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Dapagliflozin administration to a mouse model of type 2 diabetes induces DNA methylation and gene expression changes in pancreatic islets

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Abstract

Decreased pancreatic β-cell volume is a serious problem in patients with type 2 diabetes mellitus, and there is a need to establish appropriate treatments. Increasingly, sodium/glucose cotransporter 2 (SGLT2) inhibitors, which have a protective effect on pancreatic β-cells, are being prescribed to treat diabetes; however, the underlying mechanism is not well understood. We previously administered SGLT2 inhibitor dapagliflozin to a mouse model of type 2 diabetes and found significant changes in gene expression in the early-treated group, which led us to hypothesize that epigenetic regulation was a possible mechanism of these changes. Therefore, we performed comprehensive DNA methylation analysis by methylated DNA immunoprecipitation using isolated pancreatic islets after dapagliflozin administration to diabetic model mice. As a result, we identified 31 genes with changes in expression due to DNA methylation changes. Upon immunostaining, cystic fibrosis transmembrane conductance regulator and cadherin 24 were found to be upregulated in islets in the dapagliflozin-treated group. These molecules may contribute to the maintenance of islet morphology and insulin secretory capacity, suggesting that SGLT2 inhibitors' protective effect on pancreatic β-cells is accompanied by DNA methylation changes, and that the effect is long-term and not temporary. In future diabetes care, SGLT2 inhibitors may be expected to have positive therapeutic effects, including pancreatic β-cell protection.

Keywords: SGLT2 inhibitor, pancreatic islet, DNA methylation, MeDIP

1. Introduction

Cases of type 2 diabetes are increasing worldwide and are expected to exceed 1.3 billion by 2050 [1]. This dramatic rise in cases is associated with many problems, especially the serious complications caused by hyperglycemia. In recent years, drugs for diabetes that are expected to prevent the onset and progression of complications have emerged and are attracting attention. In particular, sodium/glucose cotransporter 2 (SGLT2) inhibitors are associated with a significantly reduced risk of cardiovascular death in secondary prevention as well as heart failure hospitalization in primary prevention [2,3,4]. In addition, SGLT2 inhibitors are reported to protect renal function [5], and there have also been several reports on their effects on fatty liver [6], indicating that they are protective in various organs.

We have previously studied the mechanism by which endoplasmic reticulum stress leads to pancreatic β-cell failure in type 2 diabetes, type 2 diabetes susceptibility genes, and so on [7,8]. Furthermore, we have shown that SGLT2 inhibitors have a protective effect on pancreatic β-cells in a mouse model of diabetes [9], and several laboratories have reported that SGLT2 inhibitors maintain pancreatic β-cell function and volume

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Abbreviations: Cdh24, cadherin 24; Cftr, cystic fibrosis transmembrane conductance regulator; MeDIP, methylated DNA immunoprecipitation; PCR, polymerase chain reaction; SGLT2, sodium/glucose cotransporter 2

during the pathogenesis of diabetes [10,11]. However, it is not well known when administration of SGLT2 inhibitors is most beneficial for pancreatic β-cells. Therefore, we compared the volume of pancreatic β-cells in a murine model of type 2 diabetes (db/db) treated with dapagliflozin, an SGLT2 inhibitor, in early and late administration groups after the onset of diabetes. We found that blood glucose levels were lower and pancreatic β-cell volume was significantly maintained in the early administration group, despite having the same administration period [9]. In that report, microarray analysis of gene expression in the islets of db/db mice in the early- and late-treated groups revealed that the expression of many genes was altered between both groups. We focused on epigenetic regulation as a mechanism for the modulation of gene expression according to the timing of SGLT2 administration. Epigenetics can change gene expression without sequence alteration, and it is affected by environmental factors during periods of high plasticity such as the embryonic period and young age [12]. Therefore, we hypothesized that integrating DNA methylation analysis and exhaustive gene expression analysis of pancreatic islets would allow us to identify gene expression changes with a higher degree of confidence. In the present study, we performed integrated analysis combining the microarray analysis in our previous report with methylated DNA immunoprecipitation (MeDIP), a new method for finding exhaustive DNA methylation, to elucidate the effects

of SGLT2 inhibitors on islets in a murine model of diabetes.

2. Material and methods

2.1 Animals

Male db/db mice on a C57BL/KsJ background (CLEA Japan, Inc., Tokyo, Japan) were fed normal chow and maintained on a 12-h light/dark cycle, and their blood glucose was determined as previously described [8]. The study protocol, which conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines, was approved by the Animal Ethics Committee of Kobe University Graduate School of Medicine (Approval No. P160102).

2.2. Dapagliflozin administration

The mice were randomly allocated to the three groups (n=4 each), and dapagliflozin and saline were administered as follows. In Group A, saline was administered between the ages of 9 and 20 weeks. In Group B, saline was administered between the ages of 9 and 14 weeks, while dapagliflozin (1 mg/kg) was administered orally between the ages of 15 and 20 weeks. In Group C, dapagliflozin (1 mg/kg) was administered orally between the ages of 9 and 20 weeks. and Body weight and fed blood glucose levels were measured every 2 weeks starting at the age of 8 weeks.

2.3 Immunostaining and morphometric analysis

After excision and immersion in a 4% paraformaldehyde solution, the pancreas was embedded in paraffin and sliced into 4–5-μm sections, which were subsequently stained

with antibodies to glucagon and insulin (Agilent Technologies, Santa Clara, CA). Cy3- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used for the detection of immune complexes. For cadherin 24 (Cdh24) and cystic fibrosis transmembrane conductance regulator (Cftr) staining, pancreatic sections were incubated with an anti-Cdh24 antibody (LifeSpan Biosciences, Shirley, MA) or anti-Cftr antibody (Proteintech, Rosemont, IL) and Cy3-conjugated secondary antibody.

2.4 Isolation of RNA from isolated pancreatic islets

Isolation of islets from the four db/db mice in each group was performed as previously described [13]. Extraction and purification of total RNA was performed using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and an Agilent Bioanalyzer (Agilent Technologies) were used to ascertain both the quantity and quality of the RNA, with reference to a previous study [14]. Microarray analysis was performed using the combined samples.

2.5 Reverse transcription and real-time analysis

Isolation of total RNA from the islets was performed using an RNeasy Kit (Qiagen). SYBR Green (Promega, Madison, WI) and an ABI 7900 sequencer (Life Technologies, Carlsbad, CA) were used to perform real-time polymerase chain reaction (PCR) analysis, as previously described [8]. As an invariant control, the abundance of the target mRNAs was normalized to that of cyclophilin mRNA.

2.6 Microarray data analysis

Agilent feature extraction software (ver. 11.5.1.1), which is capable of performing background subtractions, was used to quantify the intensity values of the scanned features. Only those that were identified as having no errors (i.e., Detected Flags) were used, while features that were not positive, significant, uniform, or above the background were excluded, as were those that were saturated or population outliers (i.e., Not Detected and Compromised Flags). Agilent GeneSpring software (ver. 14.8) was used to perform normalization (normalization to the 75th percentile shift per chip).

2.7 MeDIP analysis

To enrich the methylated DNA, prepare the library, and perform sequencing, 1 μg of genomic DNA from each sample was fragmented into ~200 bp using an ultrasonicator (M220 Focused-ultrasonicator; Covaris, LLC, Woburn, MA). Fragmented DNA was subjected to methylated DNA enrichment using a CpG MethylQuest DNA Isolation Kit (Merck Millipore, Burlington, MA). A KAPA HyperPlus kit (Kapa Biosystems, Wilmington, MA) was used to construct the library for Illumina sequencing. Size selection (0.2–0.6× volume of purification beads) was performed after library amplification. Then, an Agilent 2200 TapeStation High Sensitivity D1000 (Agilent Technologies) was used to assess the library quality, and a NextSeq system (Illumina, San Diego, CA) was used to sequence the pooled sample libraries in 76-bp single-end reads.

The tool Trimmomatic-0.32 [15] was used to trim the sequencing adaptors, low-quality reads, and bases, while bowtie2-2.2.6 [16] was used to align the sequence reads to the mouse reference genome (mm10). Then Picard (ver. 1.119; https://broadinstitute.github.io/picard/) was used to remove the PCR duplicates.

Downstream analysis of the aligned reads was performed using the R package MEDIPS (ver. 1.30) [17], and MEDIPS was used to calculate the short-read coverage in genome-wide 100-bp bins (extend value = 300). Differentially methylated regions were detected based on the RPKM values for genome-wide 100-bp sliding windows.

2.8 Statistical analysis

Data were compared by analysis of variance followed by a two-tailed Student's *t*-test and are presented as the mean ± standard error of the mean. statistical significance was indicated as $P < 0.05$.

3. Results

3.1 Mice treated with the SGLT2 inhibitor dapagliflozin exhibit hypoglycemia, weight gain, and DNA methylation changes in islets

Dapagliflozin and/or saline was administered to Groups A, B, and C, as shown in Fig. 1A. First, blood glucose levels and body weight were assessed to ensure that the metabolic data in these groups were similar to those of a previous study [9]. As reported previously, the weight of each group increased with growth, and Group C was significantly heavier than Group A. Group B also gained significantly more weight than Group A in the second half of dapagliflozin treatment (Fig. 1B). Blood glucose levels were 500–600 mg/dL in Group A and around 500 mg/dL in Group B in the first half of

treatment, but dropped to around 200 mg/dL in the second half of treatment due to dapagliflozin administration (Fig. 1C). The blood glucose level of Group C was 200 mg/dL, indicating that blood glucose significantly decreased after drug administration (Fig. 1C).

Next, islets were isolated from db/db mice of each group, and DNA methylation analysis was performed on extracted genomic DNA using MeDIP. First, a comparison of DNA methylation of the three groups is shown using an MA-plot in Fig. 1D. Similar trends in DNA methylation changes were observed in Groups A and B, as well as in Groups A and C. However, a comparison of changes in DNA methylation between Groups B and C showed different results to the other comparisons. This indicates that the methylation changes in Groups B and C relative to Group A were similar, suggesting that dapagliflozin administration affected islet DNA methylation, even for a short period of administration. An exhaustive comparison of the DNA methylation of genes in islets from Groups A, B, and C was then performed using MeDIP.

3.2 Effects of dapagliflozin administration on DNA methylation and gene expression in mouse pancreatic islets identified through comprehensive integrative analysis

It is clear that dapagliflozin treatment affects DNA methylation in pancreatic islets,

but we do not know how it affects gene expression as a result. Essentially, DNA hypermethylation decreases gene expression, while DNA hypomethylation increases gene expression [18]. We have previously used microarrays to analyze the changes in gene expression in mouse islets isolated from the three groups of db/db mice in a similar experimental design [9]. By integrating the results of the previous microarray analysis with those of the current MeDIP analysis, we identified a group of genes whose changes in expression were consistent with the changes in DNA methylation (Table 1). In this integrated analysis, the findings between Groups B and C were compared first. As a result, 18 genes in Group C showed decreased DNA methylation and increased expression, while one gene showed increased DNA methylation and decreased expression. Next, a comparison was made between Groups A and C. Sixteen genes were hypomethylated and showed increased expression in Group C. However, no genes were highly methylated or downregulated (Fig. 2A). Including the genes that overlapped in each comparison, there were 31 genes for which the expression changes identified by microarray analysis matched the DNA methylation changes identified by MeDIP. To confirm that these genes were expressed in islets, RNA extracted from islets was subjected to real-time PCR, and it was confirmed that 12 of the 31 genes were expressed in islets. Furthermore, the expression of six genes—Cdh24, Cftr, Spo11, Begain, Mae1, and Ddx4—was

upregulated in Group C (Fig. 2B).

3.3 Cdh24 and Cftr are upregulated in the islets of dapagliflozin-treated mice

Among these genes, we focused on Cdh24 and Cftr. Cdh24 is a member of the cadherin family and is thought to contribute to intercellular adhesion [19]. For pancreatic β-cells to secrete insulin, intercellular adhesion is important, and the importance of cadherins in islets has been previously reported [20]. However, Cdh24's role in pancreatic β-cells has yet to be fully elucidated. Cftr is a chloride channel expressed in epithelial cells throughout the body, and its genetic mutation causes cystic fibrosis [21]. Cftr is reported to affect pancreatic β-cell function [22], and we tested whether Cftr is associated with pancreatic β-cell protection by SGLT2 inhibitors. First, immunostaining for Cdh24 was performed in islets isolated from each group. Co-staining with insulin revealed increased expression of Cdh24 in pancreatic β-cells in Group B compared with Group A. Furthermore, in Group C, Cdh24 was more strongly co-expressed with insulin (Fig. 2C). These results suggest that SGLT2 inhibitor treatment enhances Cdh24 expression in pancreatic β-cells. Next, immunostaining of Cftr in pancreatic islets was performed. As observed with Cdh24, Cftr expression was upregulated more in Group C compared with Group A and Group B. However, it did not co-stain with insulin or glucagon, and was

mostly expressed in cells negative for both insulin and glucagon (Fig. 2D). These results suggest that Cftr is upregulated in cells other than pancreatic α- and β-cells.

4. Discussion

In recent years, SGLT2 inhibitors have attracted considerable attention as drugs with organ-protective effects in addition to their hypoglycemic effects. In particular, their protective effects on the heart and kidneys have been demonstrated in randomized clinical trials and they have been used in many cases [2–5]. Furthermore, SGLT2 inhibitors are also known to have pancreatic β-cell protective effects, and many laboratories have reported that they improve pancreatic β-cell volume and insulin secretory capacity [10,11].

In the present study, we investigated the methylation of DNA in pancreatic islets in order to elucidate the mechanism underlying the SGLT2 inhibitors' protective effect on pancreatic β-cells, although there are many molecules whose DNA methylation and gene expression are altered in the islets of db/db mice treated with SGLT2 inhibitors. Cdh24 is one of these molecules, and its expression was upregulated in pancreatic β-cells upon SGLT2 inhibitor treatment. Cdh24 is a member of the cadherin family and is thought to contribute to intercellular adhesion, but its role *in vivo* is unknown [19]. Genetic mutations of Cdh24 are observed frequently in gastric and rectal cancers [23]. However,

the mechanism by which Cdh24 is associated with cancer development has not been investigated, and further research is expected.

Cftr is a causative gene of cystic fibrosis and is a component of ion channels in epithelial cells [21]. Cftr is widely expressed in the trachea, pancreatic duct, and intestinal tract, and abnormalities in Cftr cause intestinal ileus, pancreatitis, and respiratory tract infections [24]. Patients with cystic fibrosis are prone to develop diabetes, but the mechanism is not well understood; in 2014, Guo et al. showed that Cftr inhibition suppresses insulin secretion and glucose-dependent potential activity in β-cells [25]. This finding is a breakthrough not only in elucidating the mechanism of Cftr-induced diabetes but also in selecting an appropriate treatment for diabetes in patients with cystic fibrosis. However, the results of the present study revealed increased DNA methylation and decreased expression of the Cftr gene region in the islets of db/db mice. In contrast, SGLT2 inhibitor administration promoted the release of DNA methylation and increased gene expression, suggesting that Cftr expression is altered in the pathogenesis of type 2 diabetes and that increased Cftr expression may be play a role in maintaining islet morphology by SGLT2 inhibitor administration. Interestingly, immunostaining revealed that Cftr expression was upregulated in non-α/β-cells. Previous studies have reported that Cftr expression is expressed predominantly in α-cells in addition to pancreatic β-cells [26],

but Cftr is not expressed in rat δ-cells under normal conditions [27]. The increased expression of Cftr in non-α/β-cells under diabetic conditions observed in the present study has not been reported previously. In future analyses, Cftr expression in δ and PP cells should be examined.

Although SGLT2 inhibitors have attracted attention for their cardio-renal protective effects, this study revealed that they also change gene expression via DNA methylation for the maintenance of islet morphology. The effect is thought to be more long-term and reproducible through epigenetic regulation. In future diabetes care, it is expected that the long-term effects of SGLT2 inhibitor treatment on patients' islets will be closely followed.

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CRediT authorship contribution statement

Aisha Yokoi: Investigation, Methodology, Writing-Original draft. Shun-ichiro Asahara: Conceptualization, Project administration, Writing-Review & Editing. Hiroyuki Inoue: Investigation, Validation. Masako Seike: Investigation, Validation. Nozomi Kido: Investigation. Hirotaka Suzuki: Investigation. Ayumi Kanno: Investigation, Funding acquisition. Maki Kimura-Koyanagi: Supervision, Visualization. Yoshiaki Kido: Supervision, Funding acquisition. Wataru Ogawa: Supervision.

Declaration of competing interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1. Characteristics of db/db mice according to the duration of dapagliflozin administration

(A) Schedule of dapagliflozin and/or saline administration. (B) Body weight for each group of db/db mice at various ages. (C) Blood glucose levels in the fed state for each group of db/db mice at various ages. (D) MA-plot of DNA methylation in pancreatic islets for each group of db/db mice. All quantitative data are the mean ± standard error of the mean for 8–10 independent populations from each group. *$P < 0.05$; **$P < 0.01$.

Figure 2. Integrated analysis of gene expression by microarray and DNA methylation by MeDIP using islets from each group of db/db mice

(A) Integrated comparison of DNA methylation and gene expression in Group B vs. Group C and Group A vs. Group B. Blue enclosure: Group B vs. Group C; Orange enclosure: Group A vs Group C. (B) Quantitative real-time PCR analysis for each of the molecules in islets isolated from each group of db/db mice. All data are presented as the mean ± standard error of the mean for more than five independent populations from each group. *$P < 0.05$; **$P < 0.01$.

Figure 3. Immunostaining of islets from each group of db/db mice for molecules with upregulated expression and hypomethylation in pancreatic islets

(A) Immunostaining for insulin (green) and Cdh24 (red) in each group of db/db mice. (B) Immunostaining for insulin (white) and Cftr (red) in each group of db/db mice. (C) Immunostaining for glucagon (green), insulin (white), and Cftr (red) in each group of db/db mice.

Table 1. Integrated analysis of gene expression by microarray and DNA methylation by MeDIP using islets from dapagliflozin-treated mice

Group C vs. A

Chromosome	Gene symbol	Methylation logFC	Expression logFC	Category
1	Mae1	-0.725181762	1.6023951	TSS
2	Crb2	-0.80734431	1.27001	Gene body
2	Spo11	-0.836597631	0.6663357	Gene body
3	Sycp1	-0.595128392	0.8156362	Gene body
5	Afm	-0.707375292	0.8503055	Gene body
6	Cftr	-0.911655238	0.92333457	TSS
7	Slc8a2	-0.885032867	0.6406193	Gene body
7	4732471J01Rik	-1.007856363	0.774509	Gene body
7	Chrna10	-0.89052566	2.1969695	Gene body
9	Eepd1	-0.647447377	0.7189095	Gene body
10	Lingo3	-0.59693135	1.116196	Gene body
10	Caps2	-0.609365717	0.9953903	Gene body
14	Jph4	-1.051913431	0.7498984	Gene body
15	Eppk1	-1.649560246	0.70220466	Gene body
18	Pcdha4	-0.718358024	0.623227	Gene body
18	Arhgef37	-0.601681233	0.6996246	Gene body

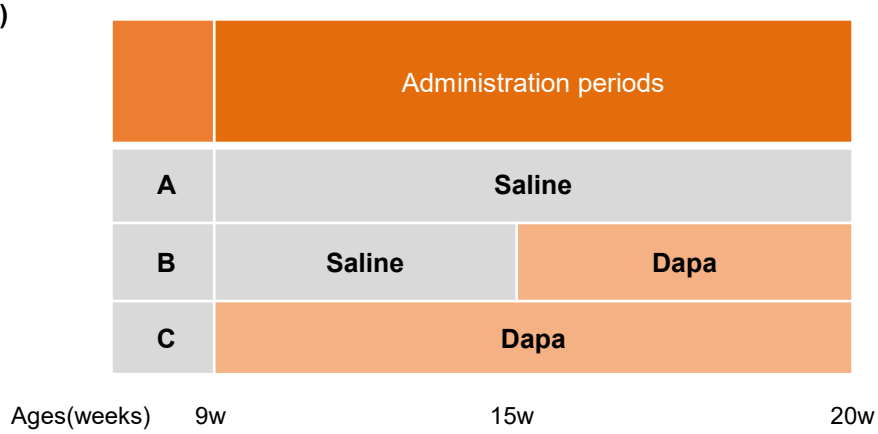
Group C vs. B

Chromosome	Gene symbol	Methylation logFC	Expression logFC	Category
1	Boll	-0.738840187	0.9967699	Gene body
1	Mae1	-0.862354493	1.5176048	Gene body
3	Sycp1	-1.082701265	2.8986793	Gene body
4	Dmrtb1	-0.613836078	0.8418693	Gene body
6	Stk31	-0.874266396	0.6677355	TSS
6	Chst13	-1.102296551	0.8783397	Gene body
6	1700030F04Rik	-0.710638779	0.9744553	Gene body
7	Zfp787	-0.840418044	1.0972704	Gene body
7	Dact3	-0.689804307	1.7687163	Gene body

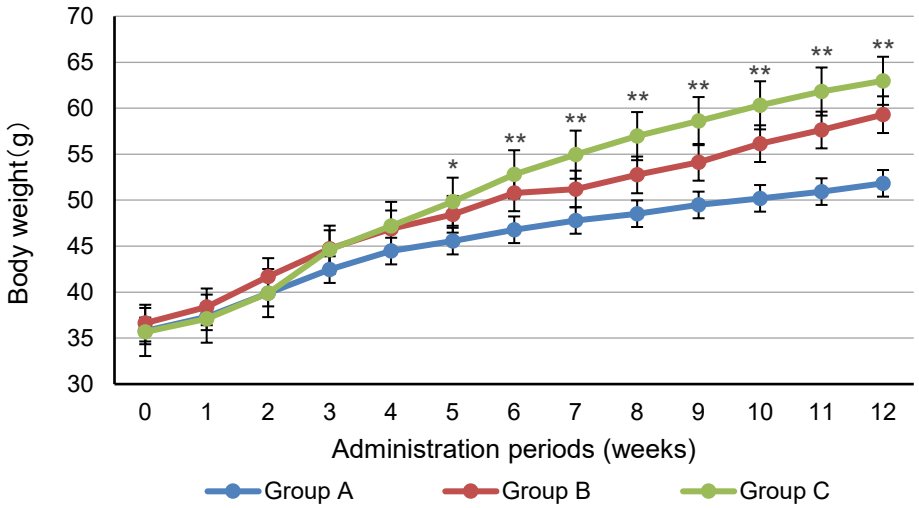
7	Agbl1	-1.023431454	0.9333637	Gene body
12	Dact1	-0.601358419	0.9232903	Gene body
12	Begain	-0.980595969	0.6255436	Gene body
12	Ankrd9	-1.301917405	1.1230879	Gene body
13	Ddx4	-0.58937198	0.9743766	Gene body
14	Cdh24	-1.301501157	2.78031255	Gene body
16	Litaf	-0.626836281	0.8823166	Gene body
18	Epb4.1l4a	-0.603430629	2.15669012	Gene body
X	C77370	0.623048489	-1.022656	Gene body

FC, fold change; TSS, transcriptional start site

(A)


Administration periods
A
Saline
B
Saline
Dapa
C
Dapa
Ages(weeks)
9w
15w
20w

(B)


Body weight(g)
70
65
60
55
50
45
40
35
30
0
1
2
3
4
5
6
7
8
9
10
11
12
Administration periods (weeks)
Group A
Group B
Group C

(C)

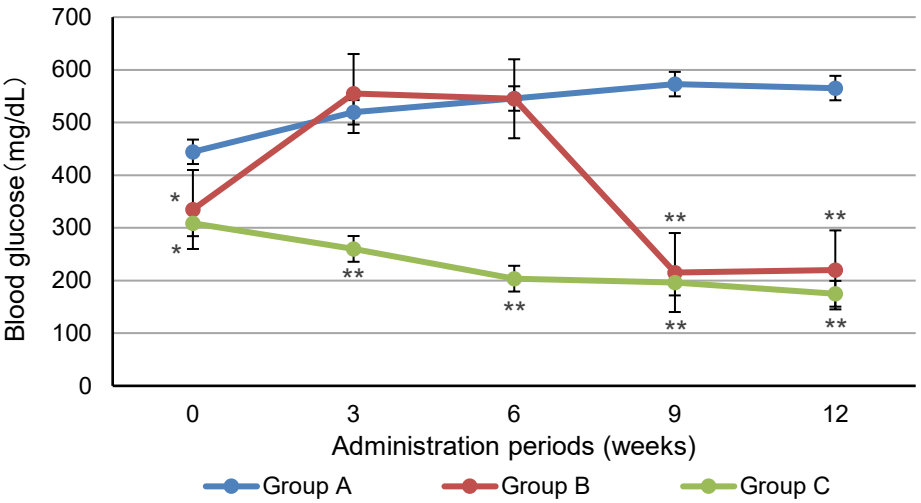

Blood glucose(mg/dL)
700
600
500
400
300
200
100
0
0
3
6
9
12
Administration periods (weeks)
Group A
Group B
Group C

Fig.1 A-C

(D)

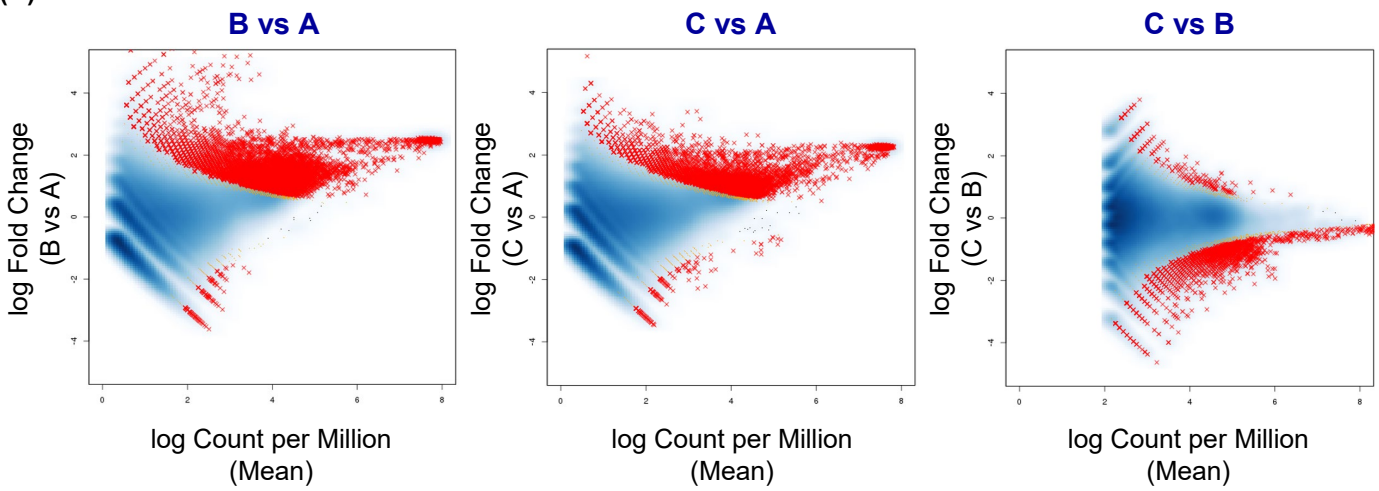

B vs A
C vs A
C vs B
log Fold Change (B vs A)
log Fold Change (C vs A)
log Fold Change (C vs B)
log Count per Million (Mean)
log Count per Million (Mean)
log Count per Million (Mean)

Fig.1 D

(A)

Low methylation & High expression	High methylation &Low expression
Boll, Dmrtb1, Stk31, Chst13, 1700030F04Rik, Zfp787, Dact3, Agbl1,Dact1, Begain, Ankrd9, Ddx4,Cdh24,Litaf, Epb4.1l4a	C77370
Mael, Sycp1	
Crb2, Spo11, Afm, Cftr, Slc8a2, 4732471J01Rik, Chrna10, Eepd1, Lingo3, Caps2,Jph4,Eppk1,Pcdha4,Arhgef37	

: Group C vs Group B

: Group C vs Group A

(B)

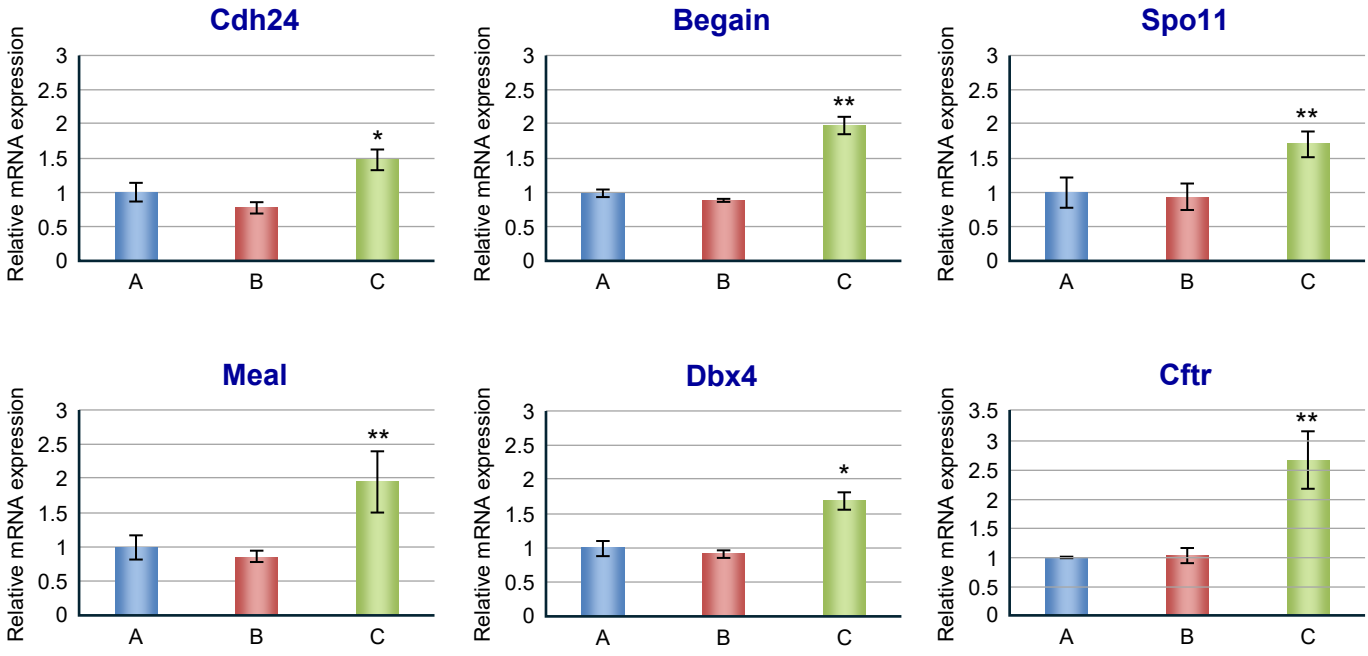


Fig.2

(A)

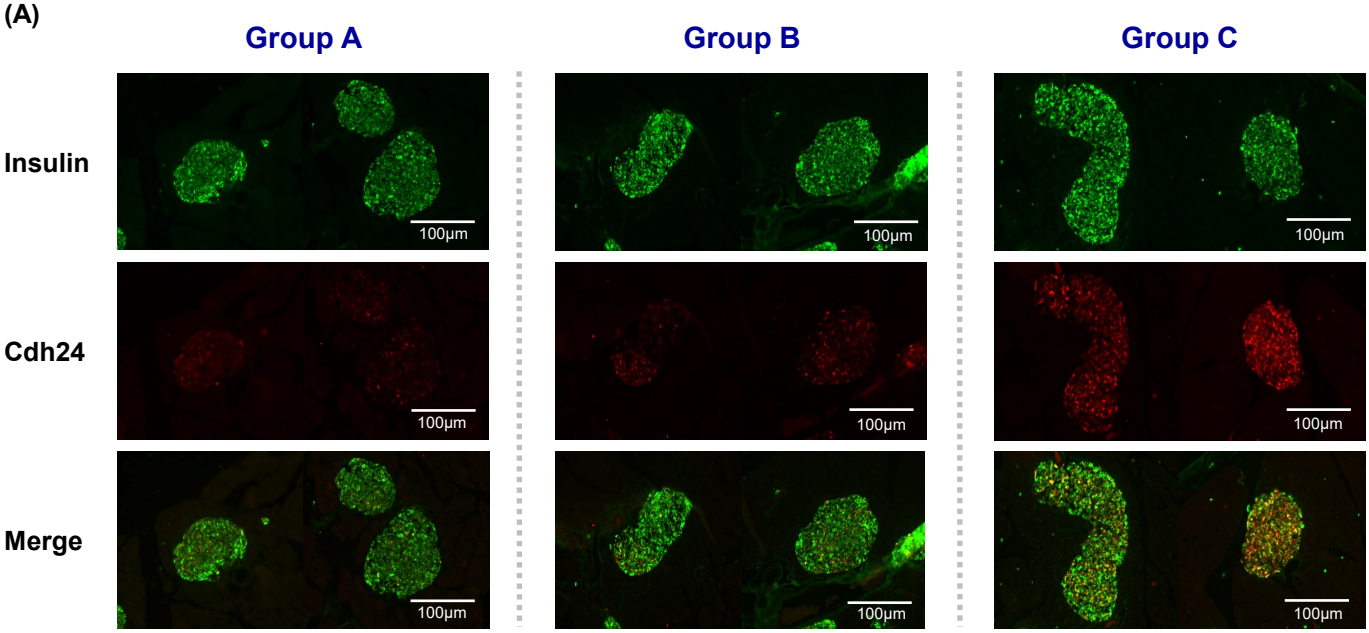


Fig.3 A

(B)

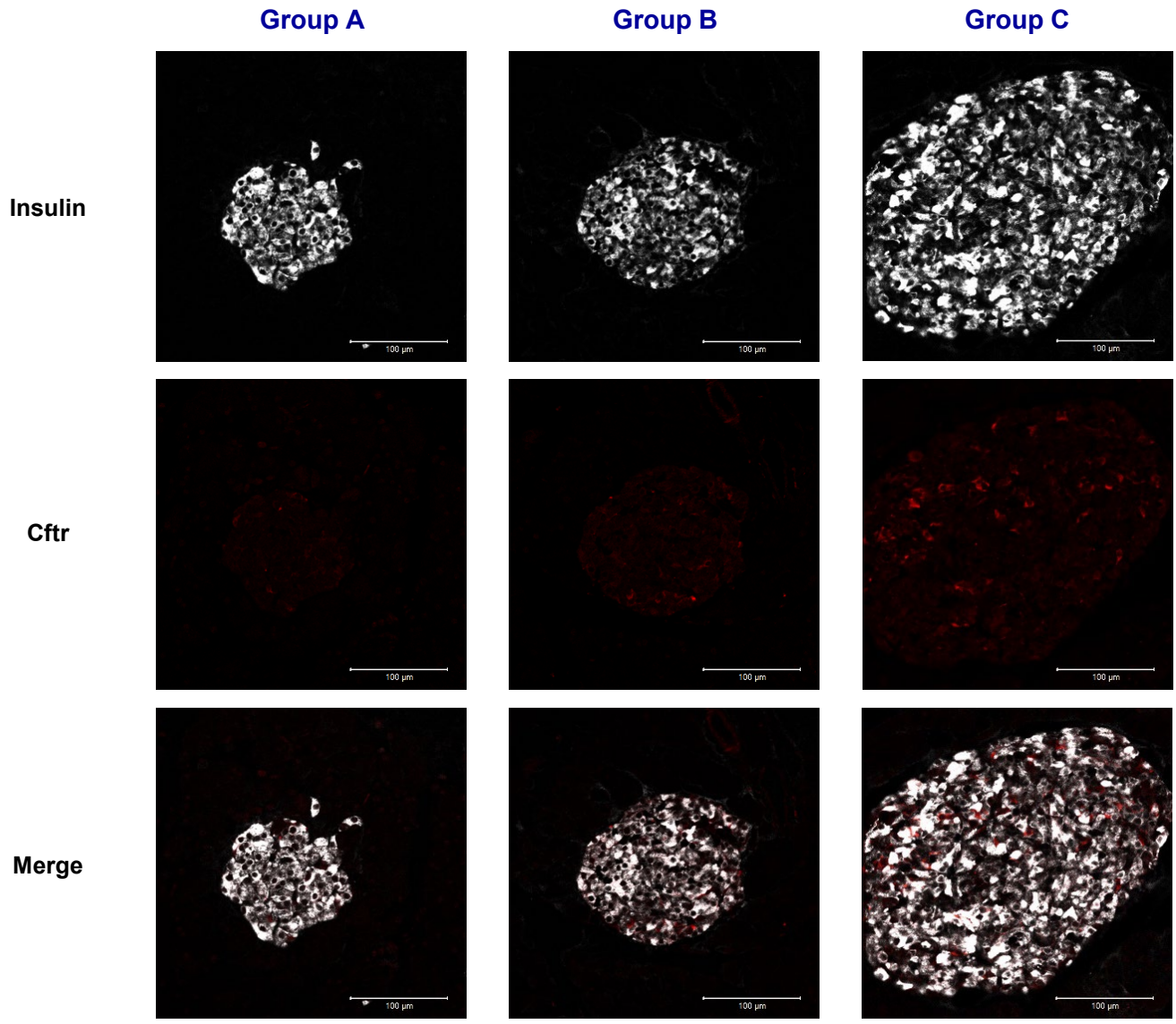


Fig.3 B

(C)

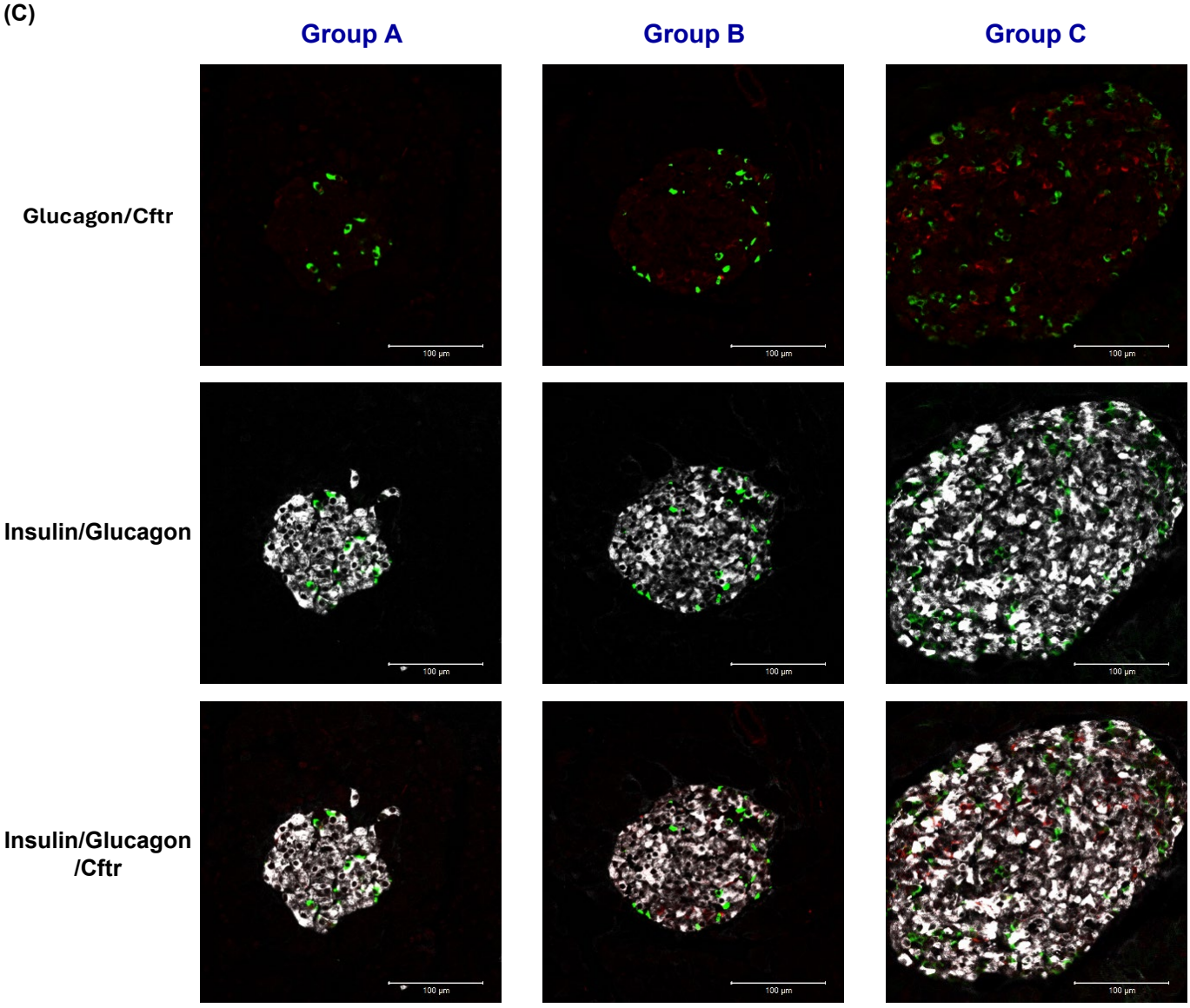

Group A
Group B
Group C
Glucagon/Cftr
Insulin/Glucagon
Insulin/Glucagon
/Cftr
100 µm

Fig.3 C