

RESEARCH ARTICLE | Gut Hormones, Metabolism, Appetite, and Obesity

Transcriptional factor Pdx1 is involved in age-related GIP hypersecretion in mice

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Submitted 16 February 2018; accepted in final form 29 April 2018

Ikeguchi E, Harada N, Kanemaru Y, Sankoda A, Yamane S, Iwasaki K, Imajo M, Murata Y, Suzuki K, Joo E, Inagaki N. Transcriptional factor Pdx1 is involved in age-related GIP hypersecretion in mice. *Am J Physiol Gastrointest Liver Physiol* 315: G272–G282, 2018. First published May 3, 2018; doi:10.1152/ajpgi.00054.2018.—Fat accumulation with aging is a serious problem; glucose-dependent insulintropic polypeptide/gastric inhibitory polypeptide (GIP) is an incretin that plays an important role in fat accumulation. GIP receptor knockout mice show reduced fat mass and improved insulin sensitivity associated with aging. Therefore, GIP is involved in fat accumulation and insulin resistance with aging. However, age-related changes of GIP secretion remain unclear. The present study aimed to elucidate age-related changes of GIP secretion and enteroendocrine K cells using GIP reporter [GIP-green fluorescent protein (GFP) knock-in heterozygous (GIP^{gfp/+})] mice. Aged 1-yr-old GIP^{gfp/+} mice exhibited a phenotype of fat accumulation, insulin resistance, and GIP hypersecretion compared with young (3–4 mo old) GIP^{gfp/+} mice. In aged mice, K-cell number in the small intestine and the mRNA expression levels of GIP and transcriptional factor pancreatic and duodenal homeobox-1 (Pdx1) in K cells were increased. K-cell number, GIP mRNA expression and content in small intestine, and GIP secretion were decreased after posteriori suppression of Pdx1 using intestine-specific gene transfer. Thus, Pdx1 positively regulates GIP mRNA and K-cell number in small intestine. Increased Pdx1 expression might be involved in GIP hypersecretion with aging.

NEW & NOTEWORTHY Age-related changes of glucose-dependent insulintropic polypeptide/gastric inhibitory polypeptide (GIP) secretion and K cells were investigated. We found that K-cell number and GIP and pancreatic and duodenal homeobox-1 (Pdx1) expression in K cells were increased in aged mice, which showed greater GIP secretion compared with young mice. In addition, we have succeeded in posteriori suppression of Pdx1 in small intestine using the method of intestine-specific gene transfer, and showed that K-cell number, GIP expression, and GIP secretion were decreased in the Pdx1-knockdown intestine.

aging; GIP; K cell; Pdx1

INTRODUCTION

Increase in fat mass and coincident decrease in muscle mass, so-called “sarcopenic obesity,” in the elderly has become a

serious global problem in an aging society (4, 29). Fat, especially visceral fat, accumulation leads to metabolic syndrome, including type 2 diabetes and cardiovascular disease based on insulin resistance (6, 18). Fat accumulation causes a decline in the activities of daily living (ADL) in the elderly and increases the burden for their caregivers (2). This is a major obstacle to the management of the ever-increasing aged population of patients with type 2 diabetes. It is therefore necessary to take measures to prevent the progress of fat accumulation. Aging is associated with a progressive increase in fat mass independent of body weight changes (40). One of the possible mechanisms of fat accumulation with aging is a decline in thermoregulation and energy expenditure through decreases in the activity and the amount of uncoupling protein 1 and brown adipose tissue (30).

Glucose-dependent insulintropic polypeptide/gastric inhibitory polypeptide (GIP) is an incretin secreted from enteroendocrine K cells in response to glucose and fat ingestion (5, 7, 39) and potentiates insulin secretion through the GIP receptor (GIPR) expressed in pancreatic β -cells (28, 33). K cells are expressed mainly in upper small intestine, and not in large intestine (13). GIP plays an important role in maintaining blood glucose levels by inducing hypersecretion of insulin in high-fat diet (HFD)-induced obesity (10). GIPR expressed in adipose tissue is involved in HFD-induced insulin resistance and hepatic steatosis (17). Thus, GIP is a key hormone for fat accumulation. GIP secretion is increased in obesity, and inhibition of GIP secretion prevents HFD-induced obesity and insulin resistance (27, 28). The previous studies on GIPR antagonists, chemical K-cell ablation, GIPR-knockout mice, and GIP antibody therapy also indicated that the inhibition of GIP action ameliorates HFD-induced obesity and insulin resistance (1, 23, 24, 26). Therefore, GIP has the possibility to become a therapeutic target in fat accumulation and obesity. It is reported that there is a positive correlation between age and GIP secretion in response to glucose ingestion in humans (37). Another report concluded that aging in people with obesity is associated with enhanced GIP responses to oral glucose (25). A previous study indicated that GIPR-knockout mice show significantly reduced fat mass and improved insulin sensitivity while wild-type mice show increased fat mass associated with aging (38). That study implied that GIP could be involved in fat accumulation and insulin resistance with aging; however, age-related changes of GIP secretion remain unclear. Furthermore, all of the previous studies sug-

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gesting that a reduction of GIP secretion prevents obesity were performed under over-nutrient conditions, such as HFD (26, 27). Thus, it has not been revealed that aging itself, besides the obesity, could change GIP secretion under normal diet conditions.

In the present study, we investigated GIP secretion in aged mice and age-related changes of enteroendocrine K cells under a normal diet condition using GIP-green fluorescent protein (GFP) knock-in (GIP-GFP) mice, which enabled us to visualize and isolate K cells by GFP.

MATERIALS AND METHODS

Animals. Analysis of K cells was achieved both in quality and quantity using a GIP-GFP mouse model characterized by truncation of the *prepro-Gip* gene and insertion of a GFP sequence (34). Male GIP-GFP heterozygous ($GIP^{GFP/+}$) mice were used in all experiments. GIP secretion in $GIP^{GFP/+}$ mice is reduced by ~50% compared with that in wild-type mice (27). We defined $GIP^{GFP/+}$ mice at the age of 3–4 mo (10–16 wk) as young mice and $GIP^{GFP/+}$ mice at the age of 1 yr (46–56 wk) as aged mice and compared the two groups. The data of Fig. 1, A and B, were acquired from one cohort of $GIP^{GFP/+}$ mice. The data of Figs. 1, C–E, 2, 3, and 4 were acquired by measuring parameters in two different cohorts of mice, one young and one aged. The mice were housed in an air-controlled 25°C room with a dark-light cycle of 10 and 14 h with free access to water and normal diet food (3.73 kcal/g; 12% fat, 23% protein, and 65% carbohydrate; Funabashi Farm, Funabashi, Japan), unless otherwise stated. Animal care and procedures were approved by the Animal Care Committee of Kyoto University (Med Kyo 16584).

Measurement of body fat composition. Young and aged mice were dissected, and the weight of visceral fat (both sides of perirenal, epididymal, and mesenteric fat), subcutaneous fat (both sides of inguinal subcutaneous fat) and right side of gastrocnemius muscle was measured. The data were expressed as the weight of fat or muscle per body weight. In addition, body fat was measured by a computed tomography (CT) scan. The mice were anesthetized with intraperitoneal injection of pentobarbital, fixed in a chamber, and scanned using a La Theta experimental animal CT system (LCT-100M, Hitachi Aloka Medical, Tokyo, Japan). Contiguous 2-mm slice images from shoulder to caudal region were used for quantitative analysis of visceral fat, subcutaneous fat, and lean body mass (visceral mass without visceral fat) by La Theta software (vs. 3.00).

Blood collection under free feeding and oral glucose tolerance test. Blood samples (50 μ l) were collected from the tail vein at 10:00 AM under free feeding. An oral glucose tolerance test (OGTT) was performed after a 16-h fasting period. Blood samples were collected at 0, 15, 30, 60, and 120 min after oral glucose (2 g/kg body wt) administration by oral gavage. Blood glucose levels were measured by the glucose oxidase method (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Plasma insulin and total GIP levels were measured using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Shibukawa, Japan) and GIP ELISA kit (MilliporeSigma, Burlington, MA), respectively. Plasma xenin levels were measured using a xenin fluorescent enzyme immunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA). Dipeptidyl peptidase IV (DPP-4) activity was measured using a previously described method (20).

Insulin tolerance test. An insulin tolerance test (ITT) was performed using 0.75 U/kg body wt human regular insulin (100 U/ml, Eli Lilly and Company, Indianapolis, IN) by intraperitoneal injection after a 4-h fasting period. Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after insulin administration. The glucose levels during ITT represent the percentage change from fasting glucose levels.

Immunohistochemistry. The gastrointestinal tract from the stomach to the anus was taken from the mice, and the length of the small

intestine (from the pylorus of the stomach to the ileocecal valve) and the large intestine (from the ileocecal valve to the anus) was measured. The small intestine was divided into two portions at the middle position, and the oral and rectal portions were defined as the upper and lower small intestine, respectively. Upper small intestine samples were fixed in 4% paraformaldehyde phosphate buffer solution, embedded in paraffin, and sectioned at 3 μ m, and some of the sections were stained with hematoxylin-eosin. The other paraffin sections were rehydrated, blocked with 3% BSA, and incubated overnight at 4°C with primary monoclonal mouse anti-GFP antibody (sc-9996, 1:100, Santa Cruz Biotechnology, Dallas, TX), polyclonal rabbit anti-chromogranin A antibody (ab15160, 1:100, Abcam, Cambridge, UK), and polyclonal rabbit anti-pancreatic and duodenal homeobox-1 (Pdx1) antibody (no. 253, 1:1000, kindly provided by Dr. Joel Habener, Harvard Medical School, Boston, MA). The sections were then incubated for 1 h at room temperature with secondary antibody. Images were taken using fluorescent microscopy with the FSX100 system (Olympus Corporation, Tokyo, Japan). More than 50 representative villi from each slide were randomly selected; their mean length was evaluated using images of hematoxylin-eosin staining and GFP-positive cells (K cells), and chromogranin A-positive cells were quantified using fluorescent microscopy images.

Isolation of K cells from murine intestinal epithelium. Upper small intestine samples were removed and washed with phosphate-buffered saline (PBS). The protocol of isolation and collection of GFP-positive cells from murine intestinal epithelium was previously described (13). The number of GFP-positive cells (per 100,000 epithelial cells) in the intestinal epithelium was analyzed using a BD FACSARIA Flow Cytometer (Becton Dickinson, San Jose, CA). Sorted K cells were collected in vials containing extraction medium at a rate of 2,000 cells per tube. Total RNA was extracted using the Picopure RNA Isolation Kit (Applied Biosystems, Foster City, CA) and treated with deoxyribonuclease (Qiagen Inc., Valencia, CA).

Quantitative RT-PCR. Total RNA of the islets, visceral fat, and intestine was extracted using RNeasy Mini Kit (Qiagen). Complementary DNA was prepared by reverse transcriptase with an oligo deoxythymidine primer (Invitrogen, Carlsbad, CA). The mRNA expression levels were measured by quantitative RT-PCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). PCR analyses were carried out using oligonucleotide primers. SYBR Green PCR Master Mix (Applied Biosystems) was prepared for the PCR run. Ribosomal protein S18 and GAPDH were used as the internal control. Data on K cells are shown using the comparative cycle threshold (Ct) methods [Ct (internal control) – Ct (target gene)]. Data on mRNA expressions in the islets, visceral fat, and intestine were standardized against GAPDH. The primers of target molecules were designed (Table 1).

Intestine-specific gene transfer. We performed intestine-specific gene transfer (iGT) in accordance with the previous report (11). Male $GIP^{GFP/+}$ mice at the age of 8–10 wk were anaesthetized with isoflurane and subjected to abdominal laparotomy. Part of the small intestine was drawn out from the peritoneal cavity and tied with nylon string to close its 6- to 9-cm region from the pyloric ring. The intestinal lumen of the closed region was washed with PBS containing 10 mM dithiothreitol and PBS; transfection solution was then injected into the intestinal lumen. We used a haemagglutinating virus of Japan envelope-based transfection reagent (GenomONE-Neo, Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) according to the manufacturer's instructions, and short interfering RNA (siRNA) or Negative Control Medium GC Duplex (Invitrogen) (control) was delivered to the intestinal epithelium. One hour after the haemagglutinating virus of Japan envelope vector was injected, the nylon string was removed. The tissue was returned to the peritoneal cavity, and the wound was sutured. Forty-eight hours after iGT, an OGTT was performed. Blood samples were collected at 0 and 15 min after oral glucose (2 g/kg body wt, administration by oral gavage). Subsequently, the small intestine (6- to 9-cm region from the pyloric ring where transfection solution

Table 1. Primers for RT-PCR

Target Gene	Forward	Reverse
RPS18	5'-TTCTGGCCAACGGTCTAGACAAC-3'	5'-CCAGTGGTCTTGGTGTGCTGA-3'
GAPDH	5'-AAATGGTGAAGTCGGTGTG-3'	5'-TCGTTGATGGCAACAATCTC-3'
GIP	5'-GTGGCTTTGAAGACCTGCTC-3'	5'-TTGTTGTGGATCTTGTCCA-3'
Pdx1	5'-GAAATCCACCAAGCTCAGC-3'	5'-TTCAACATCACTGCCAGCTC-3'
Rfx6	5'-ACAGACACGGAATCTGACAT-3'	5'-CTCTACCACAGTGTCCAACC-3'
Isl1	5'-ACGTGCTTTGTTAGGGATGG-3'	5'-CACGAAGTCGTTCTTGTGA-3'
GIPR	5'-TGCAGGCTTTGCTTCCGCCAGTG-3'	5'-GCGTCTGGTCTGAAAGCCCCATT-3'
Copa	5'-CCTGCCAGTACCACGGAAAA-3'	5'-TGCTAGCGGAAGCCTAGAGA-3'

GIP, glucose-dependent insulintropic polypeptide/gastric inhibitory polypeptide; GIPR, GIP receptor; Isl1, Islet 1; Pdx1, pancreatic and duodenal homeobox-1; Rfx6, regulatory factor X 6; RPS18, ribosomal protein S18.

was injected) was taken from the mice for RNA extraction, measurement of GIP content, and immunostaining. Samples (3 cm in length) for measurement of GIP content were extracted with 1 ml of 0.2 N perchloric acid, and GIP levels in the supernatant were measured using a GIP ELISA kit (SigmaMillipore).

The siRNA used in this study was as follows: siRNA against Pdx1, 5'-CAGUACUACGCGGCCACACAGCUCU-3'.

Statistical analysis. The results are given as means $\pm$ SE. Statistical significance was determined using Student's *t*-test. *P* < 0.05 was considered significant.

RESULTS

Aged mice have high body fat and insulin resistance. Body weight of GIP^{tg/+} mice increased with aging (Fig. 1A). Body weight of aged mice was significantly higher than that of young mice (Fig. 1B). CT scan analysis demonstrated that aged mice accumulated more body fat compared with young mice (Fig. 1C). The lean body mass of aged mice was less than that of young mice. Subcutaneous fat and visceral fat weight of aged mice were significantly higher than those of young mice (Fig. 1D). On the other hand, gastrocnemius muscle weight of aged mice was significantly lower than that of young mice. During ITT, blood glucose reduction was significantly lower in aged mice compared with young mice (Fig. 1E). Thus, aged mice exhibit the phenotype of fat accumulation and insulin resistance.

Aged mice show GIP hypersecretion. Under the greater body weight of aged mice than that of young mice (Fig. 2A), ad libitum-fed insulin and total GIP levels were significantly greater in aged mice compared with young mice, while blood glucose levels did not differ between the two groups (Fig. 2B). Xenin is known to be a gastrointestinal hormone secreted from K cells and potentiates the insulintropic action of GIP (22, 35). Ad libitum-fed xenin levels did not differ between the two groups (data not shown). During OGTT, blood glucose levels did not differ between aged mice and young mice (Fig. 2C). Insulin levels at 15, 30, 60, and 120 min and area under the curve of insulin levels were significantly higher in aged mice (Fig. 2D). These results indicate that aged mice excessively secrete insulin to preserve blood glucose after glucose ingestion. Total GIP levels at 0, 30, 60, and 120 min and area under the curve of total GIP levels were also significantly higher in aged mice (Fig. 2E), indicating that aged mice have the phenotype of GIP hypersecretion. Fasting DPP-4 activity was measured and did not differ between young and aged mice (Fig. 2F). There was no significant difference in the expression levels of GIPR mRNA in islets and visceral fat between young and aged mice (Fig. 2G).

K-cell number is increased in small intestine of aged mice.

To investigate the cause of GIP hypersecretion in aged mice, the number of K cells in the upper small intestine was estimated. The length of small intestine was shorter in aged mice compared with young mice (Fig. 3A). The length of intestinal villi of aged mice also was shorter than that of young mice (Fig. 3B). To evaluate the number of enteroendocrine cells other than K cells, we performed immunostaining using anti-chromogranin A antibody. Chromogranin A is localized in neuroendocrine cells, such as enterochromaffin-like cells. The number of chromogranin A-positive cells per one villus in aged mice was significantly less compared with young mice (Fig. 3C). In contrast, the number of GFP-positive cells (K cells) per one villus was significantly greater in aged mice than that in young mice by immunohistochemical analysis (Fig. 3D). In spite of the shorter length of small intestine and intestinal villi, the number of GFP-positive cells per one villus was greater, and thus the K-cell number was certainly increased in aged mice. Flow cytometry analysis also showed that the number of GFP-positive cells in the small intestine was significantly greater in aged mice than in young mice (Fig. 3E). The number of chromogranin A-positive cells was less in aged mice; therefore, it was found that not all enteroendocrine cells were increased with aging. These results suggest that the GIP hypersecretion in aged mice is due to the increase in K-cell number in the small intestine.

GIP and Pdx1 mRNA levels are increased in K cells of aged mice. RT-PCR showed that expression levels of GIP mRNA in isolated K cells were significantly greater in aged mice compared with those in young mice (Fig. 4A). We then evaluated the expression levels of transcriptional factors in K cells that could affect GIP production, such as Pdx1, regulatory factor X 6 (Rfx6), and Islet 1 (Isl1) (Fig. 4B). Pdx1 mRNA was highly expressed in aged mice compared with that in young mice. There was no significant difference in the expression levels of Rfx6 mRNA between the two groups. Although the expression levels of Isl1 mRNA tended to be higher in aged mice, the difference was not statistically significant. The mRNA expression levels of *Copa*, the gene responsible for synthesis of xenin, in K cells did not differ between the two groups (data not shown). Immunohistochemical analysis revealed that all nuclei of GFP-positive cells (K cells) as well as epithelial cells in the upper small intestine were stained using Pdx1 antibody (Fig. 4C). The upper small intestine of 4 mice in each group was observed, and 40 GFP-positive cells in each mouse were assessed. Immunoreactivity was higher in the aged mice than that of the young mice with the same exposure time (1/8 s).

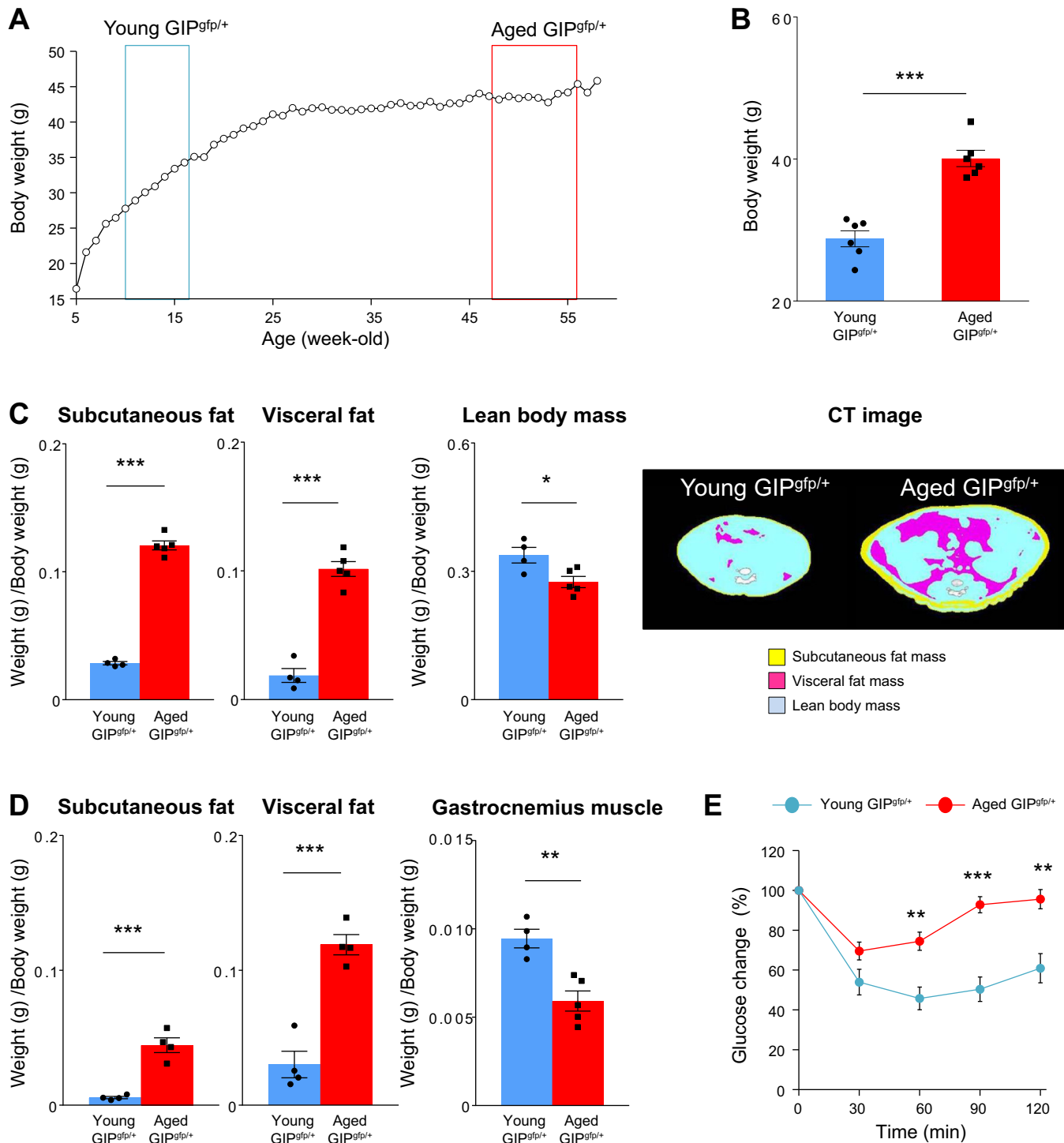


Fig. 1. Body fat composition in young and aged glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP)-green fluorescent protein (GFP) knock-in heterozygous ($GIP^{gfp/+}$) mice. **A**: body change of male $GIP^{gfp/+}$ mice ($n = 6$). **B**: body weight of young (14 wk old) and aged (56 wk old) mice ($n = 6$). **C**: subcutaneous and visceral fat weight and lean body mass estimated by computed tomography (CT) scan analysis in young (15 wk old) and aged (54 wk old) mice ($n = 4-5$). Pink, yellow, and blue areas represent visceral fat, subcutaneous fat, and lean body mass, respectively. **D**: subcutaneous and visceral fat and gastrocnemius muscle weight (per body wt) of young (16 wk old) and aged (53 wk old) mice ($n = 4-5$). **E**: blood glucose change (%) during insulin tolerance test (ITT) (insulin 0.75 U/kg body wt) in young (12 wk old) and aged (54 wk old) mice ($n = 6$). The glucose levels during ITT represent the % change from fasting glucose levels. Young mice are represented by blue circles and bars. Aged mice are represented by red circles and bars. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. young mice.

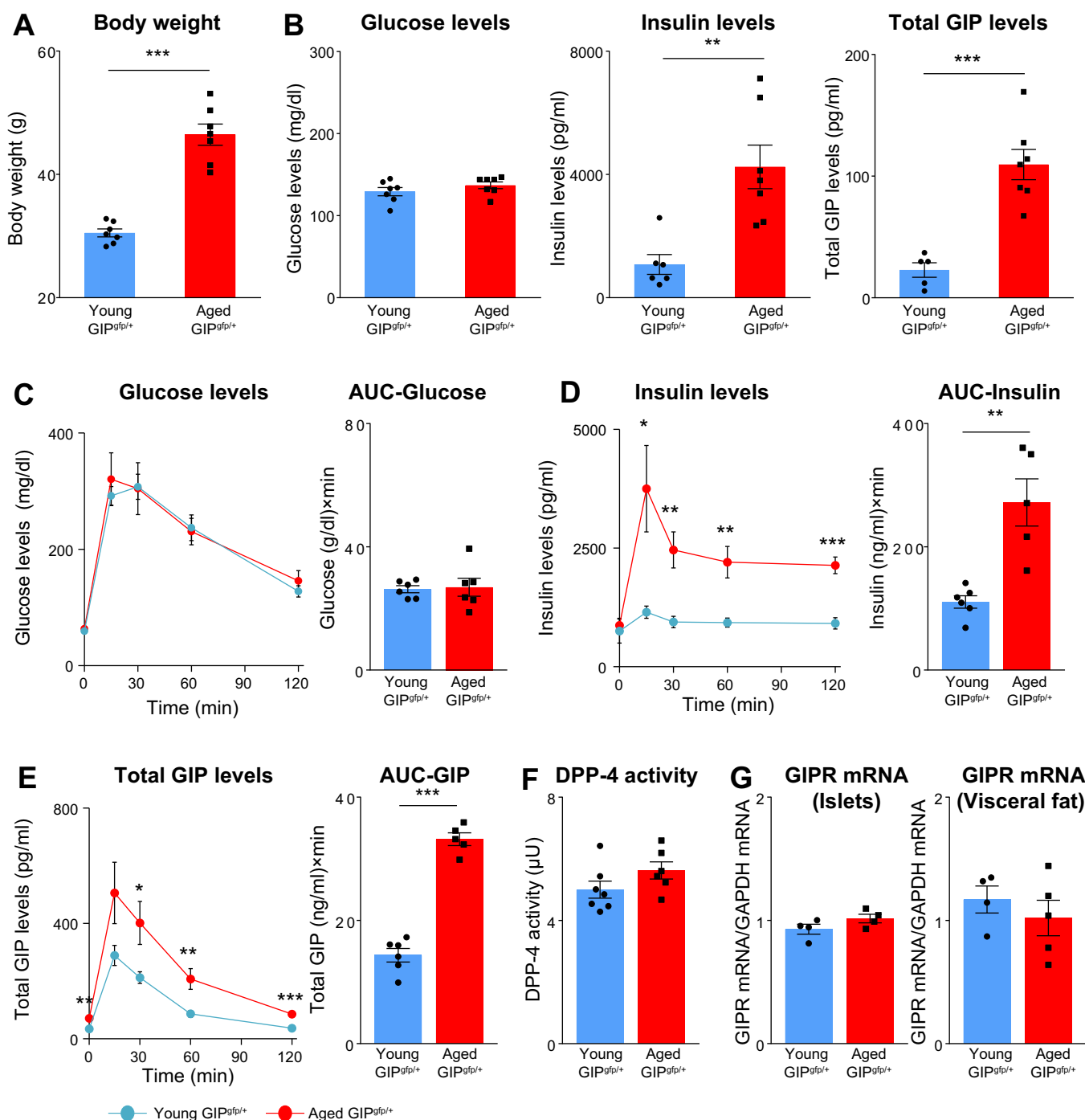
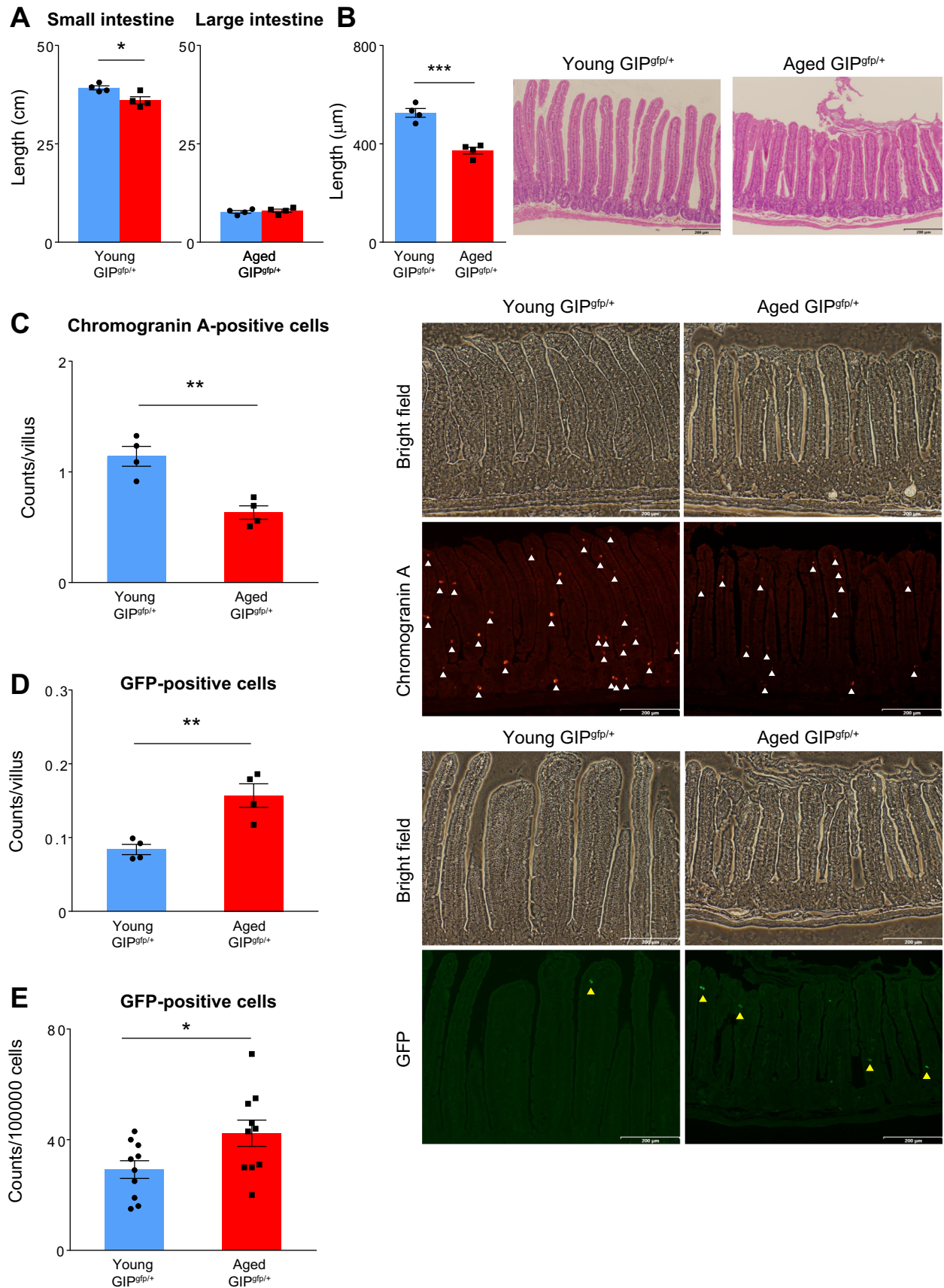


Fig. 2. Blood collection under free feeding, oral glucose tolerance test (OGTT), and insulin tolerance test (ITT) in young and aged glucose-dependent insulintropic polypeptide/gastric inhibitory polypeptide (GIP)-green fluorescent protein (GFP) knock-in heterozygous ($GIP^{gfp/+}$) mice. Body weight (A) and ad libitum-fed blood glucose, insulin, and total GIP levels (B) in young (10 wk old) and aged (46 wk old) mice ($n = 5-7$). Blood glucose (C), insulin (D), and total GIP (E) levels during OGTT (glucose 2 g/kg body wt) in young (14 wk old) and aged (56 wk old) mice ($n = 5-6$). Fasting dipeptidyl peptidase IV (DPP-4) activity in young (13 wk old) and aged (48 wk old) mice ($n = 6-7$) (F). The expression levels of GIP receptor (GIPR) mRNA in islets and visceral fat of young (16 wk old) and aged (55 wk old) mice ($n = 4-5$) (G). Young mice are represented by blue circles and bars. Aged mice are represented by red circles and bars. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. young mice. AUC, area under the curve.

These results suggest that GIP and Pdx1 expression levels are increased in the K cells of aged mice.

K-cell number is decreased by a posteriori suppression of Pdx1 expression in small intestine. We attempted to suppress Pdx1 expression in the small intestine by using the method of iGT. Introduction of Pdx1 siRNA efficiently suppressed the expression

levels of Pdx1 mRNA (Fig. 5A). Nuclei of enterocytes of the Pdx1-knockdown intestine showed lower immunoreactivity against Pdx1 antibody with the same exposure time (1/3 s) (Fig. 5B). The expression levels of GIP mRNA were significantly decreased in the Pdx1-knockdown intestine (Fig. 5C). There was no significant difference in the expression levels of Isl1 and Rfx6



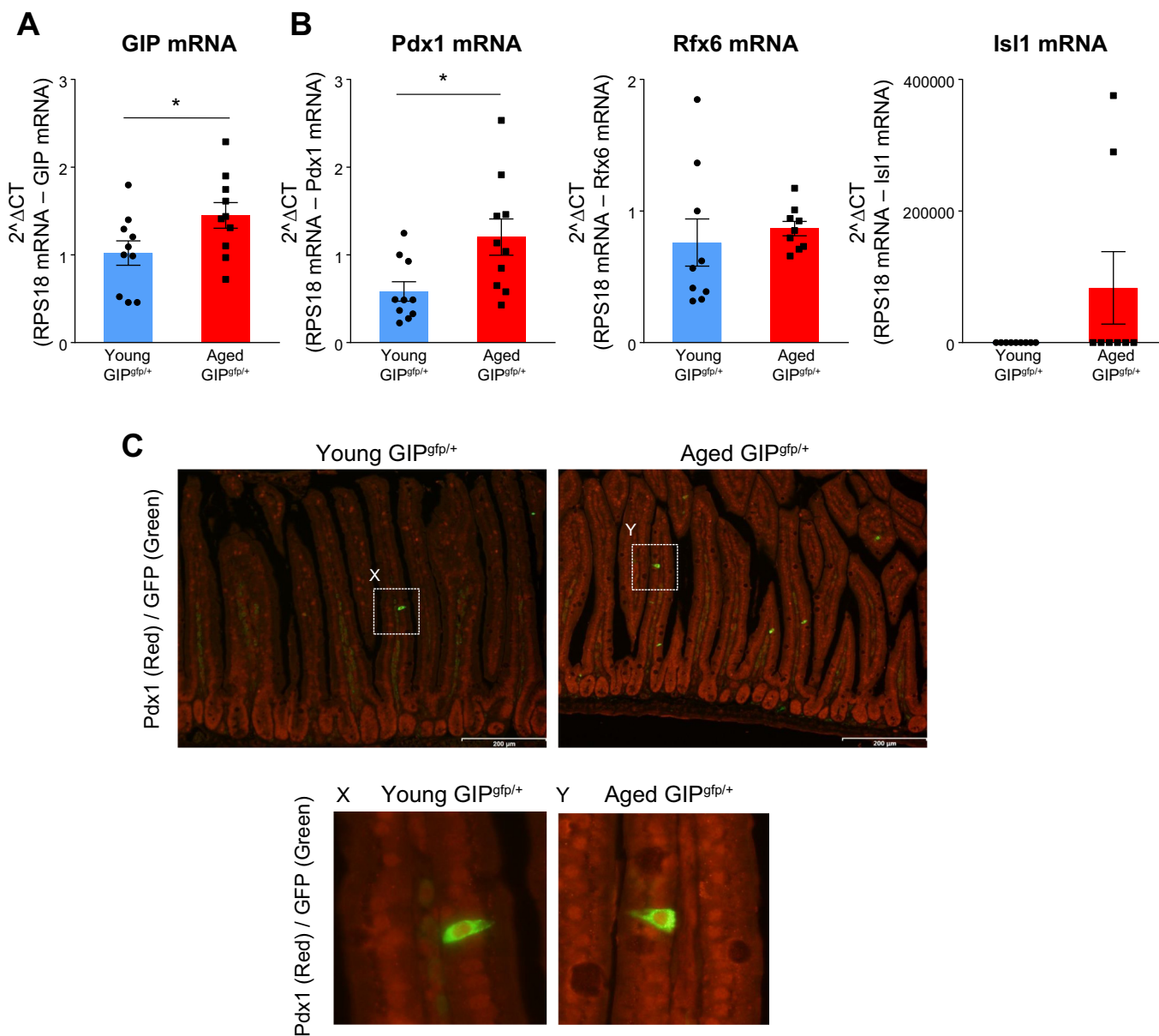
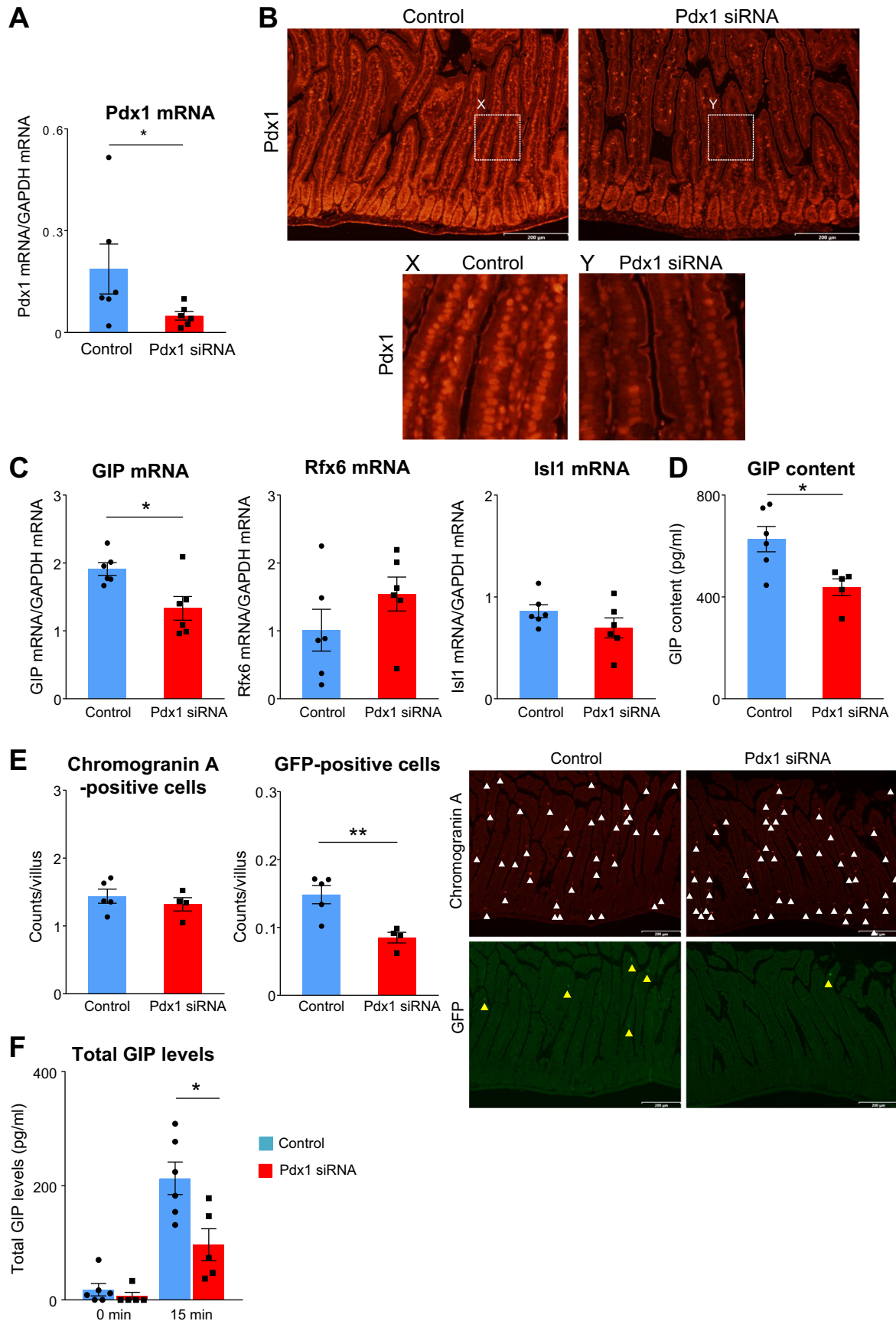


Fig. 4. Expression of glucose-dependent insulintropic polypeptide/gastric inhibitory polypeptide (GIP) mRNA and transcriptional factors in K cells of young and aged GIP-green fluorescent protein (GFP) knock-in heterozygous ($GIP^{gfp/+}$) mice. The mRNA expression levels of GIP (A) and pancreatic and duodenal homeobox-1 (Pdx1), regulatory factor X 6 (Rfx6), and Islet 1 (Isl1) (B) in K cells of young (15 wk old) and aged (54 wk old) mice ($n = 8-10$). Young mice are represented by blue bars. Aged mice are represented by red bars. $*P < 0.05$ vs. young mice. Immunohistochemical images of the upper small intestine of the young (16 wk old) and aged (55 wk old) mice (C). GFP is stained in green, and Pdx1 is stained in red. RPS18, ribosomal protein S18.

mRNA between the Pdx1-knockdown intestine and control. GIP content was significantly decreased in the Pdx1-knockdown intestine (Fig. 5D). The number of GFP-positive cells was decreased in the Pdx1-knockdown intestine while the number of chromogranin A-positive cells was not (Fig. 5E). Total GIP levels

at 15 min during OGTT were significantly decreased in the intestine of Pdx1-knockdown mice (Fig. 5F). These results suggest that a posteriori suppression of Pdx1 expression leads to the decreases in K-cell numbers, GIP mRNA expression and content in the small intestine, and GIP secretion.

Fig. 3. Analysis of K-cell numbers in the small intestine of young and aged glucose-dependent insulintropic polypeptide/gastric inhibitory polypeptide (GIP)-green fluorescent protein (GFP) knock-in heterozygous ($GIP^{gfp/+}$) mice. Length of the small and large intestine (A) and intestinal villi (B) of young (16 wk old) and aged (55 wk old) mice ($n = 4$). Number of chromogranin A-positive cells (C) and GFP-positive cells (D) per one villus by immunohistochemical analysis in young (16 wk old) and aged (55 wk old) mice ($n = 4$). White and yellow arrows indicate chromogranin A-positive and GFP-positive cells (K cells), respectively. Number of GFP-positive cells (per 100,000 cells) by flow cytometry analysis in young (15 wk old) and aged (54 wk old) mice ($n = 10$) (E). Young mice are represented by blue bars. Aged mice are represented by red bars. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. young mice.



DISCUSSION

In the present study, we demonstrate that aged mice exhibit the phenotype of fat accumulation, insulin resistance, and GIP hypersecretion. Furthermore, it was considered that GIP hypersecretion in aged mice was due to increases in K-cell number and GIP mRNA expression in K cells.

There are few reports regarding age-related changes of K cells; one of the reasons is the difficulty in isolating K cells. K cells are expressed in the intestinal mucosal epithelial cells at a very low rate, and, in addition, there is no ideal cell line. It has been reported that the number of GIP-positive cells in human duodenum are not different with age (31). Another report indicated that the number of GIP-immunoreactive cells in murine duodenum was reduced in 12-mo-old mice compared with 3-mo-old mice (32). These previous findings were reported based on immunohistochemical analyses. Our present study is the first report, to our knowledge, analyzing age-related changes of K cells and GIP secretion by using GIP reporter (GIP-GFP) mice. We have succeeded in isolating K cells by using GIP-GFP mice and have elucidated age-related changes of K-cell number and mRNA expression levels of GIP and transcriptional factors in K cells.

Various transcriptional factors are involved in GIP mRNA expression. Among them, Pdx1 and Isl1 are reported to affect the number of GIP-expressing cells in the intestine (8, 9, 16, 36), and thus we focused on these transcriptional factors in particular. Pdx1 is expressed in K cells and involved in regulating GIP expression. It has been reported that Pdx1 binds 150 bp upstream of the *Gip* promoter, activates the *Gip* promoter in murine intestinal cell line STC-1, and is essential for producing GIP in K cells (8, 16). In a previous study, Pdx1-deficient mice showed a greatly decreased number of GIP-expressing cells in the intestine (16). Another study reported that augmentation of GIP secretion in HFD conditions was due to increases in K-cell number (9). In that report, agglomerates of Pdx1 and GIP double-expressing cells were found inside the duodenal mucosa of obese rats after an HFD. It has been reported that mRNA abundance for GIP is significantly reduced in mice with intestine-specific Pdx1 inactivation (3). In that study, the number of GIP-expressing cells tended to be smaller than that of control mice, although the difference was not statistically significant. In the present study, we found that Pdx1 expression was increased in K cells as well as epithelial cells of aged mice and showed that K-cell number, GIP mRNA expression and content, and GIP secretion were decreased by posteriori suppression of Pdx1 in the small intestine by using iGT. Thus, Pdx1 positively regulates GIP mRNA and K-cell number in small intestine, and increased Pdx1 expression might be involved in GIP hypersecretion in aged mice. It has been reported that forced expression of Pdx1 in STC-1 cells induced a shift of L-type to K/L-type cells (8). Therefore, increased Pdx1 expression could be related to the differentiation to K

cells, which might be one of the factors contributing to the increase in K-cell number with aging.

We previously investigated GIP hypersecretion in HFD-induced obesity by using GIP^{tg/+} mice (34). The difference in body weight was ~10 g between HFD-fed mice and control-fat diet-fed mice, as is the case between aged mice and young mice in the present study. However, HFD-fed mice had some different characteristics compared with aged mice. The fasting GIP levels did not differ between HFD-fed mice and control-fat diet-fed mice, while the fasting GIP levels in aged mice were significantly higher compared with those in young mice in our present study. In addition, K-cell number did not change in the HFD condition, although Pdx1 mRNA expression levels were increased. In the HFD condition, Rfx6 mRNA expression levels were also increased, and we showed that Rfx6 specifically expressed in K cells played a critical role in differentiation and GIP production of K cells in collaboration with Pdx1. In the present study, Rfx6 mRNA expression levels did not differ between aged mice and young mice. The regulation of GIP gene expression is under complex control (8, 14, 15, 21). Results of our study suggest the possibility that some other transcriptional factors regulate K-cell number and GIP mRNA expression in collaboration with Pdx1 and are involved in age-related GIP hypersecretion.

Consistent with the previous study (38), aged mice in the present study showed increased insulin resistance compared with young mice. To compensate for insulin resistance with aging and maintain glucose homeostasis, insulin secretion might be potentiated via GIP hypersecretion. GIP hypersecretion leads to fat accumulation as an adverse effect, and then insulin resistance is further aggravated. Our hypothesis is that insulin resistance with aging is the driving factor that increases Pdx1 expression, which might result in age-related GIP hypersecretion and fat accumulation. The previous study showed that the administration of GIPR agonist improved insulin release in aged mice (12), suggesting that the response of GIPR in β -cells is not impaired with aging. In addition, there was no significant difference in the expression levels of GIPR mRNA in islets and visceral fat between young and aged mice in the present study. Thus, GIP hypersecretion with aging is not due to the compensation for reduced reactivity of GIPR *in vivo*.

The regulation of Pdx1 expression and accompanying GIP secretion might enable us to prevent fat accumulation with aging and thus could lead to clinical applications as an anti-obesity or an anti-insulin resistance drug (19). In addition to aging itself, fat accumulation causes a decline in ADL levels in the elderly. If this problem is solved, various benefits, such as improvement of ADL and quality of life for the elderly and reduction of burdens for their caregivers, could be obtained. Furthermore, prevention of fat accumulation with aging might also contribute to the risk reduction of type 2 diabetes via

Fig. 5. Analysis of the small intestine, K cells, and glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP) secretion after intestine-specific gene transfer (iGT) of pancreatic and duodenal homeobox-1 (Pdx1) siRNAs. A and B: mRNA expression levels of Pdx1 ($n = 6$) and immunohistochemical images of the intestine against Pdx1 antibody, respectively. Pdx1 is stained in red. C: mRNA expression levels of GIP, regulatory factor X 6 (Rfx6), and Islet 1 (Isl1) ($n = 6$). D: GIP content in the small intestine ($n = 5-6$). E: number of chromogranin A-positive cells and green fluorescent protein (GFP)-positive cells per one villus ($n = 4-5$). F: total GIP levels during oral glucose tolerance test (OGTT) (glucose 2 g/kg body wt) ($n = 5-6$). Control mice are represented by blue bars. Pdx1-knockdown mice are represented by red bars. * $P < 0.05$, ** $P < 0.01$ vs. control mice.

amelioration of insulin resistance, which could lead to the extension of healthy life expectancy.

In conclusion, transcriptional factor Pdx1 positively regulates GIP mRNA and K-cell number in the small intestine; increased Pdx1 expression might be involved in GIP hypersecretion with aging.

ACKNOWLEDGMENTS

We thank Prof. Timothy J. Kieffer, Adam Ramzy, and Ali Asadi (Dept. of Cellular and Physiological Sciences, Life Sciences Inst., Univ. of British Columbia) and Dr. Joel Habener (Harvard Medical School) for providing the Pdx1 antibody and immunostaining conditions and helpful suggestions regarding the study. We also thank Shoichi Asano (Dept. of Diabetes, Endocrinology, and Nutrition, Graduate School of Medicine, Kyoto Univ.) for technical support.

GRANTS

This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology; Japan Society for the Promotion of Science; Ministry of Health, Labour, and Welfare; Ministry of Agriculture, Forestry, and Fisheries; Japan Diabetes Foundation; Japan Association for Diabetes Education and Care; Merck Sharp & Dohme Life Science Foundation; Public Interest Incorporated Foundation; and Suzuken Memorial Foundation.

DISCLOSURES

N. Inagaki receives fees from Takeda Pharmaceutical Company, Mitsubishi Tanabe Pharma Corporation, Merck Sharp & Dohme Life Science Foundation, Sanofi, Novartis Pharma, Daiichi Sankyo, Kyowa Hakko Kirin Co., Ltd., Eli Lilly Japan, Shiraori Pharmaceutical, Roche Diagnostics, Japan Tobacco, Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma, Daiichi Sankyo, and Ono Pharmaceutical Co., Ltd. and Taisho Toyama Pharmaceutical Co., Ltd. outside the submitted work. N. Harada receives fees from Mitsubishi Tanabe Pharma Corporation, Ono Pharmaceutical Co., Ltd., and Novo Nordisk Pharma. E. Ikeguchi, Y. Kanemaru, A. Sankoda, S. Yamane, K. Iwasaki, M. Imajo, Y. Murata, K. Suzuki, and E. Joo have no conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

E.I. and N.H. conceived and designed research; E.I., Y.K., A.S., K.I., Y.M., K.S., and E.J. performed experiments; E.I. analyzed data; E.I., N.H., S.Y., and N.I. interpreted results of experiments; E.I. prepared figures; E.I. drafted manuscript; E.I., N.H., and N.I. edited and revised manuscript; E.I., N.H., Y.K., A.S., S.Y., K.I., M.I., Y.M., K.S., E.J., and N.I. approved final version of manuscript.

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