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ORIGINAL ARTICLE

Early administration of dapagliflozin preserves pancreatic β-cell mass through a legacy effect in a mouse model of type 2 diabetes

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Keywords
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ABSTRACT

Aims/Introduction: The preservation of pancreatic β-cell mass is an essential factor in the onset and development of type 2 diabetes mellitus. Recently, sodium–glucose cotransporter 2 inhibitors have been launched as antihyperglycemic agents, and their organ-protective effects are attracting attention. They are also reported to have favorable effects on the preservation of pancreatic β-cell mass, but the appropriate timing for the administration of sodium–glucose cotransporter 2 inhibitors is obscure.

Materials and Methods: In the present study, we administered a sodium–glucose cotransporter 2 inhibitor, dapagliflozin, to an animal model of type 2 diabetes mellitus, *db/db* mice, and investigated the adequate timing and duration for its administration. We also carried out microarray analysis using pancreatic islets from *db/db* mice.

Results: We found that dapagliflozin preserved pancreatic β-cell mass depending on the duration of administration and markedly improved blood glucose levels. If the duration was the same, the earlier administration of dapagliflozin was more effective in preserving pancreatic β-cell mass, increasing serum insulin levels and improving blood glucose levels. From microarray analysis, we discovered that the expression of *Agr2*, *Tff2* and *Gkn3* was significantly upregulated after the early administration of dapagliflozin. This upregulated gene expression might provide a legacy effect for the preservation of pancreatic β-cell mass.

Conclusions: We expect that the early administration of dapagliflozin would provide a long-lasting effect in preserving pancreatic β-cell mass.

INTRODUCTION

The number of patients with type 2 diabetes mellitus is increasing rapidly, and optimal treatment is required to solve this problem. The pathogenesis of type 2 diabetes mellitus is characterized by "insulin resistance"; that is, reduced peripheral insulin sensitivity, and "impaired insulin secretion"; that is, a reduction in insulin secretion from pancreatic β-cells. A variety of medical agents are used to target each of these mechanisms. Among them, sodium–glucose cotransporter 2 (SGLT2) inhibitors have recently drawn considerable attention.

SGLT2 inhibitors are antihyperglycemic agents that suppress the reabsorption of urinary glucose by inhibiting SGLT2 in the renal proximal tubules. Since they were first launched in Australia in 2012, they have been widely used mainly in Western countries, as they have a favorable influence not only on glycemic control, but also on bodyweight and blood pressure. However, in East Asian countries, including Japan, they have not been widely used compared with other agents because of concerns about dehydration. In 2015, this situation was changed drastically by the Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME), which showed a significant reduction in some adverse events, including cardiovascular mortality[1]. In 2017, the Canagliflozin Cardiovascular Assessment Study (CANVAS) trial reported a 3-point suppression in major adverse cardiovascular events[2]. SGLT2 inhibitors have shown significant benefits on

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the suppression of cardiovascular complications. In addition to the suppression of macrovascular complications, it has also been evident that SGLT2 inhibitors improve the pathogenesis of non-alcoholic steatohepatitis, preserve renal function and suppress pancreatic β-cell failure[3–5]. Pancreatic β-cell failure is one of the major mechanisms underlying the development of type 2 diabetes mellitus. The pathogenesis consists of "abnormalities in quality," which is characterized by impaired insulin secretion, and "abnormalities in quantity," which is characterized by reduced pancreatic β-cell mass. We have previously shown the importance of maintaining pancreatic β-cell mass mainly through animal studies[6–8]. In addition, we have also shown that a DPP4 inhibitor contributed to the recovery of pancreatic β-cell mass through the unfolded protein response[9,10].

Several reports have shown that SGLT2 inhibitors contribute to the preservation of pancreatic β-cell function, and almost all of them explain its mechanism through the reduction of glucose toxicity[11,12]. Others report that the molecular mechanism by which SGLT2 inhibitors preserve pancreatic β-cell function is the inhibition of inflammation or oxidative stress. However, there have been no reports discussing the adequate duration and timing for the administration of SGLT2 inhibitors. In addition, in the clinical setting, it has not been concluded whether SGLT2 inhibitors should be the first drug of choice for type 2 diabetes mellitus or should be considered as additional drugs to other antihyperglycemic agents. In the present study, we administered an SGLT2 inhibitor, dapagliflozin, to leptin receptor-deficient *db/db* mice, a model of obesity and type 2 diabetes mellitus, to examine the effect of preserving pancreatic β-cells depending on the timing of dapagliflozin administration. We divided the mice into four groups: (i) dapagliflozin administration for 12 weeks; (ii) dapagliflozin administration for the first 6 weeks; (iii) dapagliflozin administration for the last 6 weeks; and (iv) normal saline administration. We bred all groups in the same condition and analyzed the effect of dapagliflozin on pancreatic β-cell mass.

In the present study, our objective was to elucidate the appropriate timing for the initiation of SGLT2 inhibitor treatment, and to examine the changes of gene expression in pancreatic β-cells induced by an SGLT2 inhibitor.

METHODS

Animals

We obtained *db/db* and *db/m* mice on a C57BL/KsJ background from CLEA Japan, Inc. (Tokyo, Japan). The mice were maintained on a 12-h light–dark cycle and fed normal chow. Blood glucose and plasma insulin concentrations were determined as described previously[13]. Measurement of urine volume, urinary glucose excretion and food intake was carried out using individual metabolic cages (CLEA Japan) in a 24-h period. All experiments were carried out with male mice. The Animal Ethics Committee of Kobe University Graduate School of Medicine approved all protocols involving the mice. All animal experiments were carried out following the national guidelines and the relevant national laws on the protection of animals.

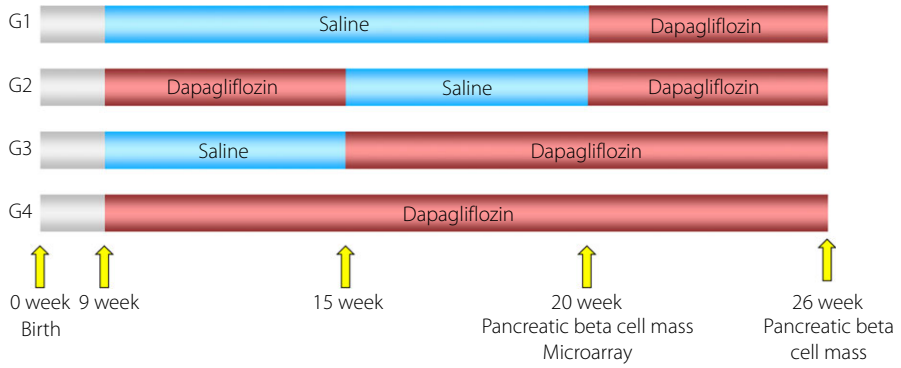
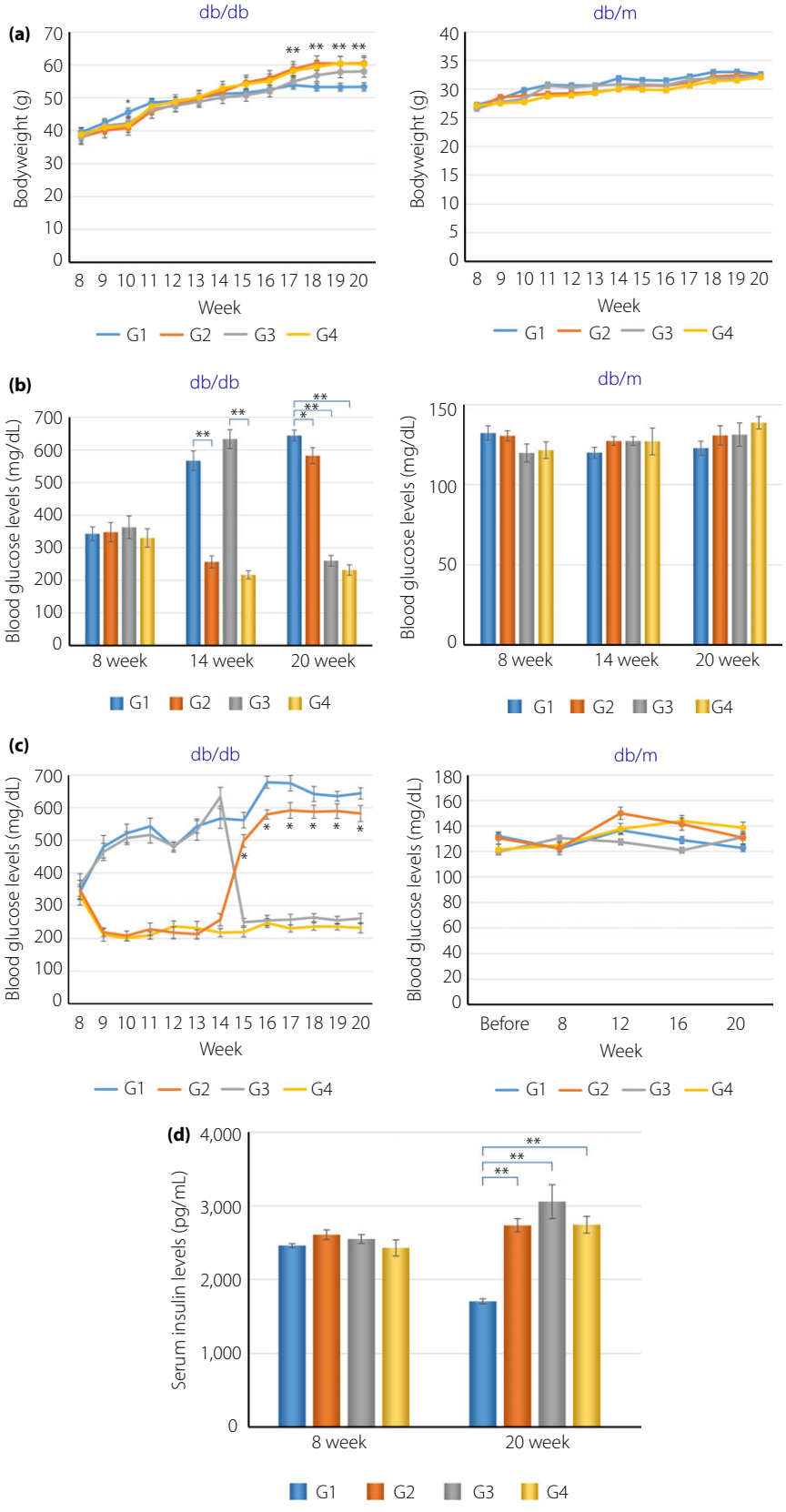


Figure 1 | Schedule of dapagliflozin administration. Pancreatic β-cell mass was measured at 20 and 26 weeks-of-age, and microarray analysis using pancreatic islets was carried out at 20 weeks-of-age. G1, group 1; G2, group 2; G3, group 3; G4, group 4.

Figure 2 | Characteristics of *db/db* mice and *db/m* mice divided according to the duration and timing of dapagliflozin administration. (a) Bodyweight for each group of *db/db* and *db/m* mice at various ages. (b) Blood glucose levels in the fed state for each group of *db/db* and *db/m* mice at 8, 14 and 20 weeks-of-age (w). (c) Blood glucose levels in the fed state for each group of *db/db* and *db/m* mice at various ages. (d) Serum insulin levels in the fed state for each group of *db/db* and *db/m* mice at 8 and 20 weeks-of-age. (e) Urine volume for each group of *db/db* mice at 8 and 20 weeks-of-age. (f) Urinary glucose excretion for each group of *db/db* mice at 8 and 20 weeks-of-age. (g) Food intake for each group of *db/db* mice at 8 and 20 weeks-of-age. G1, group 1; G2, group 2; G3, group 3; G4, group 4. All quantitative data are the mean ± standard error of the mean for (a–d) 8–10 and (e–g) four mice of each group. *$P < 0.05$, **$P < 0.01$.


(a)
db/db
db/m
Bodyweight (g)
Week
G1 G2 G3 G4
(b)
db/db
db/m
Blood glucose levels (mg/dL)
8 week
14 week
20 week
G1 G2 G3 G4
(c)
db/db
db/m
Blood glucose levels (mg/dL)
Week
Before
G1 G2 G3 G4
(d)
Serum insulin levels (pg/mL)
8 week
20 week
G1 G2 G3 G4

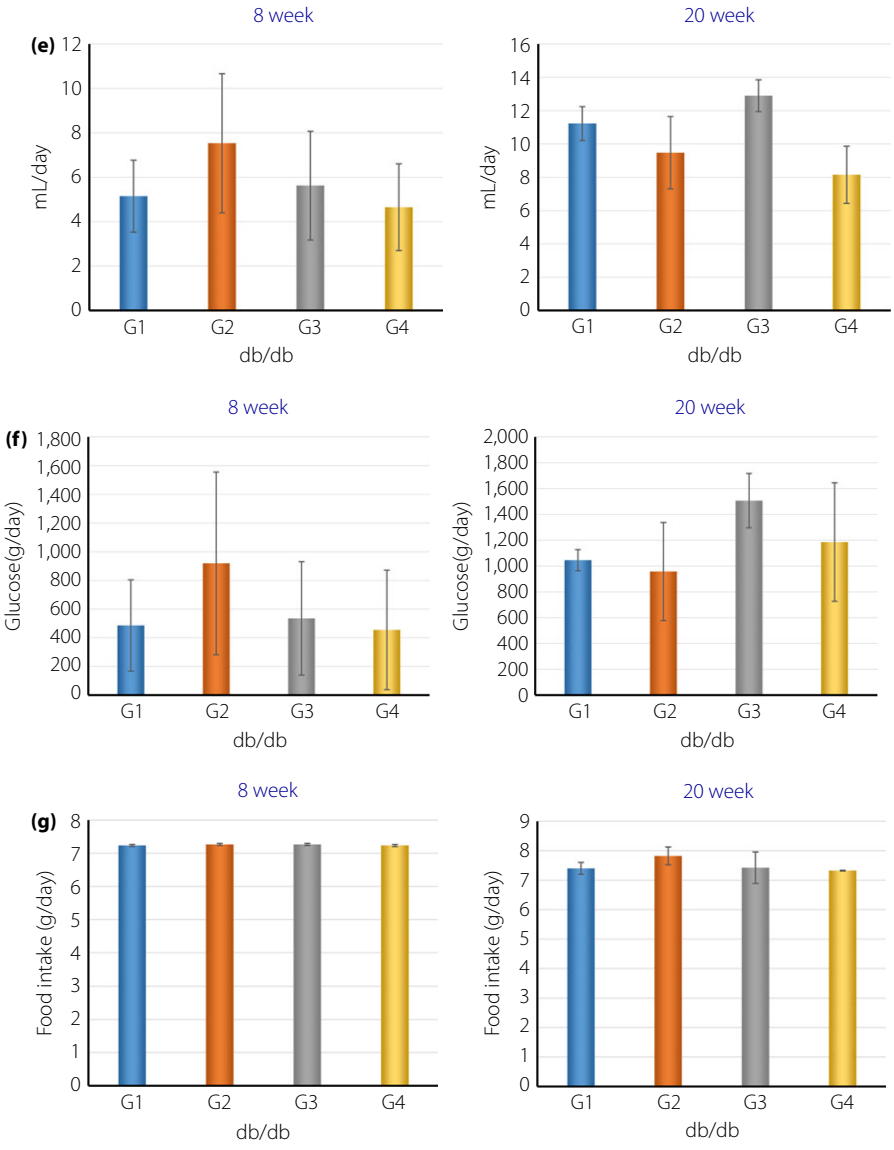


Figure 2 | Continued.

Administration of Dapagliflozin to Mice

Type 2 diabetes *db*/*db* mice were classified into the following four groups, and the SGLT2 inhibitor dapagliflozin and saline were administered accordingly: group 1, saline was administered from 9 to 20 weeks-of-age; group 2, 1 mg/kg dapagliflozin was administered orally from 9 to 14 weeks-of-age, and saline was administered from 15 to 20 weeks-of-age; group 3, saline was administered from 9 to 14 weeks-of-age and 1 mg/kg dapagliflozin was administered orally from 15 to 20 weeks-of-age; and group 4, 1 mg/kg dapagliflozin was administered orally from 9 to 20 weeks-of-age. Fed blood glucose level, fed serum insulin value and bodyweight were measured from 8 weeks-of-age at an interval of 2 weeks.

Pancreatic β-cell mass measurement and microarray analysis of isolated pancreatic islets were carried out at 20 weeks-of-age (Figure 1). Experiments were also carried out on four groups of *db*/*m* mice as the control.

All *db*/*db* mice groups were administered 1 mg/kg dapagliflozin orally for 6 weeks from 21 weeks-of-age for further analysis.

Immunostaining and Morphometric Analysis

The pancreas was immersed in Bouin's solution, embedded in paraffin and sectioned at a thickness of 4–5 μm. The sections were stained with antibodies to insulin and glucagon (Agilent Technologies, Santa Clara, CA, USA). Immune complexes were detected with secondary antibodies conjugated with either cyanine 3 or fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Quantitation of α-cell and β-cell mass was described previously[14,15]. For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining,

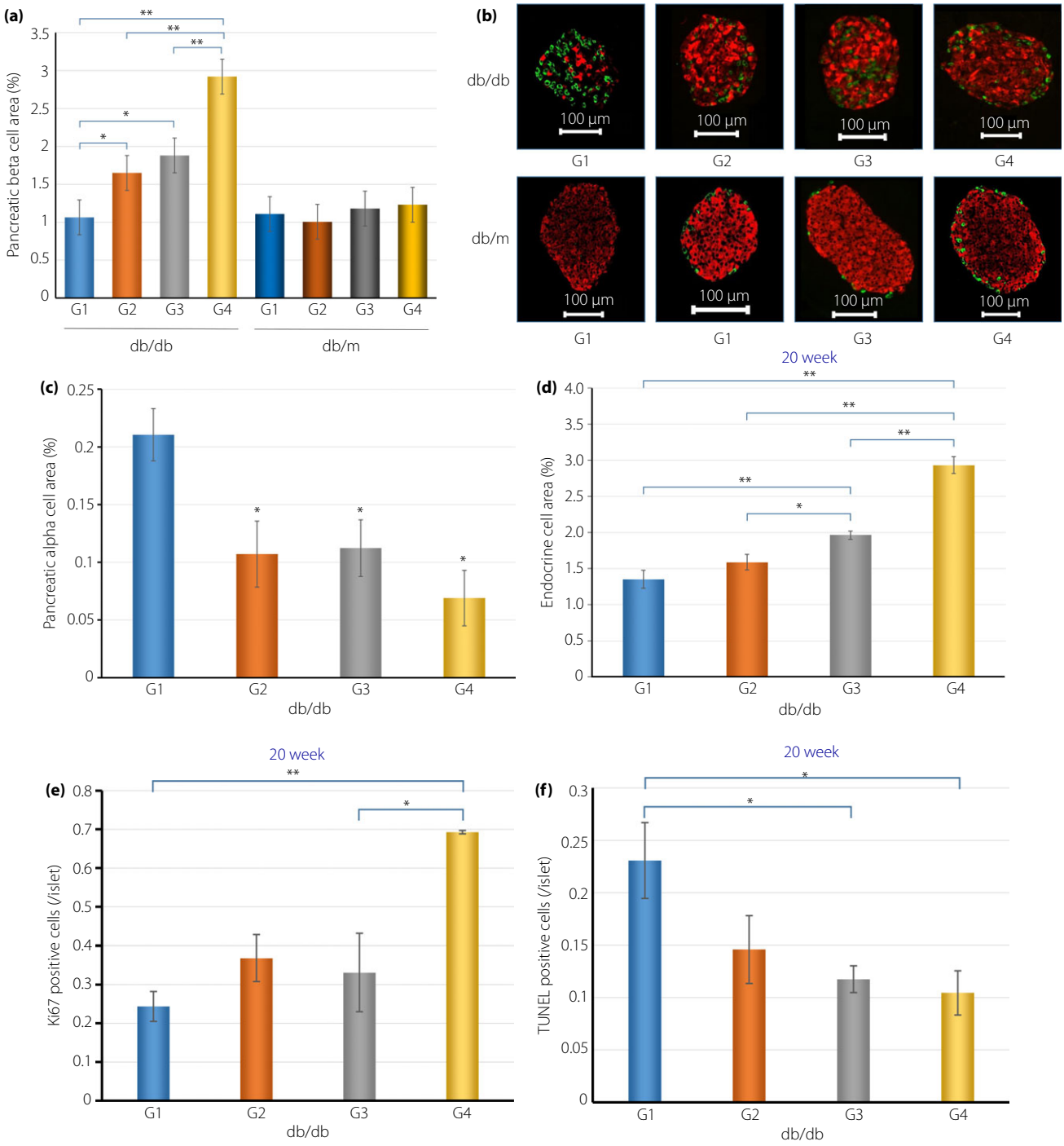


Figure 3 | Immunostaining of *db/db* mice and *db/m* mice divided according to the duration and timing of dapagliflozin administration. (a) The β-cell area was determined as the ratio of the area positive for insulin immunostaining to the total pancreatic area in sections from 20-week-old *db/db* and *db/m* mice. (b) Immunostaining of pancreatic sections from 20-week-old *db/db* and *db/m* mice with antibodies to insulin (red) and glucagon (green). Scale bars, 100 μm. (c) The α-cell area was determined as the ratio of the area positive for glucagon immunostaining to the insulin and glucagon-positive area in sections from 20-week-old *db/db* mice. (d) The islet area was determined as the ratio of the area positive for chromogranin A immunostaining to the total pancreatic area in sections from 20-week-old (20w) *db/db* mice. (e) Ratio of Ki67-positive cells in insulin-positive cells observed in 20-week-old *db/db* mice from each group. (f) Ratio of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in insulin-positive cells observed in 20-week-old *db/db* mice from each group. G1, group 1; G2, group 2; G3, group 3; G4, group 4. All quantitative data are the mean ± standard error of the mean for (a, c–f) four mice of each group. *$P < 0.05$, **$P < 0.01$.

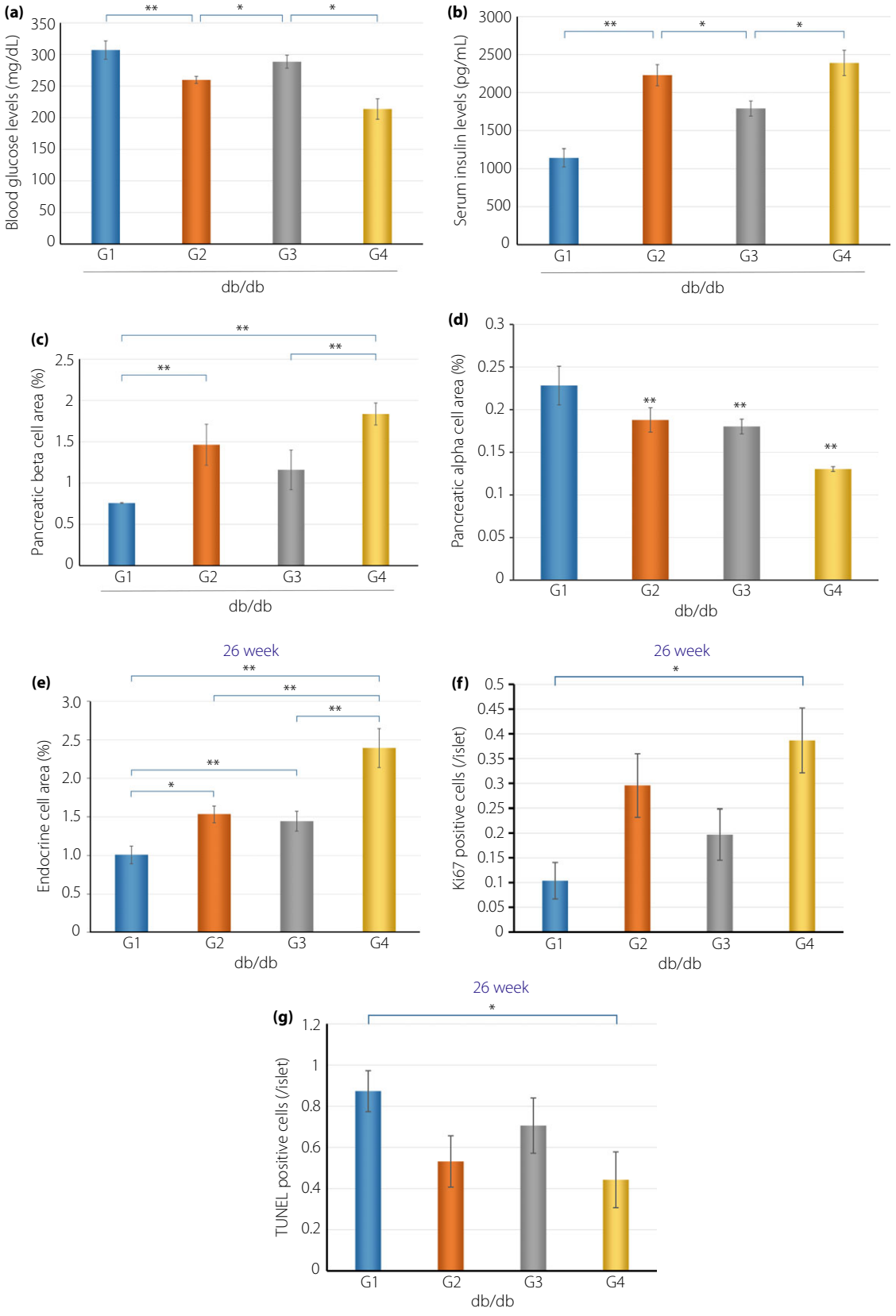


Figure 4 | Longitudinal effect of early or late dapagliflozin administration on *db/db* mice. (a) Blood glucose levels and (b) serum insulin levels in the fed state for each group of *db/db* mice at 26 weeks-of-age. (c) Pancreatic β-cell area in each group of *db/db* mice at 26 weeks-of-age. (d) The α-cell area was determined as the ratio of the area positive for glucagon immunostaining to the insulin and glucagon-positive area in sections from 26-week-old (w) *db/db* mice. (e) The islet area was determined as the ratio of the area positive for chromogranin A immunostaining to the total pancreatic area in sections from 26-week-old *db/db* mice. (f) Ratio of Ki67-positive cells in insulin-positive cells observed in 26-week-old *db/db* mice from each group. (g) Ratio of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in insulin-positive cells observed in 26-week-old *db/db* mice from each group. G1, group 1; G2, group 2; G3, group 3; G4, group 4. All quantitative data are the mean ± standard error of the mean for four mice of each group. $*P < 0.05$, $**P < 0.01$.

pancreas sections were labeled with an Apoptosis *in situ* Detection Kit (Wako, Osaka, Japan). For Ki67 staining, pancreas sections were incubated with an anti-Ki67 antibody (Abcam, Cambridge, UK) and a secondary antibody conjugated to cyanine 3 staining. For anterior gradient 2 (AGR2), trefoil factor 2 (TFF2) and gastrokine 3 (GKN3) staining, pancreas sections were incubated with an anti-AGR2 antibody (Proteintech, Chicago, IL, USA), anti-TFF2 antibody (Proteintech) or anti-GKN3 antibody (Cloud-Clone Corp., Katy, TX, USA), respectively, and a secondary antibody conjugated to cyanine 3 staining.

Ribonucleic Acid Isolation from Isolated Pancreatic Islets

Islets were isolated from four *db/db* and four *db/m* mice as described previously[16]. Total ribonucleic acid was extracted and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Ribonucleic acid quantity and quality were determined using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and an Agilent Bioanalyzer (Agilent Technologies), as recommended[17]. Combined samples were used for microarray analysis.

Analysis of Microarray Data

The intensity values of each scanned feature were quantified using Agilent feature extraction software version 11.5.1.1 (Agilent Technologies), Agilent GeneSpring software version 14.8 (Agilent Technologies), which carries out background subtractions. We only used features that were flagged as no errors (Detected Flags), and excluded features that were not positive, not significant, not uniform, not above background, saturated and population outliers (Not Detected and Compromised Flags). Normalization was carried out using Agilent GeneSpring software version 14.8 (per chip: normalization to 75th percentile shift).

Statistical Analysis

Data are presented as the mean ± standard error of the mean, and were compared by analysis of variance followed by two-tailed Student's *t*-tests. A *P*-value <0.05 was considered statistically significant.

RESULTS

Early Administration of Dapagliflozin Maintains Blood Glucose Improvement Even After Medication is Discontinued

The bodyweight of mice administered dapagliflozin from 9 weeks-of-age, groups 2 and 4, was significantly decreased at 10 weeks-of-age. However, at 20 weeks-of-age, they showed a remarkable increase in bodyweight compared with group 1 mice (Figure 2a). Ad libitum blood glucose levels were approximately 350 mg/dL in all groups of mice before 9 weeks-of-age, when dapagliflozin administration was started, and dapagliflozin decreased blood glucose levels in group 2 and group 4 mice <200 mg/dL after administration for 1 week. At 14 weeks-of-age, the blood glucose levels in group 1 and group 3 mice increased to approximately 600 mg/dL, but those in group 2 and group 4 mice were maintained at approximately 200 mg/dL. After we switched the administration of saline to dapagliflozin in group 3 mice at 15 weeks-of-age, their blood glucose levels immediately became as low as those in group 4 mice. In contrast, the blood glucose levels in group 2 mice elevated rapidly after dapagliflozin administration was stopped, but they were still significantly lower than those of group 1 mice (Figure 2b,c). No difference was seen in the blood glucose levels of any group of *db/m* mice after dapagliflozin administration. Serum insulin levels were not altered in any group of mice before dapagliflozin administration. However, when we finished dapagliflozin administration at 20 weeks-of-age, serum insulin levels were significantly lower in group 1 mice than in the other groups, and they were maintained at a higher level in group 2, 3 and 4 mice, indicating that insulin secretion was maintained in these groups of mice (Figure 2d). We also examined alterations of urine volume, urinary glucose excretion and food intake. With respect to urine volume, there was no significant difference among the groups at 8 weeks-of-age before dapagliflozin treatment. In good agreement with previous findings[5], urine volume was significantly reduced with blood glucose improvement at 20 weeks-of-age in group 4 mice (Figure 2e). There was no significant difference in urinary glucose excretion because of large individual differences (Figure 2f). Significant differences in food intake were not found at both 8 and 20 weeks-of-age, in good agreement with previous findings (Figure 2g)[5].

The Longer the Dapagliflozin Administration, the More Pancreatic β-Cell Mass is Retained

We next evaluated pancreatic β-cell mass in each group of mice. Pancreatic β-cells in younger *db/db* mice are known show compensatory hyperplasia in response to insulin resistance[17]. However, pancreatic β-cell mass in group 1 *db/db* mice was almost as small as that in *db/m* mice at 20 weeks-of-age. In contrast, pancreatic β-cell mass in group 4 mice was

maintained at almost threefold this level after 12 weeks-of-dapagliflozin administration, indicating the beneficial effect of dapagliflozin on pancreatic β-cells by reducing glucose toxicity. Interestingly, pancreatic β-cell mass in group 2 and group 3 mice, which were administered dapagliflozin for just 6 weeks, was significantly larger than that in group 1 mice (Figure 3a). Notably, in group 2 mice, their significantly improved blood glucose levels were considered to be due to the increase in pancreatic β-cell mass at 6 weeks after the termination of dapagliflozin administration (Figure 2c), suggesting that dapagliflozin administration from an early stage is beneficial for maintaining pancreatic β-cell mass and blood glucose levels.

Observations on the morphology of the islets of mice in each group showed increased pancreatic α-cell mass and collapsed islet structures in addition to reduced pancreatic β-cell mass in group 1 *db*/*db* mice. Group 4 *db*/*db* mice were found to have highly preserved islet structures, as well as preserved pancreatic β-cell mass. In group 2 and group 3 *db*/*db* mice, islet structures were preserved, but unlike group 4 *db*/*db* mice, some pancreatic α-cells were located in the center of the islets (Figure 3b). Quantitation of pancreatic α-cell mass at 20 weeks-of-age showed that the α-cell areas were significantly smaller in groups 2, 3 and 4 *db*/*db* mice compared with group 1 *db*/*db* mice (Figure 3c). To elucidate islet volume in the pancreas, we carried out chromogranin A staining of pancreatic islets. Pancreatic islets were found to be largest in group 4 mice (Figure 3d).

To elucidate the effects of dapagliflozin on pancreatic β-cell proliferation and apoptosis, we carried out Ki67 and TUNEL staining of insulin-positive cells, respectively. Ki67 staining showed that β-cell proliferation at 20 weeks-of-age was highest in group 4, whereas it was lowest in group 1 (Figure 3e). Conversely, TUNEL staining showed that the number of TUNEL-positive β-cells was highest in group 1, and it was lowest in group 4 (Figure 3f).

Early Administration of Dapagliflozin has a Positive Effect on the Retention of Pancreatic β-Cell Mass

Although it was evident that the duration and timing of dapagliflozin administration had an effect on pancreatic β-cell mass, it was difficult to compare the four groups for the longitudinal effect of dapagliflozin administration. Therefore, we administered dapagliflozin for an additional 6 weeks to the four groups of mice, measured their blood glucose levels, serum insulin levels, and pancreatic α-cell and β-cell mass at 26 weeks-of-age. We found that blood glucose levels were highest in group 1 mice, and improved the most in group 4 mice. Interestingly, compared with the blood glucose levels in group 3 mice, those of group 2 mice showed a notable improvement (Figure 4a). Serum insulin levels were prominently elevated in group 2 mice, which were considered to contribute to the improvement in blood glucose levels (Figure 4b). Quantitative measurement showed that pancreatic α-cell mass was the largest in group 1 mice, whereas it was the smallest in group 4 mice (Figure 4c). In contrast, the smallest pancreatic β-cell mass was found in group 1 mice, whereas the largest was in group 4 mice. It was notable that pancreatic β-cell mass in group 2 mice tended to be increased compared with group 3 mice (Figure 4d). Similar to pancreatic β-cell mass, pancreatic islets in group 2 mice tended to be increased compared with those in group 3 mice (Figure 4e). Ki67 staining at 26 weeks-of-age also showed that pancreatic β-cell proliferation tended to be increased in group 2 mice compared with group 3 mice (Figure 4f). In TUNEL staining, the smallest number of TUNEL-positive cells was found in group 4 mice, and the number of TUNEL-positive cells tended to be lower in group 2 mice compared with group 3 mice (Figure 4g). Reducing glucose toxicity from 20 weeks-of-age was more effective in group 2 than in group 3 for the retention of pancreatic β-cell mass, indicating that the early administration of dapagliflozin could have a positive effect on later treatment.

Increased Expression of Agr2, Tff2 and Gkn3 in Pancreatic Islets After the Early Administration of Dapagliflozin

To elucidate the molecular mechanisms underlying these phenotypic differences, we carried out microarray analysis using pancreatic islets from the four groups of mice at 20 weeks-of-age. We extracted genes whose expression was more than doubled. As a result, in group 2 mice, 209 genes were upregulated and 1,826 genes were downregulated; in group 3 mice, 237 genes were upregulated and 3,015 genes were downregulated; and in group 4 mice, 203 genes were upregulated and 547 genes were downregulated.

One of the most significant findings in the present study was that the early administration of the SGLT2 inhibitor, dapagliflozin, was exceedingly effective at maintaining pancreatic β-cell mass. Thus, the earlier administration of an SGLT2 inhibitor might be able to maintain pancreatic β-cell mass over time even though the mice were administered the drug for the same length of time. To verify this hypothesis, we tried to identify similarities between group 2 and group 4 mice, both of which were administered dapagliflozin from 9 weeks-of-age, and elucidate their differences from group 3 mice, which were administered dapagliflozin from 15 weeks-of-age. From the microarray analysis, we searched for genes whose expression was more than doubled in both group 2 and group 4 mice, and extracted nine candidate genes (Table 1). The number of extracted genes was unexpectedly small. We believe that this was because of the differences in gene expression influenced by the differences in blood glucose levels between group 2 and group 4 mice, as blood glucose levels in group 3 and group 4 mice were approximately 250 mg/dL, whereas they were as high as 582 mg/dL in group 2 mice. This means that there is a high possibility that the nine extracted genes that were commonly upregulated in group 2 and group 4 mice are related to the early administration of dapagliflozin.

From these nine genes, we searched for genes that were not upregulated in group 3 mice, and extracted three genes: *Agr2*,

Table 1 | Gene sets upregulated both in the pancreatic islets of group 2 and group 4 mice compared with group 1 mice in microarray analysis

Probe name	Gene symbol	Gene name	FC ([dbdb_4] vs [dbdb_1])	Log FC ([dbdb_4] vs [dbdb_1])	Regulation ([dbdb_4] vs [dbdb_1])	FC ([dbdb_2] vs [dbdb_1])	Log FC ([dbdb_2] vs [dbdb_1])	Regulation ([dbdb_2] vs [dbdb_1])	FC ([dbdb_3] vs [dbdb_1])	Log FC ([dbdb_3] vs [dbdb_1])	Regulation ([dbdb_3] vs [dbdb_1])
A_51_P209122	*Agr2*	Anterior gradient 2	11.806119	3.5614629	Up	2.41	1.27	Up	0.856928	−0.22275	Down
A_51_P480602	*Tff2*	Trefoil factor 2 (spasmolytic protein 1)	9.659103	3.2718892	Up	9.75	3.28	Up	0.477734	−1.06572	Down
A_51_P267700	*Gkn3*	Gastrokine 3	8.970609	3.165206	Up	13.08	3.71	Up	0.666484	−0.58536	Down
A_51_P333549	*Reg2*	Regenerating islet-derived 2	4.07349	2.0262654	Up	9.55	3.26	Up	4.488359	2.166188	Up
A_51_P510156	*Lcn2*	Lipocalin 2	2.9515853	1.56149	Up	3.65	1.87	Up	2.160748	1.111531	Up
A_52_P127569	*Pigg*	Phosphatidylinositol glycan anchor biosynthesis, class G	2.4536302	1.2949178	Up	2.80	1.49	Up	6.762811	2.757623	Up
A_66_P104361	*Gm14051*	Predicted gene 14051	2.1316068	1.0919414	Up	2.03	1.02	Up	3.74384	1.904519	Up
A_51_P511608	*Mtag2*	Metastasis associated gene 2	2.1161308	1.0814288	Up	2.89	1.53	Up	5.561634	2.475509	Up
A_51_P247928	*Clmn*	Calmin	2.0245616	1.0176096	Up	2.16	1.11	Up	1.524861	0.608677	Up

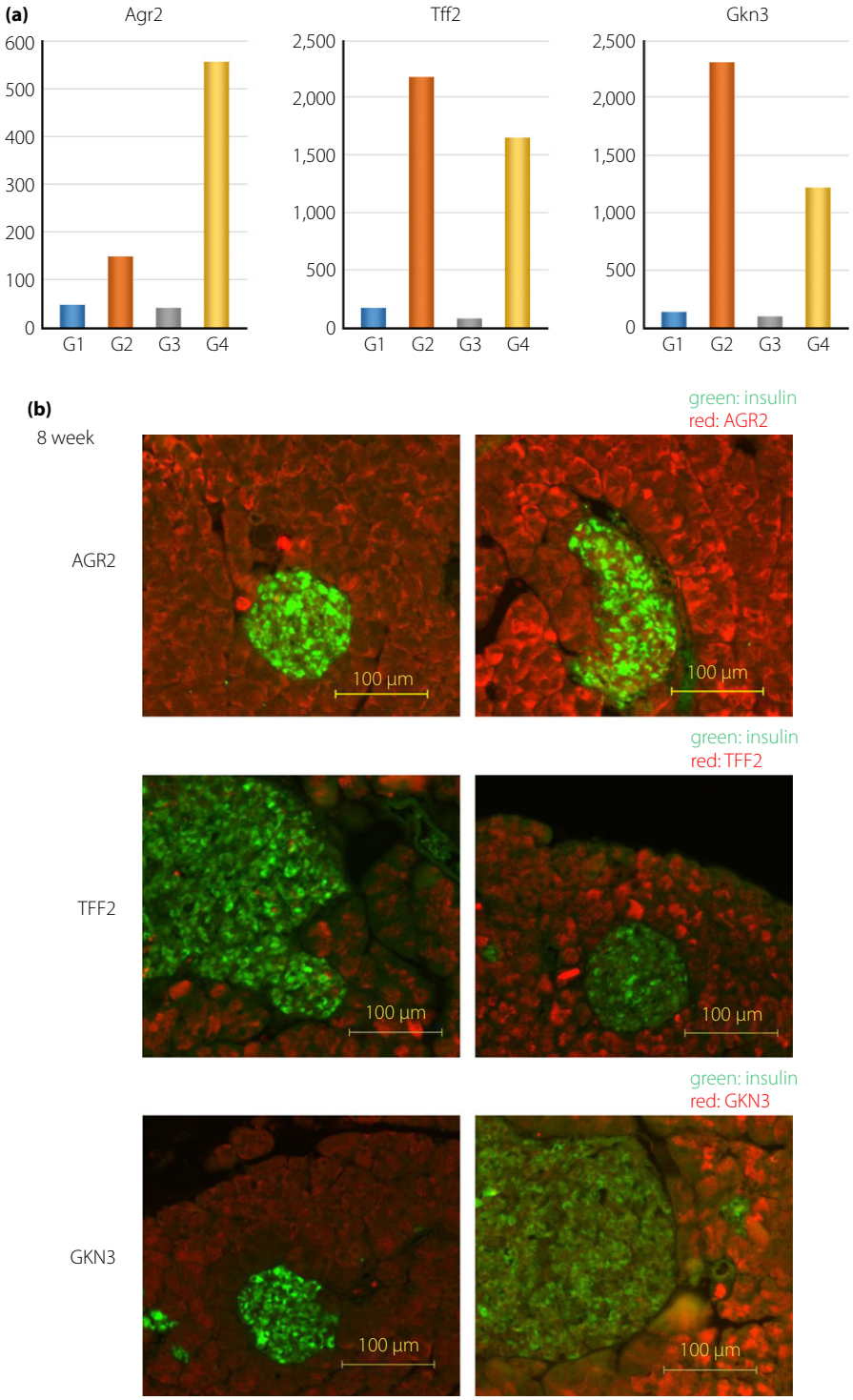


Figure 5 | *Agr2*, *Tff2* and *Gkn3* expression levels of each group of *db/db* mice administered dapagliflozin. (a) Microarray analysis for *Agr2*, *Tff2* and *Gkn3* expression levels in each group of db/db mice. (b,c) Immunostaining for anterior gradient 2 (AGR2; red), trefoil factor 2 (TFF2; red) and gastrokine 3 (GKN3; red) and insulin (green) in each group of *db/db* mice at (b) 8 and (c) 26 weeks-of-age (w). (d) Immunostaining for AGR2, TFF2 and GKN3 (red) and insulin (green) in group 1 *db/db* mice at 26 weeks-of-age. (e) Immunostaining for AGR2, TFF2 and GKN3 (red) and glucagon (green) in group 1 *db/db* mice at 26 weeks-of-age. G1, group 1; G2, group 2; G3, group 3; G4, group 4.

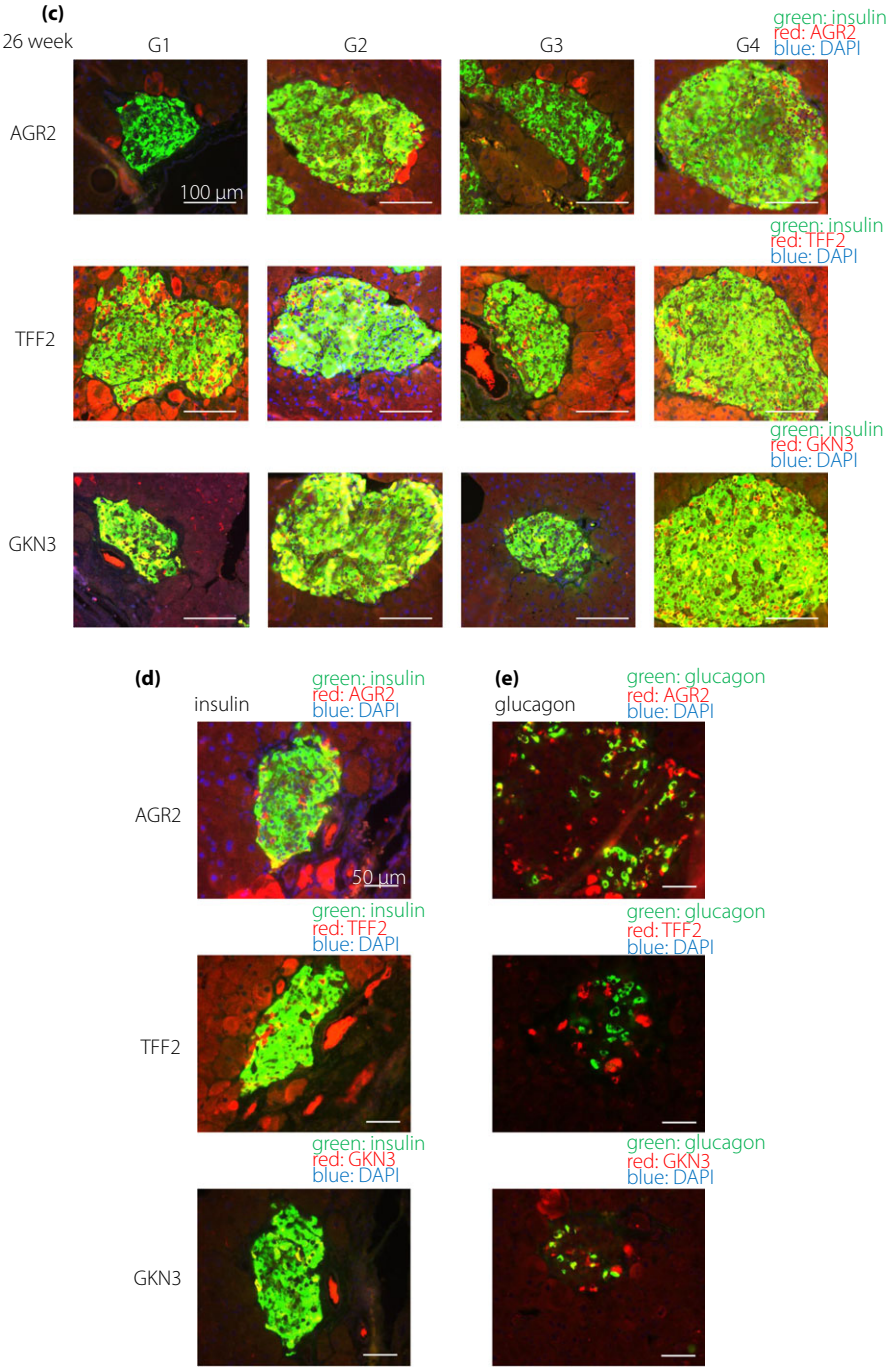


Figure 5 | Continued.

Tff2 and *Gkn3* (Figure 5a). It was previously reported that AGR2 is induced by endoplasmic reticulum (ER) stress and is involved in cell proliferation[18,19]. We believe that the other two genes are more important in relation to the effect of early dapagliflozin administration. TFF2 has been reported to positively regulate pancreatic β-cell growth[20]. GKN3 has been reported to be coexpressed and interact with TFF2 in stomach mucosal cells for cell proliferation[21]. Furthermore, it is noteworthy that *Tff2* and *Gkn3* were significantly upregulated in group 2 and group 4 mice. The upregulated expression of these three genes in the islets at 20 weeks-of-age is considered to have contributed to the increase in pancreatic β-cell mass at 26 weeks-of-age.

Immunostaining for AGR2, TFF2 and GKN3 in pancreatic islets of each group of *db*/*db* mice at 8 weeks-of-age showed they were not expressed before dapagliflozin treatment (Figure 5b). After treatment, their expression was increased in

pancreatic β-cells, especially those of group 2 and group 4 *db*/*db* mice at 26 weeks-of-age (Figure 5c). Their expression was found mainly in pancreatic β-cells and partly in pancreatic α-cells (Figure 5d,e).

DISCUSSION

The percentage of type 2 diabetes mellitus patients who are prescribed SGLT2 inhibitors is increasing rapidly due to their positive effect on macrovascular complications, which became apparent in the EMPA-REG OUTCOME and CANVAS trials[1,2]. SGLT2 inhibitors also have other favorable effects, such as blood pressure reduction, weight loss and lower risk of hypoglycemia. They are expected to play important roles in the treatment of diabetes, and their effects on pancreatic β-cell protection should be appraised thoroughly to evaluate their longitudinal glucose-lowering effects. It has already been shown that SGLT2 inhibitors improve insulin secretion in patients with type 2 diabetes mellitus and they restore pancreatic β-cell mass in animal models of diabetes[5,22]. The reduction of glucose toxicity is considered to be an important mechanism for their effect. However, there have been no reports analyzing the precise duration and timing for the administration of SGLT2 inhibitors to preserve pancreatic β-cells. We can easily presume that the early start of treatment for diabetes provides organ protection and the duration of treatment definitely influences its effectiveness, so study participants can be divided into two groups whose durations are the same, but were started at different times, namely, "early administration" and "late administration."

Recently, Takahashi *et al.*[23] administered the SGLT2 inhibitor luseogliflozin to 6-, 10-, 14- and 24-week-old *db*/*db* mice for 4 weeks, and found that *Mafa* and *Pdx1* expression levels were increased, and the increased proliferation and suppressed apoptosis of pancreatic β-cells were more evident in mice that were administered the drug earlier. These results support the present findings and are of great importance, because they showed that SGLT2 inhibitors protect pancreatic β-cells more effectively when administered at the early phase. However, it was not investigated whether such an effect of early administration persisted over time. Thus, in the present study, we investigated the long-term effect of the administration of SGLT2 inhibitors.

In the present study, we administered the SGLT2 inhibitor, dapagliflozin, to a representative mouse model of type 2 diabetes mellitus, *db*/*db* mice, and investigated its effects on pancreatic β-cell mass. For 12 weeks from 9 weeks-of-age, we administered dapagliflozin for the first 6 weeks to group 2 mice, the last 6 weeks to group 3 mice and the entire period to group 4 mice, and compared their pancreatic β-cell mass with those in group 1 mice, which were administered saline. As a result, in group 4 mice, pancreatic β-cell mass was threefold higher than that of group 1 mice. In group 3 mice, pancreatic β-cell mass was 1.6-fold as high as that of group 1 mice. Interestingly, in group 2 mice, pancreatic β-cell mass was almost the same as that in group 3 mice. These results showed that the earlier administration of dapagliflozin had an impact on pancreatic β-cell proliferation, apoptosis and transdifferentiation.

From these results, it is evident that pancreatic β-cells were prominently preserved by the early start of dapagliflozin administration, as expected. In contrast, when dapagliflozin was started earlier and discontinued, a certain level of effect remained. As the pancreatic β-cell mass of group 2 mice was the same as that of group 3 mice at 20 weeks-of-age, we subsequently bred two groups of mice in the same condition and investigated the differences that developed between the groups. As a result, we discovered a greater improvement in insulin secretion, blood glucose levels and pancreatic β-cell mass in group 2 mice.

In recent studies, the fact that dedifferentiation plays an important role in the regulation of pancreatic β-cell mass has drawn attention. Pancreatic endocrine cells are considered to be associated with a high degree of plasticity, and stresses, such as glucose toxicity, greatly affect their plasticity[24,25]. It has been reported that environmental stresses, such as hyperglycemia and undernutrition, influence epigenetic modifications, and affect pancreatic β-cell mass and function[26–28]. From the present results, in group 2 mice, which were administered dapagliflozin for 6 weeks from 9 weeks-of-age, pancreatic β-cell mass was increased by the reduction of glucose toxicity at a younger age when plasticity is high. As a legacy effect of the early administration of SGLT2 inhibitors was shown to be important, we carried out microarray analysis to determine the changes in gene expression that contributed to this effect. After we analyzed pancreatic islets from all groups of mice at 20 weeks-of-age, we examined the genes that were commonly upregulated in group 2 and group 4 mice, which were administered dapagliflozin from 9 weeks-of-age, and extracted nine genes. The number of extracted genes was unexpectedly small, which might be because the blood glucose levels in both groups of mice at 20 weeks-of-age were considerably different. Furthermore, to identify genes that were important for early administration from those involved in late administration, out of the nine genes, we searched for genes that were not upregulated in group 3 mice, and extracted just three genes, namely, *Agr2*, *Tff2* and *Gkn3*.

AGR2 is a member of the disulfide isomerase family that is reported to be induced by ER stress[18]. Higa *et al.*[19] reported that AGR2 expression was partly regulated by IRE1α in HepG2 cells. It is considered to play a role in regulating the unfolded protein response, and alleviates ER stress through the activity of ATF6 and XBP1[19]. Research groups, including ours, have reported that pancreatic β-cells in diabetes conditions are exposed to an enormous level of ER stress, leading to pancreatic β-cell failure[29,30]. Therefore, it is important that pancreatic β-cells are resistant to ER stress for their protection. The maintained pancreatic β-cell mass in group 2 and group 4 *db*/*db* mice might be attributed to the increased expression of the ER

stress-resistant protein, AGR2, after the early administration of dapagliflozin.

TFF2 is a peptide secreted from the gastrointestinal tract and is dominantly expressed on the surface epithelium of the gastrointestinal tract[31]. Tu *et al.*[32] reported that gastrin regulated TFF2 transcription through PKC-, MEK1- and PI3K-dependent pathways. It has also been reported to be expressed in pancreatic β-cells, and its messenger ribonucleic acid expression levels in the normal state are very low. However, it has been reported that the forced expression of TFF2 in pancreatic islets or pancreatic β-cells upregulates cell proliferation[20]. Orime *et al.* showed that TFF2 enhances pancreatic β-cell proliferation through CX-chemokine receptor 4-mediated extracellular signal-regulated kinase 1/2 phosphorylation. Together with the present data, these observations suggest that the early reduction of glucose toxicity might upregulate TFF2 expression in pancreatic β-cells, which could lead to the retention of pancreatic β-cell mass through an autocrine mechanism.

GKN3 is a gastrokine that is expressed specifically in the stomach, but its function is obscure. GKN3 expression has been found to be upregulated in gastric antral tumorigenesis and Japanese encephalitis virus-infected brain tissues[21,33]. It was reported that GKN2 forms heterodimers with TFF1 in stomach mucosal cells and regulates the extracellular function of TFFs[34]. GKN3 was also shown to colocalize with TFF2[21]. It is interesting that both GKN3 and TFF2 expression was upregulated in group 2 and group 4 mice in our microarray data. However, the underlying mechanism has not been identified yet, and further study is require.

In the present study, we showed that the early administration of dapagliflozin generated highly protective effects on pancreatic β-cells through a "legacy effect." SGLT2 inhibitors might become the first-choice drug for the treatment of diabetes not only because they have beneficial effects on the cardiovascular system, but also because they are expected to exert protective effects on pancreatic β-cells.

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DISCLOSURE

The authors declare no conflict of interest.

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