

Ectopic Expression of GIP in Pancreatic β -Cells Maintains Enhanced Insulin Secretion in Mice With Complete Absence of Proglucagon-Derived Peptides

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Glucagon and glucagon-like peptide-1 (GLP-1) are produced in pancreatic α -cells and enteroendocrine L-cells, respectively, in a tissue-specific manner from the same precursor, proglucagon, that is encoded by glucagon gene (*Gcg*), and play critical roles in glucose homeostasis. Here, we studied glucose homeostasis and β -cell function of *Gcg*-deficient mice that are homozygous for a *Gcg*-GFP knock-in allele (*Gcg^{afp/afp}*). The *Gcg^{afp/afp}* mice displayed improved glucose tolerance and enhanced insulin secretion, as assessed by both oral glucose tolerance test (OGTT) and intraperitoneal glucose tolerance test (IPGTT). Responses of glucose-dependent insulinotropic polypeptide (GIP) to both oral and intraperitoneal glucose loads were unexpectedly enhanced in *Gcg^{afp/afp}* mice, and immunohistochemistry localized GIP to pancreatic β -cells of *Gcg^{afp/afp}* mice. Furthermore, secretion of GIP in response to glucose was detected in isolated islets of *Gcg^{afp/afp}* mice. Blockade of GIP action in vitro and in vivo by cAMP antagonist and genetic deletion of the GIP receptor, respectively, almost completely abrogated enhanced insulin secretion in *Gcg^{afp/afp}* mice. These results indicate that ectopic GIP expression in β -cells maintains insulin secretion in the absence of proglucagon-derived peptides (PGDPs), revealing a novel compensatory mechanism for sustaining incretin hormone action in islets. *Diabetes* 62:510–518, 2013

The glucagon gene encodes proglucagon, a precursor of multiple peptides including glucagon, GLP-1, oxyntomodulin, and GLP-2 (1,2). Glucagon is produced in pancreatic α -cells, whereas GLPs are found in intestinal L-cells (1,2). Glucagon has been recognized as a major counteracting hormone to insulin in regulating glucose homeostasis (3,4). The main action of glucagon is to stimulate hepatic glucose production by promoting gluconeogenesis and glycogenolysis

while inhibiting glycogen synthesis and glycolysis in response to hypoglycemia (4,5). Dysregulation of glucagon secretion contributes to the pathophysiology of diabetes mellitus through increased hepatic glucose production (6,7). Furthermore, experimental suppression of hyperglucagonemia corrects postprandial hyperglycemia in individuals with type 2 diabetes (7). Therefore, inhibition of glucagon action represents one potential approach to the treatment of type 2 diabetes (4,8).

The importance of glucagon in regulating glucose homeostasis has been demonstrated by using genetically modified mouse models and by pharmacological interventions that suppress glucagon signaling (9–15). In such models, suppression of glucagon signaling increases circulating levels not only of glucagon but also of GLP-1. The increased GLP-1 levels, in turn, contribute to improved function of pancreatic β -cells. Mice with targeted deletion of the glucagon receptor gene (*Gcgr^{-/-}*) exhibit increased plasma GLP-1 levels, enhanced insulin secretion in vivo, and resistance to streptozotocin-induced destruction of pancreatic β -cells (14,16). It also has been demonstrated that treatment with *Gcgr* antisense oligonucleotides improves glucose tolerance and increases circulating levels of active GLP-1 in rodent diabetic models (13). In addition, treatment with *Gcgr* antisense oligonucleotides increases both GLP-1 and the insulin content of islets in *db/db* mice (13).

GLP-1 and GIP, which is produced in intestinal K-cells, both have been recognized as incretins (1,17). Both GLP-1 and GIP stimulate insulin secretion and are secreted by intestinal endocrine cells in response to ingestion of nutrients, including carbohydrates, lipids, and proteins. In addition to insulinotropic effects, both GLP-1 and GIP promote β -cell proliferation and inhibit apoptosis (1,18). However, these peptides exert differential effects on glucagon secretion. GLP-1 inhibits the postprandial glucagon response, whereas GIP enhances it in a glucose-dependent manner (1,19,20).

To determine the consequences of loss of glucagon action in the absence of concomitant upregulation of GLP-1 production, we recently established a mouse model in which the entire proglucagon gene is disrupted by insertion of GFP. Both GFP and PGDPs are expressed in pancreatic α -cells and intestinal L-cells in heterozygous *Gcg^{afp/+}* mice. The homozygous (*Gcg^{afp/afp}*) mice lack all PGDPs and display hyperplasia of GFP-positive “ α ”-cells in pancreatic islets but not of “L”-cells in the intestine (2,21). In the current study, we characterized glucose tolerance and β -cell function in *Gcg^{afp/afp}* mice to elucidate the consequences of PGDP deficiency on islet function and glucose homeostasis.

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RESEARCH DESIGN AND METHODS

Animal studies. The establishment of the glucagon-GFP knock-in mouse has been described previously in detail (21). *Gcg^{afp/+}* and *Gipr^{+/-}* mice (22), which had been backcrossed to C57BL/6J background for at least eight generations, were provided by the RIKEN BRC through the National Bio-Resource Project of the MRXT (Japan). Double heterozygote *Gcg^{afp/+}* and *Gipr^{+/-}* mice were intercrossed to obtain *Gcg^{afp/+}Gipr^{+/-}*, *Gcg^{afp/afp}Gipr^{+/-}*, and *Gcg^{afp/+}Gipr^{-/-}* single knockout littermates and *Gcg^{afp/afp}Gipr^{-/-}* double knockout (DKO) mice. All mice were housed in a temperature-controlled room under a standard 12-h light/dark cycle. All procedures were performed according to a protocol approved by the Nagoya University Institutional Animal Care and Use Committee.

Glucose tolerance test and measurement of insulin and GIP. After 16 h of food deprivation in 12- to 26-week-old male mice, 2 g/kg body weight D-glucose was administered in OGTT or IPGTT. Blood was collected at the indicated times to measure glucose, insulin, and GIP levels. Blood glucose levels were measured with Antsense II (Horiba, Kyoto, Japan). Plasma levels of insulin and GIP were determined using a mouse insulin enzyme-linked immunosorbent assay kit (Morinaga, Tokyo, Japan) and a rat/mouse GIP (TOTAL) enzyme-linked immunosorbent assay kit (Millipore, Billerica, MA), respectively.

Islet isolation and measurement of insulin and GIP secretion and GIP content. Pancreatic islets of 5- to 7-month-old male mice were isolated using the collagenase digestion method (23), and isolated islets were cultured for 2 h in RPMI 1640 medium containing 10% (volume/volume) fetal bovine serum under humidified conditions of 95% air and 5% CO₂. The islets were incubated for 30 min with Krebs-Ringer buffer containing 2.8 mmol/L glucose. Thereafter, five size-matched islets were collected from each plate and then incubated in 100 μ L of buffer containing 2.8, 4.2, or 16.7 mmol/L glucose for 30 min. In some experiments, 500 μ mol/L Rp-cAMP was included in the incubation medium. Concentrations of insulin released into the medium and cellular insulin content were measured by radioimmunoassay (Eiken Chemical, Tokyo, Japan), and insulin secretion was normalized to cellular insulin content. To analyze GIP secretion, 10 islets were incubated in 100 μ L RPMI 1640 medium containing 16.7 mmol/L glucose for 16 h and GIP concentration in the medium was determined by enzyme-linked immunosorbent assay as described. For determination of insulin and GIP content, islets were lysed in acid ethanol, and cell extracts were collected. GIP content was normalized to insulin content.

Immunohistochemistry, morphometric analysis, and electron microscopy. The pancreata of *Gcg^{+/+}*, *Gcg^{afp/+}*, *Gcg^{afp/afp}*, *Gcg^{afp/afp}Gipr^{-/-}*, *Gcgr^{-/-}*, GLP-1 receptor knockout (*Glp1r^{-/-}*), and *Gcgr^{-/-}Glp1r^{-/-}* mice were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were incubated overnight at 4°C with primary antibodies against insulin (1:500; Abcam, Tokyo, Japan), glucagon (1:500; Abcam), and GIP (1:500; Peninsula, San Carlos, CA), followed by 90-min incubation in Alexa Fluor-conjugated secondary antibody (1:300; Alexa Fluor 488 or Alexa Fluor 594; Invitrogen, Grand Island, NY) at room temperature. Fluorescent images of Alexa Fluor and GFP were taken using an HS BZ-9000 fluorescent microscope system (Keyence, Osaka, Japan) or LSM710 confocal microscope (Zeiss, Birkerd, Denmark). For morphometric analyses, the pancreata were resected from 9-week-old *Gcg^{+/+}* and *Gcg^{afp/afp}* male mice. Serial sections of 4- μ m thickness were cut from each paraffin block at 200- μ m intervals, and 5 sections were randomly selected from each mouse and immunostained for insulin and glucagon. The number of islets analyzed was 192 in *Gcg^{+/+}* mice and 745 in *Gcg^{afp/afp}* mice. The total area of islets, glucagon-positive or GFP-positive cells (α -cells), and insulin-positive cells (β -cells) relative to the sectional area or total pancreas area was determined by using the HS BZ-II analysis application. For conventional transmission electron microscopy, animals were perfused intracardially with 2% paraformaldehyde/2.5% glutaraldehyde. Pancreata were postfixed with 1% OsO₄ and then processed using a standard method. Ultra-thin sections were examined by a JEOL-1210 electron microscope.

Isolation of tissue RNA and quantitative real-time RT-PCR. Total RNA was isolated from the intestine and isolated islets using TRIzol reagent and the RNeasy Plus Kit (Qiagen, Tokyo, Japan), respectively. Synthesis of complementary DNA and quantitative real-time RT-PCR were performed with the ABI 7300 Real Time PCR System using a Power SYBR Green RNA to C_T 1-Step kit (Applied Biosystems, Carlsbad, CA). The following oligonucleotide primers were used: mouse GIP, 5'-ATCCGACAACAAGACTTCGT-3' (sense) and 5'-ATCATCACTGAGGCTCTGG-3' (antisense); and mouse GIP receptor, 5'-GGATCTTGGAGAGACCACACTC-3' (sense) and 5'-TAAGATGAGTAGGGCTA GCAGCAG-3' (antisense). The sequences of the primers used for mouse insulin 1 and glyceraldehyde-3-phosphate dehydrogenase have been described previously (21). The mRNA levels were normalized with respect to those of insulin 1 or glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis. Data are presented as mean \pm SEM. Significance was evaluated using Student *t* test or ANOVA followed by posttest comparisons when applicable. *P* < 0.05 was regarded as statistically significant.

RESULTS

The *Gcg^{afp/afp}* mouse exhibits enhanced insulin secretion. We first evaluated glucose tolerance and insulin secretion in *Gcg^{afp/afp}* mice. As shown in Fig. 1A and 1C, *Gcg^{afp/afp}* mice displayed improved oral and intraperitoneal glucose tolerance. Insulin secretion during the OGTT, in which secretion of incretins is stimulated, was significantly enhanced to a much greater extent despite lack of GLP-1 in *Gcg^{afp/afp}* compared with control mice (Fig. 1B). Significantly increased insulin responses in *Gcg^{afp/afp}* mice also were observed during the IPGTT, suggesting that compensatory mechanisms have evolved to upregulate β -cell function independent of the requirement for enteral glucose exposure (Fig. 1D). Because these results suggested that *Gcg^{afp/afp}* mice may have development of enhanced β -cell function in an autonomous manner, we examined glucose-induced insulin secretion from isolated islets. Insulin secretion from islets of *Gcg^{afp/afp}* mice at 2.8 mmol/L glucose was significantly higher than in islets from control mice (Fig. 1E). Insulin secretion from *Gcg^{afp/afp}* islets was significantly greater across a range of glucose levels, from 2.8 to 16.7 mmol/L (Fig. 1E).

β -Cell mass is not increased in *Gcg^{afp/afp}* mice. To understand the mechanisms by which *Gcg^{afp/afp}* mice exhibit improved islet function, we analyzed the morphology of pancreatic islets. Although by definition α -cells classically contain glucagon, we refer to GFP-positive cells in *Gcg^{afp/afp}* mice as α -cells for simplicity throughout this article. As shown in Fig. 2A, increased islet number and hyperplasia of GFP-positive " α "-cells were conspicuous in *Gcg^{afp/afp}* mice. Morphometric analysis revealed increases in both islet area and islet number in *Gcg^{afp/afp}* mice (Fig. 2B, C). Whereas the α -cell area in the pancreata of *Gcg^{afp/afp}* mice was significantly increased (Fig. 2D), the insulin-positive (β -cell) area in the pancreata of *Gcg^{afp/afp}* mice was similar to that in *Gcg^{+/+}* mice (Fig. 2E). Accordingly, the ratio of α -cell area to β -cell area was increased in *Gcg^{afp/afp}* mice (Fig. 2F). In electron microscopic analysis, the distribution of dense-core vesicles in β -cells, which contain insulin, was unchanged between *Gcg^{afp/afp}* and control mice (Fig. 2G). Hence, the increased β -cell function observed in *Gcg^{afp/afp}* mice in vivo is not secondary to increased numbers of β -cells.

***Gcg^{afp/afp}* mice exhibit enhanced GIP secretion and increased GIP expression in pancreatic islets.** Because GIP secretion is increased in *Glp1r^{-/-}* mice (24), we analyzed plasma GIP levels during OGTT and IPGTT in *Gcg^{afp/afp}* mice. As shown in Fig. 3A and 3B, plasma GIP levels at baseline in *Gcg^{afp/afp}* mice were modestly, yet significantly, higher than those in control mice. In the OGTT, plasma GIP levels at 15 min were increased both in the *Gcg^{afp/afp}* and in the control mice; however, GIP levels in the *Gcg^{afp/afp}* mice were significantly higher (Fig. 3A). Although circulating glucose levels do not classically influence intestinal GIP secretion, plasma GIP levels at 15 min were significantly increased in *Gcg^{afp/afp}* mice but not in control mice during the IPGTT (Fig. 3B). We then investigated whether GIP expression in the *Gcg^{afp/afp}* intestine is increased; however, levels of GIP mRNA expression in the intestine were comparable between *Gcg^{afp/afp}* and control mice (Fig. 3C).

Recently, it has been reported that GIP is expressed in and secreted from pancreatic α -cells (25); therefore, we examined GIP expression in isolated islets. The expression levels of both GIP and GIPR mRNA in *Gcg^{afp/afp}* mice were

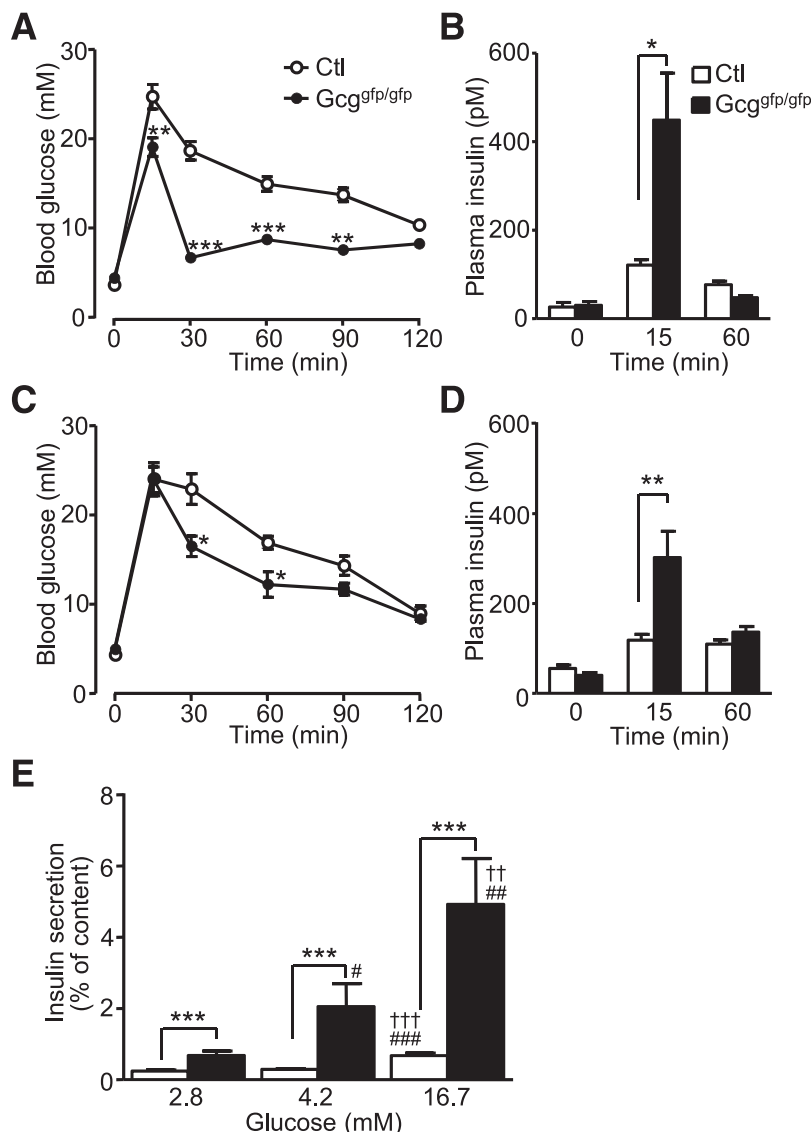


FIG. 1. Disruption of PGDPs improves glucose tolerance and enhances insulin secretion. Blood glucose levels (A) and plasma insulin levels (B) in the OGTT in 12- to 15-week-old control (Ctl, *Gcg^{+/+}* and *Gcg^{gfp/gfp}*) and *Gcg^{gfp/gfp}* mice ($n = 5-6$ mice per group). Blood glucose levels (C) and plasma insulin levels (D) in the IPGTT in 11- to 22-week-old mice ($n = 9-10$ mice per group). E: Glucose-induced insulin secretion from isolated islets. Pancreatic islets were isolated from 5- to 7-month-old control (*Gcg^{+/+}* and *Gcg^{gfp/gfp}*, $n = 13-18$ in each group) and *Gcg^{gfp/gfp}* ($n = 8-9$ in each group) mice and incubated with the indicated concentration of glucose for 30 min. Insulin secretion is expressed as the ratio of insulin released into the medium relative to insulin content. Values are expressed as means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. Ctl. †† $P < 0.01$. ††† $P < 0.001$ vs. 4.2 mmol/L glucose. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs. 2.8 mmol/L glucose.

markedly higher than those in control mice (Fig. 3D). GIP content in islets of *Gcg^{gfp/gfp}* mice also was significantly higher than in control islets (Fig. 3E). The GIP secreted from *Gcg^{gfp/gfp}* islets was detected after incubation in the presence of 16.7 mmol/L glucose for 16 h, whereas GIP was not detected in medium from *Gcg^{gfp/gfp}* islets (Fig. 3F). The GIP secretion from *Gcg^{gfp/gfp}* islets also was undetectable when islets were incubated in 2.8 mmol/L glucose (data not shown). To elucidate the contribution of islet-derived GIP to augmented insulin secretion in *Gcg^{gfp/gfp}* mice, we analyzed the effect of blocking GIP signaling in isolated islets. Because GIP signaling is mostly mediated through intracellular cAMP signaling, we used Rp-cAMP, a cAMP antagonist that blocks activation of protein kinase A and Epac (26). Treatment with 500 μ mol/L Rp-cAMP significantly attenuated glucose-induced insulin secretion from isolated islets of *Gcg^{gfp/gfp}* mice, whereas the insulin

response to glucose in the control islets was not suppressed by Rp-cAMP (Fig. 3G). These results indicate that cAMP signaling plays a pivotal role in enhanced insulin secretion in *Gcg^{gfp/gfp}* islets and is consistent with a role for islet-derived GIP in augment islet function.

GIP is expressed in pancreatic β -cells in *Gcg^{gfp/gfp}* mice. We next assessed the cellular localization of GIP in the islets. The GIP immunoreactivity was distributed in the islet periphery and colocalized with GFP expressed in α -cells in the islets of *Gcg^{gfp/gfp}* mice as previously reported (25) (Fig. 4A). In contrast, GIP was predominantly present in insulin-positive cells in islets of *Gcg^{gfp/gfp}* mice (Fig. 4A); the localization of GIP immunoreactivity in β -cells was independently confirmed using another antibody for GIP (Supplementary Fig. 1). Interestingly, GIP expression in β -cells was observed at embryonic day 18.5 in both control and *Gcg^{gfp/gfp}* mice (Supplementary Fig. 2). To address the

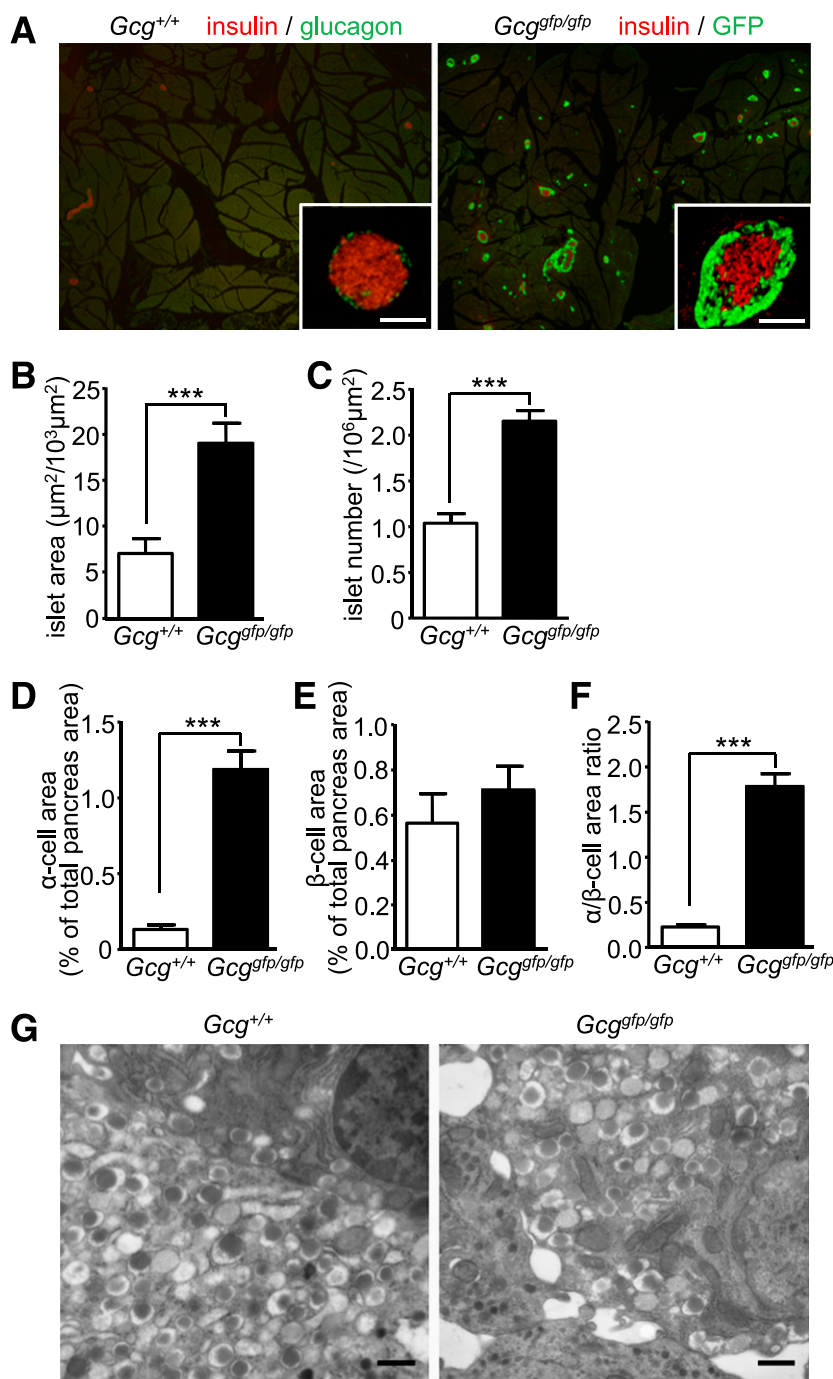


FIG. 2. *Gcg*^{gfp/gfp} mice exhibit α -cell hyperplasia. **A:** Representative pancreatic sections from 9-week-old *Gcg*^{+/+} (left) and *Gcg*^{gfp/gfp} (right) mice. In the left panel, sections were immunostained for glucagon (green) and insulin (red). In the right panel, sections were immunostained for insulin (red) and shown with autofluorescence of GFP. Scale bar, 100 μ m. **B–F:** Morphometric analysis of islets. **B:** Islet area shown relative to a 10^3 - μ m² sectional area. **C:** Islet number shown relative to a 10^6 - μ m² sectional area. **D:** The α -cell area shown as glucagon-positive or GFP-positive area relative to total pancreas area. **E:** The β -cell area shown as insulin-positive area relative to total pancreas area. **F:** The α -cell/ β -cell area ratio shown as a ratio of glucagon-positive or GFP-positive area relative to insulin-positive area. **G:** Electron microscopy of islets. Scale bar, 500 nm. Values are expressed as means \pm SEM. ****P* < 0.001. (A high-quality digital representation of this figure is available in the online issue.)

possible contribution of loss of glucagon and/or GLP-1 signaling in the altered intraislet expression pattern of GIP, we analyzed GIP expression in islets from mice deficient in GCGR (*Gcgr*^{-/-}), GLP1R (*Glp1r*^{-/-}), or both (*Gcgr*^{-/-}*Glp1r*^{-/-}) (27). As shown in Fig. 4B, GIP immunoreactivity was localized in α -cells in these animals, suggesting that induction of β -cell GIP expression in islets of *Gcg*^{gfp/gfp} mice does not arise secondary to loss of GCGR or GLP1R

signaling. We also performed immunohistochemical localization of two transcription factors, namely Pdx1 and Pax6, which have been shown previously to regulate GIP expression in intestinal K-cells (28). However, no differences in expression patterns of these transcription factors in β -cells of *Gcg*^{gfp/gfp} or *Gcg*^{gfp/+} mice (Supplementary Fig. 3) were observed, suggesting that transcription factors other than Pdx1 and Pax6 regulate GIP expression in β -cells.

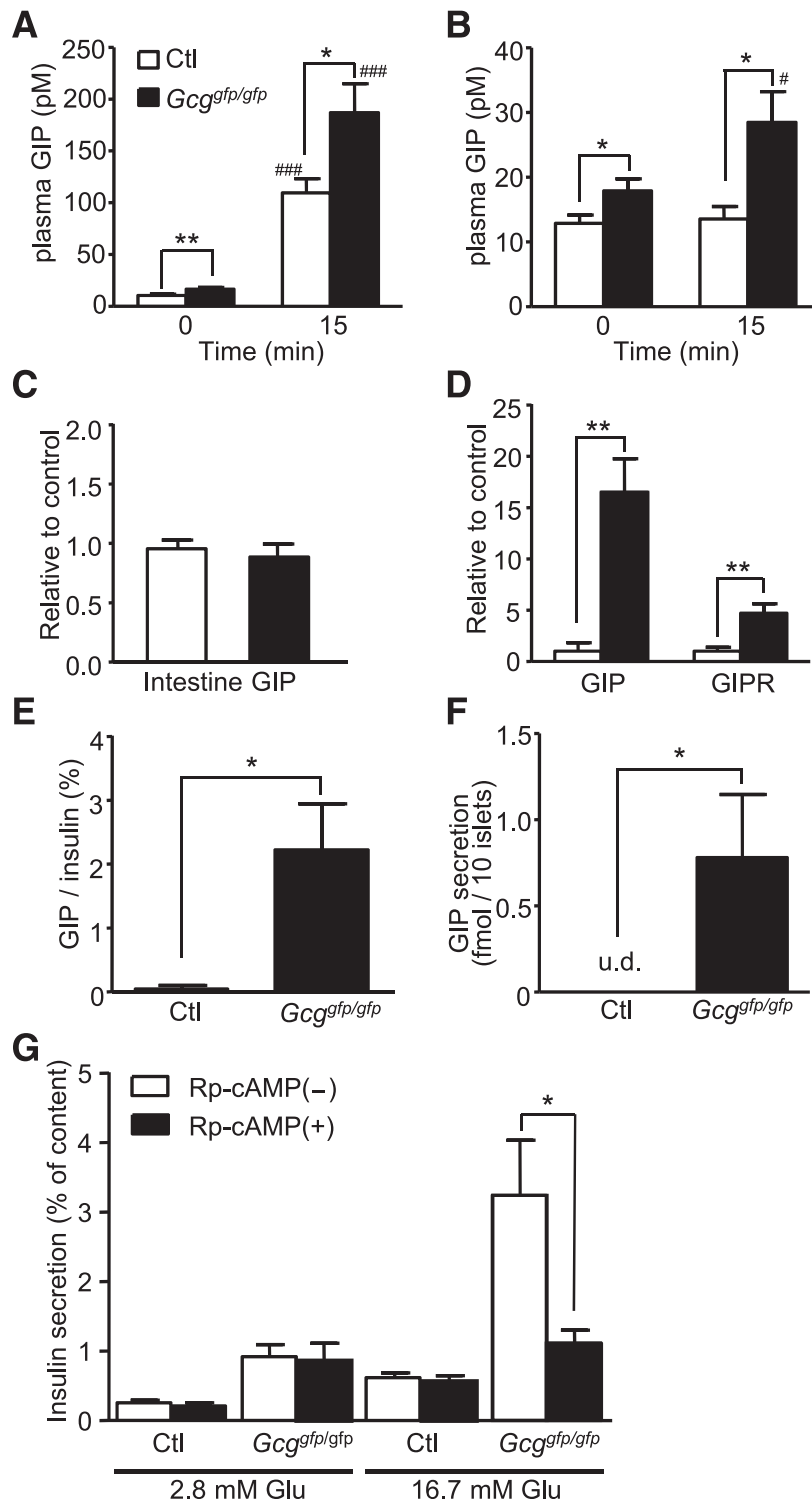


FIG. 3. *Gcg^{ΔP/ΔP}* mice display enhanced GIP secretion and GIP expression in pancreatic islets, and enhancement of glucose-induced insulin secretion is blocked by Rp-cAMP in *Gcg^{ΔP/ΔP}* mice. Plasma GIP levels at 0 and 15 min after oral (A) or intraperitoneal (B) glucose administration in 12- to 17-week-old control (Ctl, *Gcg^{+/+}*, and *Gcg^{ΔP/+}*) and *Gcg^{ΔP/ΔP}* mice ($n = 6-11$ mice per group). C: mRNA expression of GIP in the intestine ($n = 6-8$). The expression level of GIP mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA. D: mRNA expression of GIP and GIP receptor (GIPR) in isolated islets ($n = 4$). The expression levels normalized to that of insulin 1 are shown. E: GIP content in isolated islets ($n = 5-7$). GIP content was normalized to insulin content. F: GIP secretion from islets ($n = 5-8$). Isolated islets were incubated with 16.7 mmol/L glucose for 16 h. G: Effect of Rp-cAMP on glucose-induced insulin secretion. Isolated islets from control ($n = 14-22$ in each group) and *Gcg^{ΔP/ΔP}* ($n = 13-22$ in each group) mice were incubated with the indicated concentration of glucose and 500 μ mol/L Rp-cAMP for 30 min. Insulin secretion is expressed as the ratio of insulin released into the medium relative to insulin content. Values are expressed as means \pm SEM. * $P < 0.05$; ** $P < 0.01$. # $P < 0.05$; ### $P < 0.001$ vs. 0 min. u.d., undetectable.

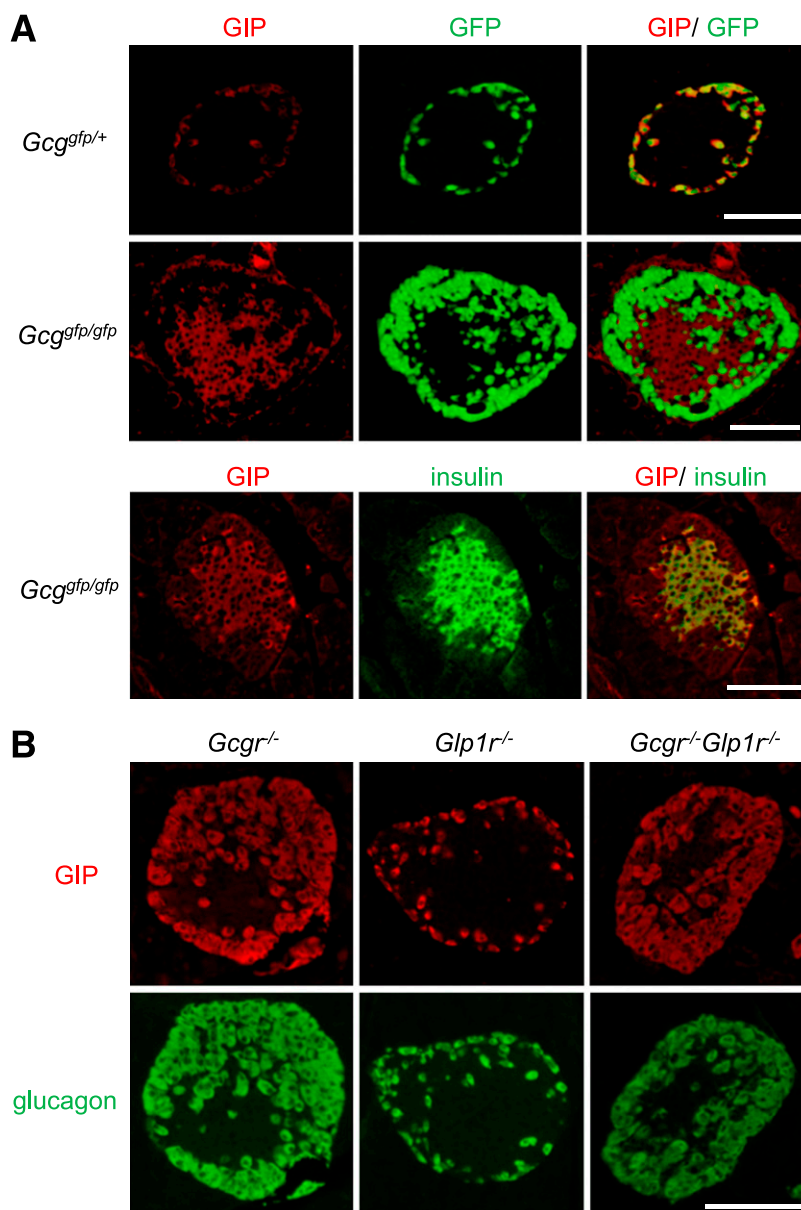


FIG. 4. **GIP is expressed in pancreatic β -cells in $Gcg^{gfp/gfp}$ mice, but not in $Gcgr^{-/-}$, $Glp1r^{-/-}$, or $Gcgr^{-/-}Glp1r^{-/-}$ mice. **A:** Representative immunostaining for GIP (red) and insulin (green), and GFP fluorescence (green) of islets from $Gcg^{gfp/gfp}$ mice and $Gcg^{gfp/+}$ mice. **B:** Representative immunostaining for GIP (red) and glucagon (green) of islets from $Gcgr^{-/-}$, $Glp1r^{-/-}$, and $Gcgr^{-/-}Glp1r^{-/-}$ mice. Scale bar, 100 μ m. (A high-quality digital representation of this figure is available in the online issue.)**

Disruption of the *Gipr* gene abrogates enhanced insulin secretion in $Gcg^{gfp/gfp}$ mice. To clarify the contribution of enhanced GIP action in the phenotype of $Gcg^{gfp/gfp}$ mice, we generated and analyzed $Gcg^{gfp/gfp}Gipr^{-/-}$ DKO mice. As observed in $Gcg^{gfp/gfp}$ mice, immunohistochemical analyses localized GIP to β -cells of $Gcg^{gfp/gfp}Gipr^{-/-}$ DKO mice (Fig. 5A), thus ruling out the possibility that the action of GIP itself is required for the altered expression pattern of GIP in islets. The hyperplasia of GFP-positive α -cells in the $Gcg^{gfp/gfp}Gipr^{-/-}$ DKO mice was comparable with that in the $Gcg^{gfp/gfp}$ mice (data not shown), indicating that GIPR signaling is not required for hyperplasia of " α "-cells. We then examined glucose tolerance and insulin secretion in the $Gcg^{gfp/gfp}Gipr^{-/-}$ DKO mice in comparison with $Gcg^{gfp/gfp}$ and control mice. Blood glucose levels in the $Gcg^{gfp/gfp}Gipr^{-/-}$ DKO mice at 15 min after oral glucose loading were higher than those in $Gcg^{gfp/gfp}$ mice (Fig. 5B), and increased insulin

levels in $Gcg^{gfp/gfp}$ mice were comparatively diminished in $Gcg^{gfp/gfp}Gipr^{-/-}$ mice to a similar degree to levels in control mice (Fig. 5C). During the IPGTT, blood glucose levels were comparable between all three groups (Fig. 5D), whereas plasma insulin levels at 15 min after intraperitoneal glucose administration were lower in $Gcg^{gfp/gfp}Gipr^{-/-}$ mice and similar to those in control mice (Fig. 5E). Furthermore, the enhancement of glucose-induced insulin secretion from isolated islets of $Gcg^{gfp/gfp}$ mice was markedly attenuated in $Gcg^{gfp/gfp}Gipr^{-/-}$ mice (Fig. 5F). These findings indicate that islet-derived GIP potentiates glucose-induced insulin secretion.

DISCUSSION

In the current study, we characterized β -cell function in mice with absence of all PGDPs, including glucagon and

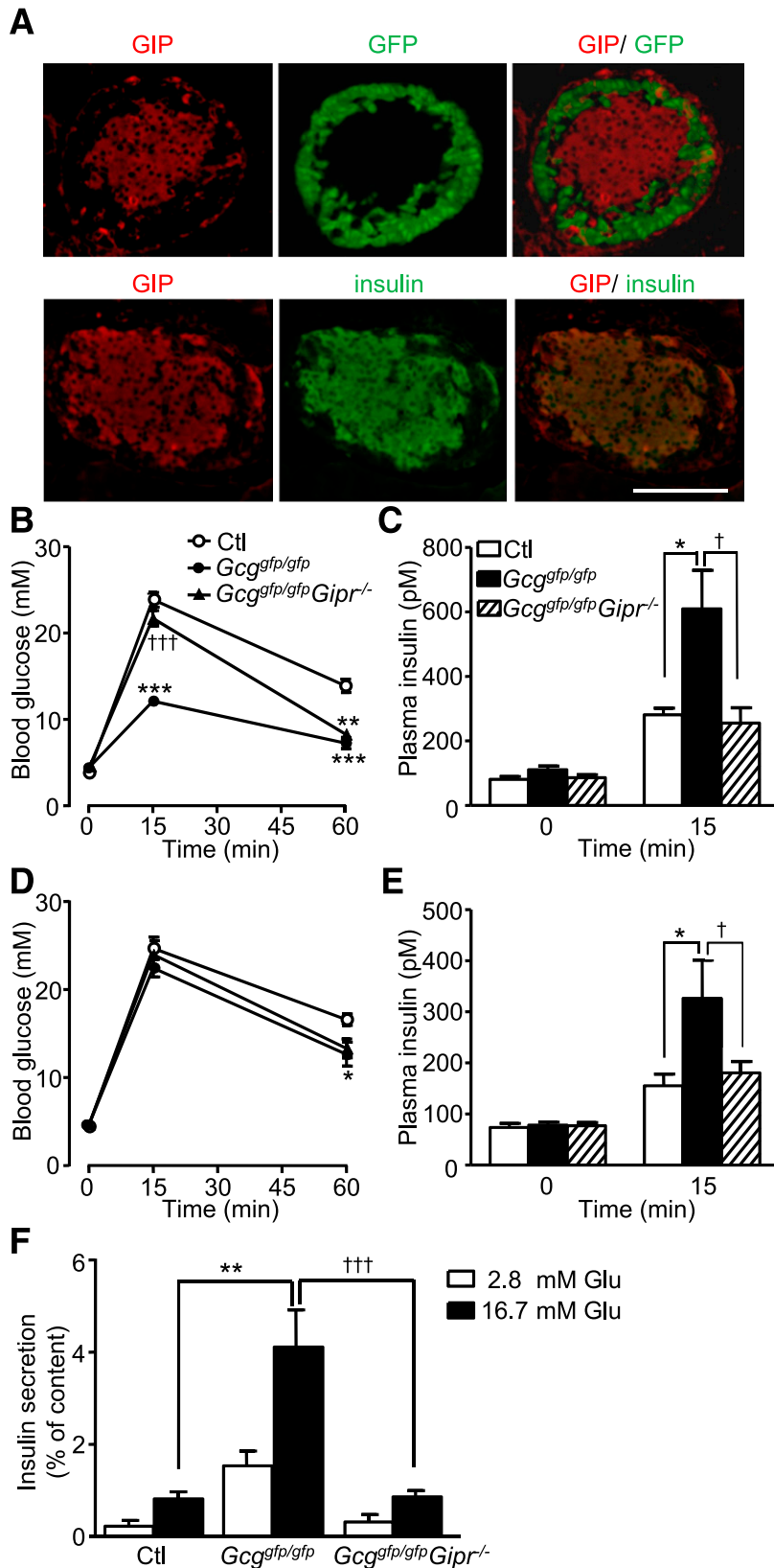


FIG. 5. Lack of GIP receptor signaling abrogates enhanced insulin secretion in *Gcg^{alpa/alpa}* mice. **A:** Representative immunostaining for GIP (red) and insulin (green), and GFP fluorescence (green) of islets from *Gcg^{alpa/alpa} Gipr^{-/-}* mice. Scale bar, 100 μ m. **B:** Blood glucose levels during the OGTT in 15- to 24-week-old control (Ctl), *Gcg^{alpa/alpa}*, and *Gcg^{alpa/alpa} Gipr^{-/-}* mice ($n = 5-7$ mice per group). **C:** Plasma insulin levels at 0 and 15 min after oral glucose loading. **D:** Blood glucose levels during the IPGTT in 13- to 22-week-old mice ($n = 6-8$ mice per group). **E:** Plasma insulin levels 0 and 15 min after intraperitoneal glucose administration. **F:** Glucose-induced insulin secretion from isolated islets. Pancreatic islets were isolated from 5- to 7-month-old control (Ctl), *Gcg^{alpa/alpa}*, and *Gcg^{alpa/alpa} Gipr^{-/-}* mice, and then incubated with the indicated concentration of glucose for 30 min. Insulin secretion is expressed as the ratio of insulin released into the medium relative to insulin content. Values are expressed as means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. Ctl. † $P < 0.05$; ††† $P < 0.001$ vs. *Gcg^{alpa/alpa}*, Ctl, *Gcg^{alpa/alpa} Gipr^{-/-}*; *Gcg^{alpa/alpa}*, *Gcg^{alpa/alpa} Gipr^{+/+}*, and *Gcg^{alpa/alpa} Gipr^{-/-}*. (A high-quality digital representation of this figure is available in the online issue.)

GLP-1. Several studies using genetic and pharmacological suppression of glucagon signaling in animal models have demonstrated improvement not only of hepatic glucose metabolism but also of β -cell function (9–15). Deletion of the GCGR appears to increase GLP-1 production in islets, resulting in improved β -cell function (13,14,16). Although the $Gcg^{afp/afp}$ mice lack glucagon, it was assumed that β -cell function in the $Gcg^{afp/afp}$ mice would be impaired because of concomitant lack of GLP-1. Unexpectedly, however, we observed improved glucose tolerance and enhanced β -cell function in $Gcg^{afp/afp}$ mice. These findings strongly suggest the emergence of novel compensatory mechanisms that maintain β -cell function in $Gcg^{afp/afp}$ mice. Because GIP secretion was enhanced in $Gcg^{afp/afp}$ mice (Fig. 4), the possible involvement of GIP was further explored in this study.

$Glp1r^{-/-}$ mice exhibit increased GIP secretion and sensitivity, which compensates for the lack of GLP-1 action (24). Mice lacking both the GLP1R and the GCGR show enhanced GIP sensitivity in islets, even though GIP levels are not increased (27). Interestingly, upregulation of GIPR mRNA levels was observed in $Gcg^{afp/afp}$ islets, suggesting that enhanced GIP action serves as one of the compensatory mechanisms for lack of GLP-1 signaling. $Gcgr^{-/-}Glp1r^{-/-}$ mice are similar to $Gcg^{afp/afp}$ mice in that they lack both glucagon and GLP-1 signaling, but they exhibit preferential preservation of insulin secretion during the OGTT but not the IPGTT (27). In contrast, our findings of enhanced insulin secretion during both OGTT and IPGTT suggested that unlike findings in $Gcgr^{-/-}Glp1r^{-/-}$ mice, enteral glucose-stimulated gut-derived factors were not likely to account for the enhanced β -cell function in $Gcg^{afp/afp}$ mice.

Recently, it has been reported that GIP is expressed in pancreatic α -cells (25); however, whether GIP secretion from α -cells is stimulated by glucose remains unclear. It has been demonstrated that GIP secretion from intestinal K-cells is not stimulated by intraperitoneal glucose loading (1,18). The current study demonstrates that serum GIP levels are increased during the IPGTT in $Gcg^{afp/afp}$, but not in control mice. Furthermore, we confirmed enhanced GIP secretion from $Gcg^{afp/afp}$ islets and GIP expression in β -cells in $Gcg^{afp/afp}$ islets (Figs. 3F and 4A). These results suggest that glucose stimulates GIP secretion from β -cells of $Gcg^{afp/afp}$ islets but not from normal islets, and are consistent with the functional observations. The mechanisms underlying emergence of GIP expression in β -cells remain unclear. Because GIP expression is observed in α -cells but not β -cells of $Gcgr^{-/-}$, $Glp1r^{-/-}$, and $Gcgr^{-/-}Glp1r^{-/-}$ mice, as well as in controls, the loss of glucagon or GLP-1 action does not contribute to β -cell GIP expression. In addition, GIP expression was observed in β -cells in $Gcg^{afp/afp}Gipr^{-/-}$ mice, demonstrating that GIP itself is not required to induce GIP expression in β -cells.

Both blockade of GIP signaling by a cAMP antagonist in vitro (Fig. 3F) and elimination of the GIPR in vivo (Fig. 5) blunted the enhanced insulin response to glucose in $Gcg^{afp/afp}$ mice. These results indicate that islet-derived GIP augments glucose-induced insulin secretion in an autocrine and/or paracrine manner in $Gcg^{afp/afp}$ mice. However, enhanced endogenous GIP action does not appear to increase β -cell mass in $Gcg^{afp/afp}$ islets. This observation may reflect a permissive need for elevated glucose levels to reveal the proliferative actions of GIP and is in accord with reports that GLP-1 promotes proliferation and survival of β -cells more strongly than GIP under hyperglycemic conditions (29).

In the current study, we demonstrate that glucose-induced insulin secretion by $Gcg^{afp/afp}Gipr^{-/-}$ islets is comparable with that of control islets both in vivo and in vitro. These results contrast with observations in another model deficient in actions of both GLP-1 and GIP. Mice deficient in receptors for both GLP-1 and GIP ($Glp1r^{-/-}Gipr^{-/-}$) showed impaired insulin secretion in response to oral glucose administration compared with controls (30). During the course of the current study, it was reported that blockade of glucagon receptor expression improved oral glucose tolerance in $Glp1r^{-/-}Gipr^{-/-}$ mice (27). It was also shown that islet expression of cholecystokinin A receptor and G-protein-coupled receptor 119 are increased on blockade of glucagon receptor expression and are involved in improved glucose tolerance (27). Such mechanisms also may contribute to the relative improvement in glucose tolerance in $Gcg^{afp/afp}Gipr^{-/-}$ mice compared with $Glp1r^{-/-}Gipr^{-/-}$ mice.

In animal models deficient in glucagon activity, hyperplasia of α -cells or GFP-positive cells in $Gcg^{afp/afp}$ islets has been observed. However, the mechanisms underlying hyperplasia remain largely unelucidated. Although glucagon itself may directly suppress proliferation of α -cells, several findings indicate that indirect signals arising from glucagon target organs also may play important roles in the development of hyperplasia. It has been reported that mice with liver-specific $G_{S\alpha}$ deficiency display pancreatic α -cell hyperplasia (31), indicating that the direct effect of glucagon on α -cells is insufficient to block aberrant α -cell proliferation. Recently, Imai et al (32) have demonstrated that signals from the liver mediated through the autonomic nervous system regulate β -cell proliferation. In addition, it has been demonstrated that hepatic metabolism, especially amino acid metabolism, is remodeled in animal models deficient in glucagon action (33,34). Such metabolic and/or neural signals from extra-islet organs also may be involved in altered proliferation and function of α -cells. These indirect mechanisms also may account for the aberrant expression of GIP observed in β -cells of $Gcg^{afp/afp}$ islets.

Glucagon is believed not to cross the placenta (35). Because hyperplasia of α -cells gradually develops after birth in $Gcg^{afp/afp}$ mice, this is consistent with loss of glucagon signaling indirectly contributing to the development of α -cell hyperplasia, as suggested by Chen et al. (31). GIP expression in β -cells is observed at embryonic day 18.5 in both control and $Gcg^{afp/afp}$ mice (Supplementary Fig. 2). These findings differ from a previous report that showed that GIP was generally coexpressed with glucagon by immunostaining (36). Although $Gcg^{afp/+}$ mice expressed GIP in both insulin-positive β -cells and in insulin-negative cells that may be α -cells, GIP is expressed only in β -cells in $Gcg^{afp/afp}$ mice. This difference in GIP distribution between $Gcg^{afp/+}$ and $Gcg^{afp/afp}$ can be detected by embryonic day 18.5, when α -cell hyperplasia has not developed in $Gcg^{afp/afp}$ mice. Therefore, these findings indicate that α -cell hyperplasia is not a prerequisite for altered GIP expression in islets.

In conclusion, we demonstrate that elimination of PGDPs results in enhancement of β -cell function that can be attributed to induction of GIP expression in β -cells. It is recognized that GIP is one of the most important factors in the regulation of insulin secretion when blood glucose levels increase after ingestion of nutrients (18). Our results show that GIP produced by β -cells is secreted when blood glucose levels increase, even if nutrients are not ingested

via the gastrointestinal tract. Future investigations should elucidate the conditions required for and mechanisms underlying the ectopic expression of GIP in β -cells.

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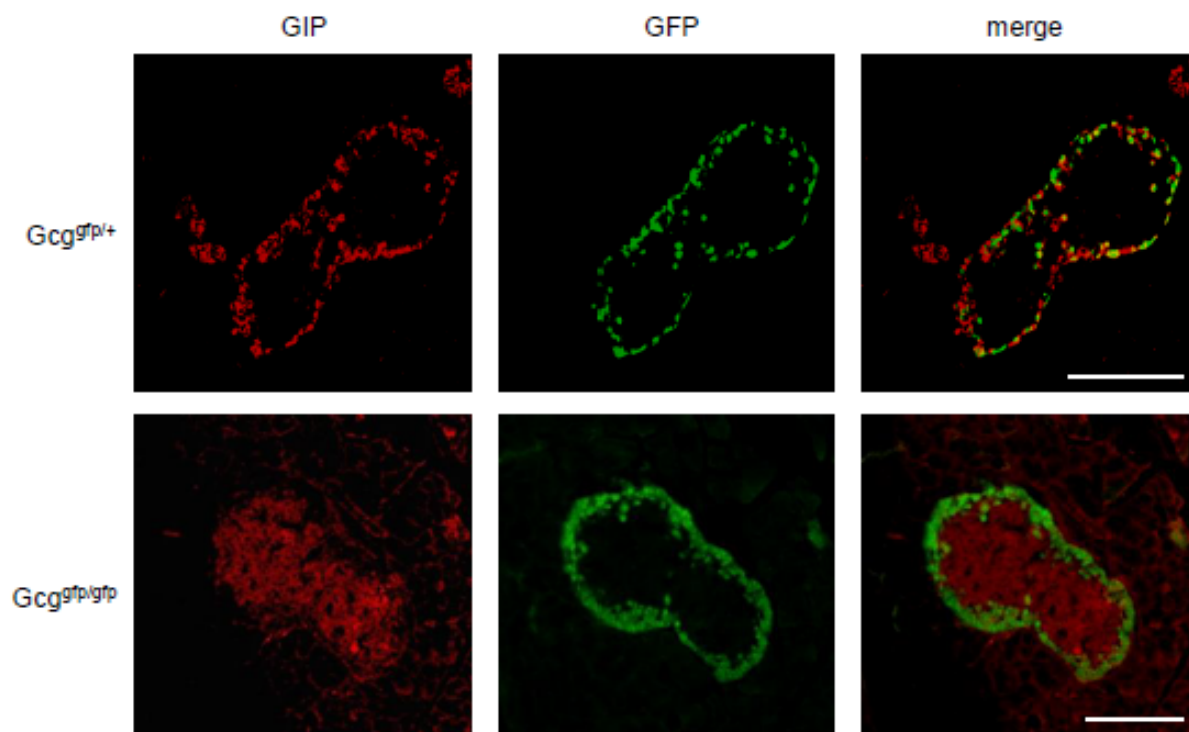
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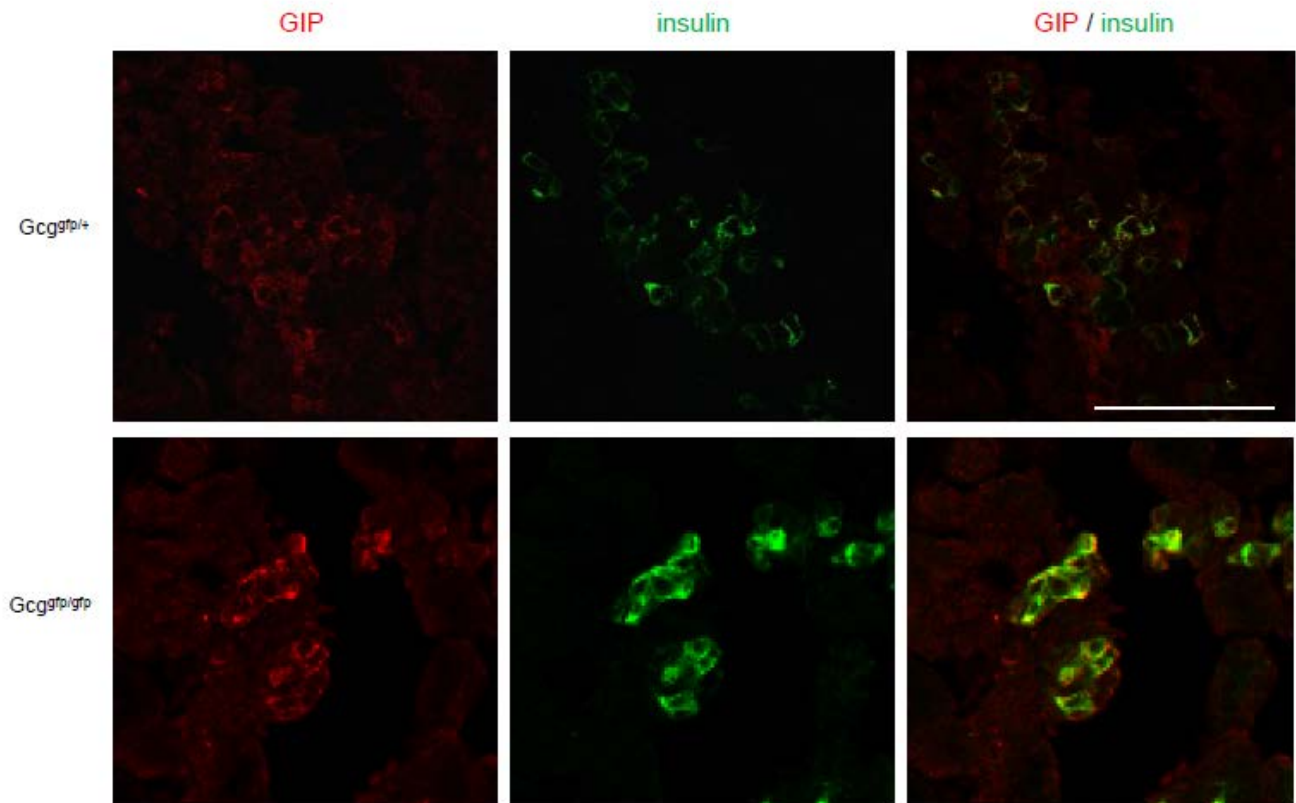
SUPPLEMENTARY DATA

Supplementary Figure 1. Immunohistochemical analyses were performed using another antibody against GIP (1:500, Yanaihara, Shizuoka, Japan). Representative immunostaining for GIP (red) and GFP fluorescence (green) in the islets of *Gcggfp/gfp* and *Gcggfp/+* mice. The distribution of GIP immunoreactivity was similar to the results shown in Figures 4A and 5A. Scale bar, 100 μ m.



SUPPLEMENTARY DATA

Supplementary Figure 2. Immunohistochemical analyses of embryonic pancreata (E18.5). Representative immunostaining for GIP (red) and insulin (green) of the islets of *Gcggfp/gfp* and *Gcggfp/+* mice. GIP is expressed in insulin-positive cells in both strains of mice. In *Gcggfp/+* mice, GIP immunoreactivity was observed in both insulin-positive and insulin-negative cells, possible α -cells. In *Gcggfp/gfp* mice, GIP was observed exclusively in insulin-positive cells. Scale bar, 50 μ m.



SUPPLEMENTARY DATA

Supplementary Figure 3. Immunohistochemical studies for Pdx1 (1:500, Millipore, Temecula, CA, USA), Pax6 (1:300, Covance, Emeryville, CA, USA) and insulin, and GFP fluorescence of the islets of *Gcggfp/gfp* and *Gcggfp/+* mice. Pdx1 is colocalized in β -cells. Pax6 is expressed both in α -cells and b-cells. Distribution of Pax6 and Pdx1 is not different between *Gcggfp/+* and *Gcggfp/gfp*. Serial sections were used for immunostaining and GFP fluorescence.

