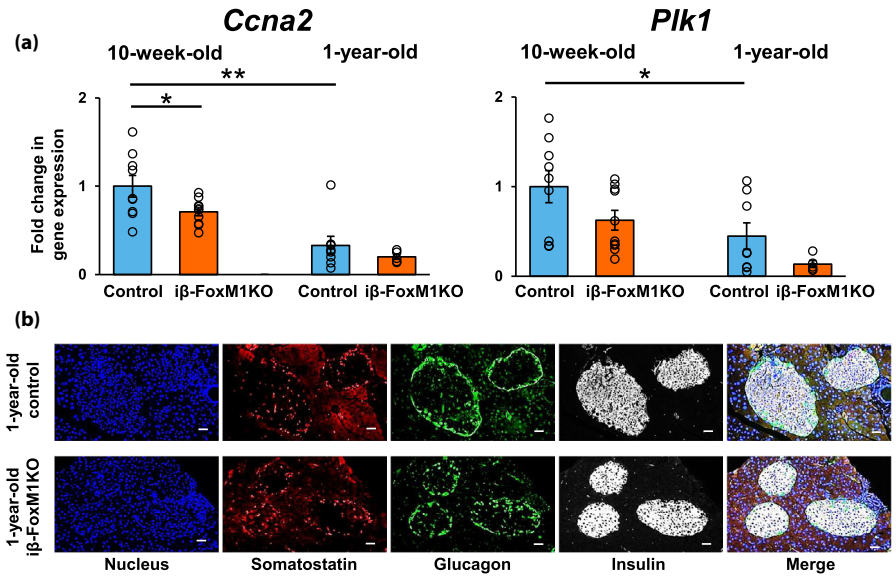


Figure 2 | (a) Relative expression levels of *Ccna2* and *Plk1* genes in islets isolated from control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old, control $n = 9$, KO $n = 10$. 1-year-old, control $n = 8$, KO $n = 5$). (b) Representative histological images of pancreases from 1-year-old control and iβ-FoxM1KO mice, immunostaining with DAPI (blue), anti-somatostatin (red), anti-glucagon (green), anti-insulin (white) antibody. Scale bars indicate 50 μm. Data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$, as assessed by one-way ANOVA followed by Holm's *post hoc* test.

of these ages (Figure 3a). Intraperitoneal glucose tolerance tests revealed that, at 10 weeks of age, elevations of both blood glucose and plasma insulin after glucose challenge were similar in iβ-FoxM1KO and control mice (Figure 3b). Thus, short-term (2 weeks) loss of FoxM1 in β-cells may exert minimal effects on plasma insulin levels and glucose tolerance. In contrast, blood glucose levels after glucose loading were significantly higher in 1-year-old iβ-FoxM1KO mice than in control mice (Figure 3c). Notably, plasma insulin levels were markedly decreased in 1-year-old iβ-FoxM1KO mice compared with control mice (Figure 3c). To examine the insulin sensitivity of iβ-FoxM1KO mice, we next performed insulin tolerance tests in mice of each age. Glucose levels after insulin administration were similar in iβ-FoxM1KO and control mice at both ages (Figure 3d), suggesting that β-cell FoxM1 deficiency does not affect insulin sensitivity at either age. Thus, the glucose intolerance observed in 1-year-old iβ-FoxM1KO mice is mainly caused by impaired insulin secretion, not by the development of insulin resistance.

Long-term β-cell FoxM1 deficiency does not impair β-cell function

We next examined whether long-term FoxM1 deficiency affects β-cell function. First, we analyzed the expression of genes involved in insulin secretion employing islets isolated from 1-year-old control and iβ-FoxM1KO mice. Expression of genes involved in transcription of the insulin gene, such as v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A *(Mafa)*[30,31] and pancreatic and duodenal homeobox 1 *(Pdx1)*[30,31] (Figure 4a), and insulin II *(Ins2)* (Figure 4b) as well as solute carrier family 2 (facilitated glucose transporter) member 2 *(Slc2a2)* (Figure 4c) in islet cells did not differ between the two groups, at 10 weeks and 1 year of age, suggesting minimal impacts of long-term FoxM1 deficiency on the expression of insulin secretion-related genes.

Finally, to examine directly whether β-cell function is impaired in 1-year-old iβ-FoxM1KO mice, we evaluated insulin secretion from β-cells in the *ex vivo* setting using isolated islets. Insulin secretion from isolated islets under both low glucose (1.67 mM) and high glucose (16.7 mM) conditions were similar in islets from iβ-FoxM1KO and control mice (Figure 4d). Thus, the insulin secretory function of β-cells, whether under basal or glucose stimulated conditions, is unlikely to be impaired even after long-term FoxM1 deficiency. Collectively, these data indicate that reduction of β-cell mass, rather than β-cell dysfunction, is the primary cause of decreased plasma insulin levels and deteriorating glucose tolerance observed in 1-year-old iβ-FoxM1KO mice. Considering that short-term (2 weeks) FoxM1 deficiency altered neither plasma insulin levels nor β-cell mass, low-level and persistent β-cell proliferation in basal states is important for the long-term maintenance of β-cell mass and glucose homeostasis. FoxM1 plays a major role in the molecular mechanism(s) underlying such basal β-cell proliferation.

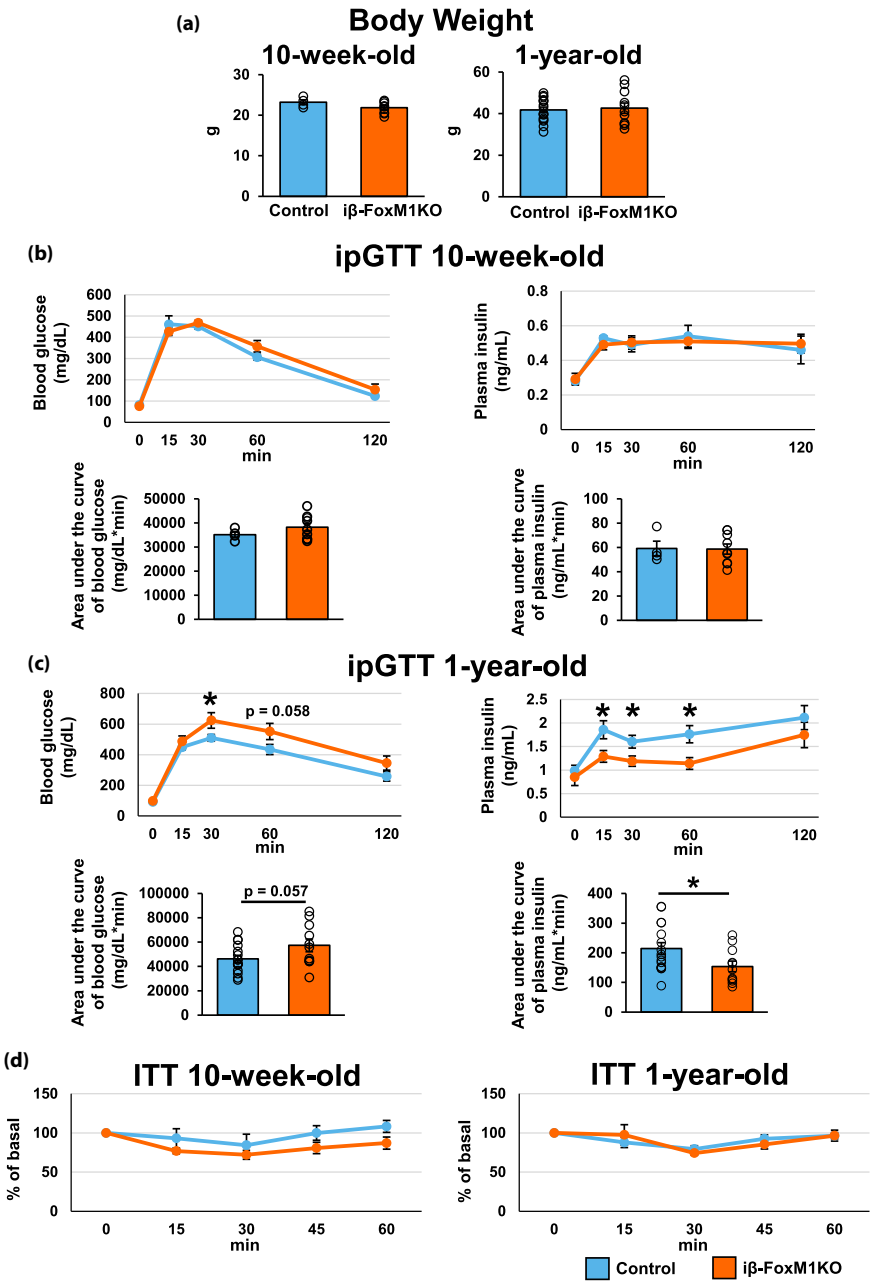


Figure 3 | (a) Body weights of control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 4$, KO $n = 9$. 1-year-old; control $n = 16$, KO $n = 11$). (b) Intraperitoneal glucose tolerance tests (ipGTT) of 10-week-old control and iβ-FoxM1KO mice (control $n = 4$, KO $n = 9$). (c) ipGTT of 1-year-old control and iβ-FoxM1KO mice (control $n = 16$, KO $n = 11$). (d) Insulin tolerance tests (ITT) of control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 4$, KO $n = 9$. 1-year-old; control $n = 9$, KO $n = 4$). Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$, as assessed by the unpaired *t*-test.

DISCUSSION

In this study, by analyzing iβ-FoxM1KO mice at 1 year of age, we clarified that β-cell FoxM1 plays an important role in maintaining basal proliferation of β-cells and sufficient β-cell mass to prevent the deterioration of glucose tolerance during adulthood. The inducible knockout model used in this study allowed us to clarify the role of β-cell FoxM1 in the maintenance of β-cell mass while avoiding the influences of β-cell FoxM1 deficiency during the organogenesis period.

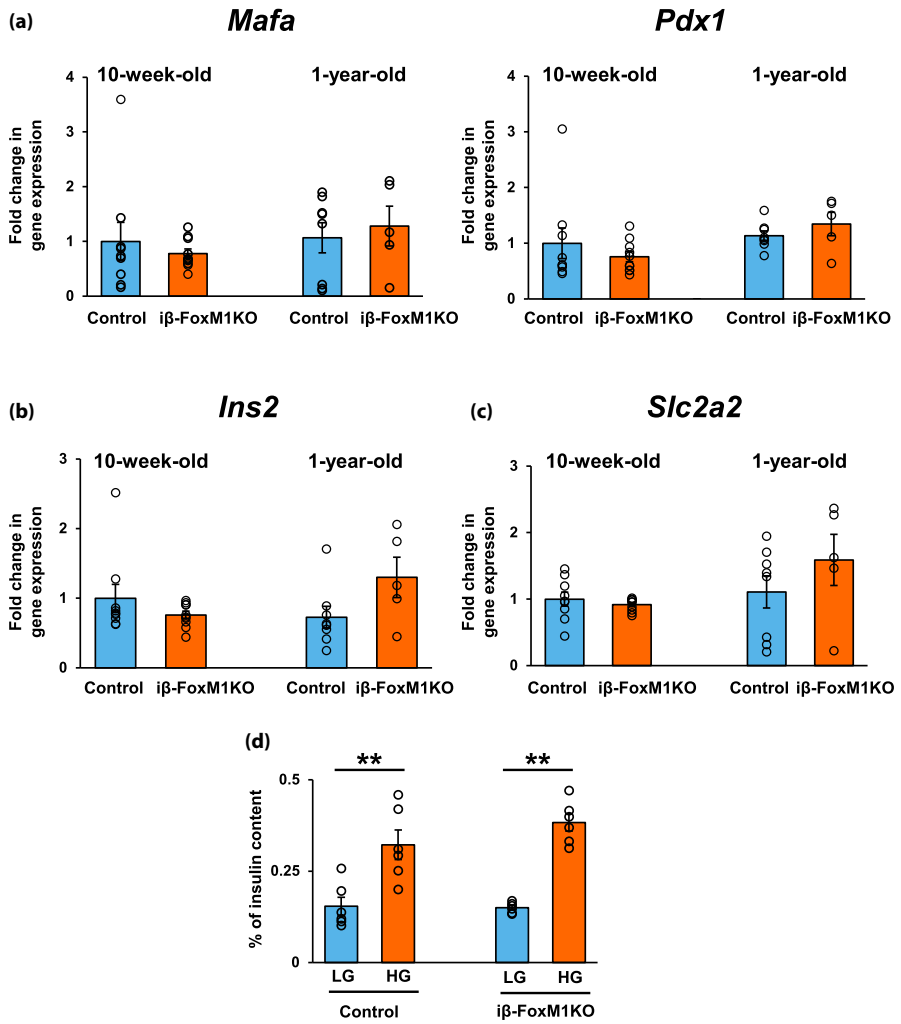


Figure 4 | (a) Relative expression levels of *Mafa* and *Pdx1* genes in islets isolated from control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 9$, KO $n = 10$. 1-year-old; control $n = 8$, KO $n = 5$). (b) Relative expression levels of *Ins2* gene in islets isolated from control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 9$, KO $n = 10$. 1-year-old; control $n = 8$, KO $n = 5$). (c) Relative expression levels of *Slc2a2* gene in islets isolated from control and iβ-FoxM1KO mice at both 10 weeks and 1 year of age (10-week-old; control $n = 9$, KO $n = 10$. 1-year-old; control $n = 8$, KO $n = 5$). (d) Insulin secretion in islets isolated from 1-year-old control and iβ-FoxM1KO mice at different glucose concentrations (LG; low glucose 1.67 mM, HG; high glucose 16.7 mM, $n = 6$ per group). Data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; N.S., not significant, as assessed by one-way ANOVA followed by Holm's *post hoc* test.

β-Cell FoxM1 is necessary for the induction of adaptive β-cell proliferation.[8,23,24] Our results indicate that FoxM1 is also required for basal persistent β-cell proliferation during adulthood. In iβ-FoxM1KO mice, β-cell mass expansion was suppressed at 1 year of age, but not at 10 weeks of age. Sufficient β-cell mass is maintained throughout adulthood[32] despite low rates of β-cell proliferation,[5,9] suggesting a long lifespan and slow turnover of β-cells. Indeed, the long lifespan of β-cells was reported in both mice[9] and humans.[33,34] Therefore, long-term observation of iβ-FoxM1KO mice enabled us to demonstrate the critical role of FoxM1-driven basal β-cell proliferation in the maintenance of β-cell mass and glucose homeostasis during adulthood.

Both the β-cell FoxM1 pathway and basal β-cell proliferation rates were shown to be decreased markedly in 1-year-old control mice. The β-cell FoxM1 expression and β-cell proliferative capacity also reportedly declines with aging in mice through 1 year of age.[22,35] For instance, compared with young mice, i.e., those 6–8 weeks of age, β-cell proliferation in middle-aged mice, i.e., those 7–8 months old, was impaired in several models, such as after partial pancreatectomy[36] and after the administration of GLP1 agonists.[37] These results suggest that down-regulation of the β-cell FoxM1 pathway is involved in the reduction of β-cell proliferative capacity with advancing age. However, the mechanism(s) whereby activity of the β-cell FoxM1 pathway in middle-aged mice are attenuated remain

largely unknown. Growth hormone reportedly increased expressions of FoxM1 and its target genes, thereby promoting cell proliferation in hepatocytes.[38] In addition, the circulating level of growth hormone is known to decrease with aging.[39] Therefore, aging-associated attenuation of FoxM1 pathway activity in β-cells might be attributable to decrements in humoral factors. Alternatively, neural factors might be involved in the decrement of β-cell FoxM1 pathway activation that occurs with aging. We showed previously that vagal nerve signals increased the expressions of FoxM1 and its target genes, indicating activation of the FoxM1 pathway, thereby promoting adaptive β-cell proliferation during the development of obesity.[7,8] Meanwhile, impairment of the autonomic nervous system with advancing age is often observed as one of the clinical features of the elderly, along with changes such as the disappearance of the respiratory variation of heart rates.[40] In this regard, it would be interesting to explore the role of vagal nerve signals in reducing β-cell FoxM1 activity and β-cell proliferative capacity during the processes of aging.

As for the RIP-CreER mice used in this study, there are several potential problems, such as tamoxifen-independent recombination[41] and ectopic expression of CreER in certain portions of the brain.[42] However, 10-week-old iβ-FoxM1KO mice exhibited no abnormalities of either plasma insulin or glucose levels with markedly decreased FoxM1 expression in β-cells. Therefore, it is unlikely that tamoxifen-independent recombination affected the metabolic observations in this study, although we cannot exclude the possibility that CreER expression in the brain[42] affected the β-cell phenotypes observed herein. Further study using other β-cell-specific Cre lines might be required to confirm our conclusion.

In conclusion, β-cell FoxM1 plays critical roles in not only adaptive, but also basal, proliferation of β-cells under normal conditions, resulting in the maintenance of sufficient β-cell mass and glucose tolerance during adulthood. Preserving β-cell FoxM1 activity might be an efficient strategy for preventing the gradual deterioration of glucose tolerance that occurs with aging.

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DISCLOSURE

The authors declare no conflict of interest.
Approval of the research protocol: Ethics approval was obtained from the Institutional Animal Care and Use Committee of the Tohoku University Environmental & Safety Committee (No. 2019MdLMO-200-03 and No.2019MdA-337-05).
Informed consent: N/A.
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Uncropped immunoblotting images for Figure 1b.