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The Role of Preproglucagon Peptides in Regulating B-Cell Morphology and Responses to Streptozotocin-Induced Diabetes

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Insulin secretion from β-cells is tightly regulated by local signaling from preproglucagon (Gcg) products from neighboring α-cells. Physiological paracrine signaling within the microenvironment of the β-cell is altered after metabolic stress, such as high-fat diet or the β-cell toxin, streptozotocin (STZ). Here, we examined the role and source of Gcg peptides in β-cell function and in response to STZ-induced hyperglycemia. We used whole body Gcg null (GcgNull) mice and mice with Gcg expression either specifically within the pancreas (GcgΔPanc) or the intestine (GcgΔIntest). With lower doses of STZ exposure, insulin levels were greater and glucose levels were lower in GcgNull mice compared with wild-type mice. When Gcg was functional only in the intestine, plasma glucagon-like peptide-1 (GLP-1) levels were fully restored but these mice did not have any additional protection from STZ-induced diabetes. Pancreatic Gcg reactivation normalized the hyperglycemic response to STZ. In animals not treated with STZ, GcgNull mice had increased pancreas mass via both α- and β-cell hyperplasia and reactivation of Gcg in the intestine normalized β- but not α-cell mass, whereas pancreatic reactivation normalized both β- and α-cell mass. GcgNull and GcgΔIntest mice maintained higher β-cell mass after treatment with STZ compared with control and GcgΔPanc mice. Although in vivo insulin response to glucose was normal,
global lack of Gcg impaired glucose-stimulated insulin secretion in isolated islets. Congenital replacement of Gcg either in the pancreas or intestine normalized glucose-stimulated insulin secretion. Interestingly, mice that had intestinal Gcg reactivated in adulthood had impaired insulin response to KCl. We surmise that the expansion of β-cell mass in the GcgNull mice compensated for decreased individual β-cell insulin secretion, which is sufficient to normalize glucose under physiological conditions and conferred some protection after STZ-induced diabetes.

NEW & NOTEWORTHY We examined the role of Gcg on β-cell function under normal and high glucose conditions. GcgNull mice had decreased glucose-stimulated insulin secretion, increased β-cell mass, and partial protection against STZ-induced hyperglycemia. Expression of Gcg within the pancreas normalized these endpoints. Intestinal expression of Gcg only normalized β-cell mass and glucose-stimulated insulin secretion. Increased β-cell mass in GcgNull mice likely compensated for decreased insulin secretion normalizing physiological glucose levels and conferring some protection after STZ-induced diabetes.

INTRODUCTION
The preproglucagon (Gcg)-derived peptides, glucagon-like peptide-1 (GLP-1), and glucagon are critical glucoregulatory peptides. Generally, these peptides are considered to have distinct physiological functions, yet expanding work suggests that depending on nutrient conditions glucagon and GLP-1 can have overlapping roles in regulating insulin secretion. Specifically, both glucagon and GLP-1 stimulate insulin secretion postprandially via the β-cell GLP-1 receptor (GLP-1R) (1–3). Conversely, elevated glucagon stimulates hepatic glucose production and has been proposed to contribute hyperglycemia in patients with type 2 diabetes (4, 5).

Intact glucagon signaling is suggested to be necessary for hyperglycemia in diabetic mouse models (6, 7). In support of this, several papers have demonstrated that genetic ablation of the glucagon receptor (GcgR KO) generates mice that are protected from streptozotocin (STZ)-induced diabetes (7–10). However, genetic ablation of the GcgR results in increased plasma levels of GLP-1 (11). In fact, one prevailing hypothesis is that it is the compensatory increase in GLP-1, and not the absence of glucagon signaling that contributes to the protection from hyperglycemia (10). STZ exposure itself increases plasma GLP-1 levels suggesting that GLP-1 is protective during metabolic stress. In addition, rats treated with STZ have acute increases in islet PC1/3 and Gcg expression, and increased translation of proglucagon to GLP-1 (12). Indeed, GcgR knockout (KO) mice administered STZ concurrent with exendin 9–39, a GLP-1 receptor antagonist, have the expected hyperglycemic response to STZ (10). Interestingly, GcgR deletion 1 wk after STZ only partially protected mice from hyperglycemia, but this effect was still reversed with pharmacological blockade of the GLP-1R (7). Whether this conditional GcgR KO mouse had limited developmental compensation leading to only partial protection from hyperglycemia is unknown. Furthermore, since STZ increases plasma GLP-1 and glucagon levels, it is not clear whether the partial protection of this mouse is due to the lack of glucagon receptor signaling or increased STZ-induced GLP-1 signaling.

If increased GLP-1 is not protecting GcgR KO mice from STZ-induced diabetes, then what is the function of increased GLP-1 with STZ-induced diabetes, and does it relate to its function in response to other types of metabolic stress including models of diabetes where pancreatic production of GLP-1 is
increased? GLP-1 is an insulin secretagogue but also has reported effects on β-cell protection (13) and as an anti-inflammatory agent (14), although the mechanism for the latter is unclear. Here, we explore the source and function of Gcg peptides that contribute to changes in β-cell morphology and function in response to STZ-induced diabetes. To do this, we used mice null for Gcg and animals that had a reactivation of Gcg either specifically within the pancreas or the intestine. We find that lack of Gcg confers a small amount of islet protection that does not always contribute to changes in overall glucose after STZ. Replacement of Gcg expression in the intestine fully restores the plasma GLP-1 response to STZ and results in higher levels of plasma insulin and β-cell mass compared with controls. Finally, lack of Gcg impairs islet function, ex vivo, and replacement of Gcg either in the pancreas or intestine normalizes islet function.

MATERIALS AND METHODS

Animals

All mice were bred in-house at the University of Cincinnati or the University of Michigan. Mice were single-housed under a 12-h light/dark cycle with ad libitum access to water and standard chow (Envigo Teklad; Cat. No. 7012). The animal room was maintained at 25°C with 50%–60% humidity. For all studies, male mice were used, groups were age-matched, and all controls were littermates. Genetically modified mice used in this study were previously validated and described (2, 15). Briefly, a mouse model with a loxP-flanked transcriptional blocking cassette that included an eGFP construct was inserted between exons 2 and 3 of the Gcg gene, and the resulting mouse is null for Gcg expression (GcgNull). In some experiments, GcgNull mice were crossed with C57BL/6J mice to generate offspring that were either GcgNull or littermate wild-type controls. We also crossed the GcgNull mice with the developmental VilCre (Stock No. 004586; Jackson Laboratory) or Pdx1Cre (Stock No. 014647; Jackson Laboratory) mice to reactivate endogenous Gcg expression within the intestine (GcgΔIntest) or pancreas/duodenum (GcgΔPanc). All reactivated mice were compared with littermate mice expressing Cre alone. We generated the intestinal Gcg-inducible mouse model (iGcgΔIntestERT) by crossing the GcgNull mouse with a tamoxifen-inducible VilCre (VilCre-ERT2) mouse. iGcgΔIntestERT mice were all administered tamoxifen (Sigma-Aldrich; Cat. No. T5648) by intraperitoneal injection every other day (120 mg/kg/day) for three administrations. All studies were approved by, and performed, according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati or the University of Michigan.

Ex Vivo Glucose-Stimulated Insulin Secretion Test

Immediately following euthanasia, the pancreas was inflated with 1 mg/mL collagenase P (Sigma-Aldrich, St. Louis, MO) in Hanks balanced salt solution (HBSS) without salts. Pancreata were incubated in a water bath at 37°C for 12–15 min with intermittent shaking. Islets were washed three times, filtered, and hand-picked into Roswell Park Memorial Institute media (RPMI) 1640 with 2.5 mL of 1 M glucose, 50 mL fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), and 5 mL penicillin/streptomycin (Life Technologies, Carlsbad, CA) After an overnight “recovery,” 40 islets were picked and incubated at 37°C for 30 min in Krebs-ringer bicarbonate buffer (KRBH) (20 mL 10× HBSS with salts, 150 mL dH2O, 4 mL 1 M HEPES, 74 mg CaCl2, 428 mg NaHCO3, pH to 7.4, 400 mg lyophilized BSA) with 2 mM glucose. Ten islets were then picked and incubated for 1 h at 37°C followed by incubation in KRBH with 22 mM glucose for 30 min. For glucagon experiments, islets were incubated in
0.1 nM glucagon in KRBH with 4 mM glucose for 1 h at 37°C followed by 1 h in KRBH with 0.1 nM glucagon and 22 mM glucose. Finally, islets were incubated in either 30 mM KCl in KRBH with 22 mM glucose or exendin-4 (Sigma-Aldrich, St. Louis, MO) in KRBH with 22 mM glucose for 30 min at 37°C. Media was collected and frozen after each incubation. Following this, 100 µL of islet lysis buffer was added to the islets for insulin content and DNA quantitation.

α- and β-Cell Mass
Pancreata from STZ-naïve mice and STZ-treated mice (2 wk after two doses of STZ at 160 mg/kg) were dissected, weighed, and then placed in a cassette suspended in 10% normal buffered formalin overnight before being transferred to 70% ethanol. Pancreata were paraffin-embedded and sectioned at 20 µm and mounted. Images were acquired with a Zeiss 880 imaging scope and islet mass was determined on randomly selected sections throughout the entire pancreas after immunostaining for insulin (polyclonal guinea pig anti-insulin; Agilent IR00261-2; FITC secondary in PDX1Cre and GcgΔPanc and Cy3 in GcgNull and GcgΔIntest mice) and immunostaining for either GFP fluorescent in Gcg-positive cells (GcgNull and GcgΔIntest mice) or for glucagon in PDX1Cre and GcgΔPanc (abcam92517; cy3 secondary) (2). The endocrine area (in µm²) was determined by intensity thresholding using the morphometry measurement tools of ImageJ v. 1.39p [National Institutes of Health, freely available at http://rsb.info.nih.gov/ij/index.html; (16)]. The α- and β-cell mass was calculated by a ratio between the weight of the pancreas and the proportion of insulin or glucagon immunoreactive cells in the samples. Insulin and glucagon cell counting was measured using ImageJ, as done previously (17).

Plasma Collection
Blood samples were obtained 10 min after an oral gavage of dextrose (2.5 g/kg) 15 days after STZ treatment before tissue collection. Blood was collected in heparinized syringes and placed in a tube with a mixture of DPP-4 inhibitor (Millipore, Burlington, MA), heparin, EDTA, and aprotinin. Insulin (Crystal Chem) and total GLP-1 (Mesoscale) was assayed from plasma samples using a commercially available kit according to the manufacturer’s instructions.

Streptozotocin (STZ) Studies
Animals were injected with STZ (Millipore Sigma) dissolved in diluted sodium citrate. Mice in the dose-response study were injected twice, 1 day apart, with either 30 mg/kg, 80 mg/kg, or 130 mg/kg of STZ (Fig. 1, A–F). In all subsequent studies, two doses of 160 mg/kg STZ were administered 1 wk apart. STZ doses vary according to the study and are dosed based on body weight. Animals were injected in the morning and were not fasted before STZ injection. Ad libitum blood glucose was monitored frequently (rate varied by study) in mice by glucometer in the morning (within a 2-h window) via tail snip.
Figure 1. Response to STZ in GgcNull vs. WT mice. No difference in ad lib glucose was seen between littermate WT (n = 4–7) and GgcNull (n = 5 or 6) mice administered two doses of 30 mg/kg (A), 80 mg/kg (B), or 130 mg/kg of STZ (C). D: the glucose response 10 min after a dextrose gavage (2.5 g/kg) was significantly lower in GgcNull mice compared with WT mice (P < 0.05, main effect of genotype). E: plasma insulin levels 10 min after a dextrose gavage were significantly lower with higher doses of STZ (P < 0.0001, main effect of STZ dose) and trended higher in GgcNull mice compared with WT (P = 0.06; main effect of genotype). Left y-axis represents the STZ doses of 30 mg/kg and 80 mg/kg STZ, whereas the right y-axis represents the 130 mg/kg STZ dose. F: total plasma GLP-1 levels were greater in the mice administered 130 mg/kg vs. 30 mg/kg and 80 mg/kg of STZ in wild-type mice (*P < 0.05, **P < 0.01). G: both GgcNull and WT mice lowered blood glucose levels in response to leptin (P < 0.0001, time × leptin interaction). Data are reported as means ± SE, RM-two-way or one-way ANOVA. GLP-1, glucagon-like peptide-1; ND, nondetectable; RM, repeated measures; STZ, streptozotocin; WT, wild type.

Leptin Injections
GgcNull and WT littermate mice were given two doses of 160 mg/kg of STZ 1 wk apart. Five days later, mini-osmotic pumps were implanted after the second STZ injection to deliver 10 μg of leptin or vehicle (PBS) per day and per mouse over the course of 16 days.

Statistics
Statistical analysis was performed using GraphPad Prism v. 9.0 (GraphPad Software, San Diego, CA). Statistical significance was determined either by unpaired Student’s t test, one-way ANOVA followed by Tukey’s multiple comparison post hoc test, two-way ANOVA followed by Bonferroni’s multiple comparison post hoc test, or a repeated measures ANOVA followed by Bonferroni’s multiple comparison post hoc test as referenced in the text. Results were considered statistically significant when P < 0.05.

RESULTS
Dose-Response to STZ in GgcNull versus WT Mice
Both GgcNull and WT mice showed greater decreases in body weight with increasing STZ doses (Supplemental Fig. S1A). GgcNull and WT mice had similar ad lib glucose levels at all STZ doses (Fig. 1, A–C), but only the 130 mg/kg total dose elicited a significant increase in ad lib blood glucose. Ten minutes following a glucose load, the plasma glucose levels were not significantly different between the 30 and
80 STZ doses but this increase was lower in GcgNull versus WT mice (Fig. 1D). Data for the 130 mg/kg dose are not shown as the glucose values in response to the glucose load surpassed the highest detection levels of the glucometer (maximum detection is 600 mg/dL). Despite observing no change in ad lib glucose levels with the lower STZ doses, glucose-stimulated insulin levels decreased with increasing doses of STZ in both WT and GcgNull mice (Fig. 1E). There was also a tendency (P = 0.06) for the GcgNull mice to have greater insulin levels. Plasma GLP-1 increased with increasing STZ dose in the WT mice and was undetectable in the GcgNull mice (Fig. 1F).

Because previous data demonstrated that leptin is protective against STZ-induced diabetes due to suppression of glucagon (18), we administered STZ to mice and 5 days later implanted mini-osmotic pumps filled with leptin. Both WT and GcgNull mice decreased blood glucose levels similarly in response to leptin (Fig. 1G). Overall, insulin levels trended higher suggesting β-cells are more protected with smaller doses of STZ in GcgNull mice resulting in mild protection from hyperglycemia.

Intestinal versus Pancreatic Gcg Reactivation and Response to STZ
We see an increase in plasma GLP-1 with increasing doses of STZ, and previous work has suggested that pancreatic production of GLP-1 increases with STZ (19). Thus, we next investigated if reactivating the Gcg gene specifically within the pancreas (GcgΔPanc) or intestine (GcgΔIntest) would impact the effect of STZ on glucose and insulin levels. Body weight decreased similarly between all genotypes with STZ administration (Supplemental Fig. S1, C and D). Ad lib blood glucose levels were significantly lower 3 days after STZ throughout the end of the study between the VilCre control and GcgΔIntest mice (Fig. 2A). Glucose-stimulated plasma insulin levels were significantly increased in GcgΔIntest mice compared with VilCre controls (Fig. 2B). Total plasma GLP-1 levels were not detectable in GcgNull mice but were similar between VilCre controls and GcgΔIntest mice (Fig. 2C). Restoring Gcg in the pancreas resulted in no difference in ad lib fed blood glucose or glucose-stimulated insulin response to STZ (Fig. 2, D and E). Total plasma GLP-1 levels were decreased in GcgΔPanc mice compared with PDX1Cre controls (Fig. 2F). Overall, Gcg expression within the intestine, but not pancreas, had a mild protective effect on insulin and glucose response to STZ.
Figure 2. Intestinal vs. pancreatic Gcg reactivation and response to STZ. A: VilCre control (n = 7), GcgNull (n = 8), and VilCre Gcg reactivated (n = 8; GcgΔIntest) mice were injected twice with STZ (160 mg/kg) 7 days between injections. VilCre control mice had higher ad lib blood glucose levels from day 3 to day 15 compared with GcgΔIntest (*P < 0.05) and at day 10 compared with GcgNull mice (†P < 0.05). B: insulin levels 10 min after a dextrose gavage (2.5 g/kg) were greater in GcgΔIntest compared with VilCre control mice (*P < 0.05). C: total plasma GLP-1 levels were not detectable in GcgNull mice, and not significantly different between VilCre control and GcgΔIntest mice. D: PDX1Cre control (n = 8), GcgNull (n = 7), and PDX1Cre Gcg reactivated (n = 7; GcgΔPanc) mice were injected twice with STZ (160 mg/kg) 7 days apart. Ad lib glucose levels were not different between PDX1Cre control, GcgNull, and GcgΔPanc over the 15-day testing period. E: plasma insulin levels were unchanged between PDX1Cre controls, GcgNull, and GcgΔPanc mice 10 min after a dextrose gavage (2.5 g/kg). F: total plasma GLP1 10 min were undetectable in GcgNull mice, and lower in GcgΔPanc compared with PDX1Cre control mice (***P < 0.001). Data are reported as means ± SE, RM-two-way, one-way ANOVA, or Student’s t test. ND, nondetectable; RM, repeated measures; STZ, streptozotocin.

Because we see some protective effect in GcgNull and GcgΔIntest mice on insulin levels, we next examined changes in islet morphology under control and STZ-treated conditions. As previously seen (2), pancreas mass is increased in the GcgNull mice compared with WT littermates (Fig. 3A). We also saw an increase in both α- and β-cell mass (Fig. 3, B and C) compared with WT controls. Although α-cell mass remained elevated in GcgΔIntest mice, α-cell mass in GcgΔPanc mice was similar to controls (Fig. 3B). Reactivation of Gcg in both the intestine and pancreas normalized β-cell mass (Fig. 3C). This resulted in a significantly greater β- to α-cell mass ratio in GcgΔPanc versus GcgΔIntest mice (Fig. 3D). Differences in untreated islet size and ratio are shown in representative images for each treatment group (Fig. 3E).
The Gcg<sup>Null</sup> mice maintained significantly greater pancreatic and α-cell mass compared with controls after STZ treatment, but the difference in β-cell mass was no longer statistically different after STZ (P = 0.07). Interestingly, although the Gcg<sup>ΔIntest</sup> mice had lower pancreatic mass compared with the Gcg<sup>Null</sup> mice without STZ treatment, pancreatic mass was similar to Gcg<sup>Null</sup> animals after STZ treatment (Fig. 3F). Like with nontreated animals, α-cell mass was greater in both Gcg<sup>Null</sup> and Gcg<sup>ΔIntest</sup> mice compared with control and Gcg<sup>ΔPanc</sup> mice after STZ treatment (Fig. 4G). However, both Gcg<sup>Null</sup> and Gcg<sup>ΔIntest</sup> mice had greater β-cell mass after STZ treatment compared with control and Gcg<sup>ΔPanc</sup> groups.
This resulted in a significantly greater β- to α-cell mass ratio in GcgΔIntest mice compared with controls (Fig. 3I). Differences after STZ treatment are shown in representative images in Fig. 3J.

Figure 4. Ex vivo islet function. A: insulin response to 22 mM glucose and 30 mM KCl was lower in GcgNull (n = 21) compared with WT (n = 12) control mice (**P < 0.001, ****P < 0.001). B: there was no difference in insulin response to 2 mM or 22 mM glucose between VilCre control (n = 11), GcgΔIntest (n = 3), and inducible iGcgΔIntestERT (n = 5) mice. iGcgΔIntestERT isolated islets had a blunted insulin response to KCl compared with VilCre control mice (**P < 0.01). C: there was no difference in insulin response between PDXCre control (n = 5) mice and GcgΔPanc (n = 3) mice after 2 mM glucose, 22 mM glucose, or 30 mM KCl stimulation. D: insulin response to low glucose (2 mM) and high glucose (22 mM) was decreased in GcgNull (n = 4) compared with VilCre control (n = 4) mice (*P < 0.05, **P < 0.01). There was no difference between GcgNull and VilCre controls in response to high glucose and administration of the GLP-1 agonist exendin-4. E: the insulin response to low glucose (2 mM) or high glucose (22 mM) with glucagon was not different between VilCre control (n = 4) and GcgNull (n = 4) mice. Data are reported as means ± SE, two-way ANOVA for each stimulus condition followed by Tukey’s post hoc test or Student’s t test. GLP-1, glucagon-like peptide-1; WT, wild type.

Ex Vivo Islet Function
To understand whether the baseline phenotype of increased β-cell mass in the GcgNull mice impacts the function of the islets, we performed glucose-stimulated insulin secretion (GSIS) on isolated islets. Islets from GcgNull mice had similar insulin responses to low glucose but exhibited a blunted insulin response
to high glucose (22 mM) and KCl (Fig. 4A). There was no significant difference in insulin secretion between VilCre control and GcgΔIntest mice, indicating that intestinal Gcg is sufficient to normalize islet function. To determine whether there is a developmental compensation for this effect, we also used a tamoxifen-inducible VilCre ERT mouse to reactivate Gcg in the intestine in the adult mouse (iGcgΔIntestERT). Interestingly, the iGcgΔIntestERT mice maintained blunted insulin secretion in response to KCl compared with VilCre controls (Fig. 3B), indicating that there are developmental contributions of intestinal Gcg to islet function. Glucose- and KCl-stimulated insulin responses were similar between PDX1Cre controls and GcgΔPanc (Fig. 4C). Thus, congenital reactivation of Gcg in both the intestine and pancreas was sufficient to normalize β-cell function while reactivation in the adult intestine was less effective. These data indicate a developmental component of Gcg to β-cell function and together with the changes in islet morphology suggest that to some extent, the islet function changes are dissociated from the impact on β-cell mass.

In a different set of experiments, we performed GSIS on isolated islets in VilCre control and GcgNull mice after combinatorial glucose and GLP-1 or glucagon treatment. Although islets from GcgNull mice had a blunted insulin response to low and high glucose compared with controls, GcgNull and control mice had a similar increase in insulin in response to high glucose combined with the GLP-1 agonist, exendin-4 (Fig. 4D). Insulin secretion in GcgNull islets tended to be lower compared with controls after combined glucose and glucagon treatment at lower, but was similar at higher, glucose concentrations (Fig. 4E). These data suggest that at least in combination with high glucose, insulin secretion from GcgNull mice is normalized when stimulated with either propraglucagon peptide.

DISCUSSION
Both glucagon and GLP-1 are critical glucoregulatory peptides. Glucagon has been proposed to prevent hyperglycemia under postprandial conditions via β-cell GLP-1R action and to defend against falling glucose levels via hepatic glucagon receptors. Although GLP-1 is thought to be primarily an insulin secretagogue, the increase in α-cell produced GLP-1 in times of metabolic stress has been proposed to protect β-cell mass and function. Plasma GLP-1 is elevated in GcgR KO and this increase has been implicated in the protection from STZ-induced diabetes observed in these mice (1–3). Here, we show that Gcg-derived peptides regulate β-cell morphology and function and that there is a critical confound of developmental compensations across genetic mouse models that have contributed to an inadequate description of the physiology of the system. Our data suggest the possibility that the protective effect of GLP-1 in STZ-induced diabetes stems from the contribution of GLP-1 β-cell protection through a developmental compensation for the lack of Gcg-derived peptides.

In the GcgNull mouse that lacks both glucagon and GLP-1, we observed only a small amount of protection on β-cell mass after STZ-induced diabetes, yet this protection was not sufficient to statistically improve ad lib glucose levels. In GcgNull animals not treated with STZ, we observe preserved in vivo nutrient-stimulated insulin responses, blunted ex vivo GSIS, and an increase in β-cell mass. We speculate that the increased number of β-cells in the GcgNull mouse compensates for the fact that individual β-cell function is less than normal. This compensation would explain why, while β-cell mass is better retained with STZ, it is not sufficient to improve glucose levels.
Replacement of \textit{Gcg} in the intestine or pancreas during development fully restores GSIS. Furthermore, intestinal reactivation fully restores plasma GLP-1 levels (including in response to STZ) and preserves β-cell mass, but the degree to which these mice are distinguished from the \textit{Gcg}^{Null} mice is less clear. We do see that plasma insulin levels are higher and glucose levels are statistically lower with STZ-induced diabetes in the \textit{Gcg}^{\Delta\text{Intest}} compared with controls, yet there is no statistical difference when the \textit{Gcg}^{\Delta\text{Intest}} mice are compared with the \textit{Gcg}^{Null} mice. Minimal impact of intestinal reactivation of \textit{Gcg} would be consistent with our previous work suggesting that intestinal \textit{Gcg} does not regulate insulin response to nutrients (2, 20).

Elevated (or unopposed) glucagon has long been implicated as the cause of hyperglycemia in T2DM and in elevated hepatic glucose production in T1DM (4, 5) This idea was perpetuated by the fact that \textit{GcgR} KO mice are protected from the hyperglycemic response to STZ. However, \textit{GcgR} KO mice have increased plasma levels of GLP-1 (10). STZ is known to further increase plasma GLP-1 even in WT animals [Fig. 1F and (7)], and pharmacological blocking of GLP-1R signaling prevents the protection from hyperglycemia in some studies (10). Interestingly, \textit{GcgR} deletion 1 wk after STZ only partially protected mice from hyperglycemia, but this effect was still reversed with pharmacological blockade of the GLP-1R (7). Genetic ablation of both \textit{GcgR/GLP-1R} signaling (21) or glucagon and GLP-1 peptides in the \textit{Gcg}^{Null} mice concurs with the pharmacological data, i.e., that GLP-1R signaling is necessary for the full extent of the protection from STZ-induced diabetes with glucagon ablation. Specifically, double \textit{GcgR/GLP-1R} KO mice have significantly greater glucose excursions in response to STZ compared with \textit{GcgR} KO mice, but still have lower ad lib glucose levels compared with either GLP-1R KO or WT mice treated with STZ (21). This is similar to our \textit{Gcg}^{Null} mice that are only slightly protected from STZ-induced diabetes. Near total ablation of α-cells in adult mice using a diphtheria toxin approach significantly reduces glucagon and GLP-1 in the islet. Furthermore, these mice were not protected from STZ-induced diabetes (22), suggesting developmental compensation(s). Finally, although glucagon has also been postulated to play a role in mediating the protective effect of leptin on STZ-induced diabetes (18), we see that leptin is fully able to protect against STZ-induced diabetes in the \textit{Gcg}^{\Delta\text{Null}} mice. Altogether with our current data, the conclusion is that the removal of glucagon signaling alone is not sufficient to protect animals from STZ-induced hyperglycemia.

The increase in plasma and pancreatic-produced GLP-1 has been implicated as protective in wild-type and \textit{GcgR} KO animals with STZ administration (12). If this were the case, then GLP-1R KO mice should have even more impaired glycemia with STZ. However, genetic models targeted to GLP-1R signaling conflict on whether these models are more hyperglycemic in response to STZ (23) versus (21). Pharmacological activation of the GLP-1R does not consistently protect against STZ-induced hyperglycemia (7 vs. 23). Thus, GLP-1R signaling alone also does not seem sufficient to protect against STZ-induced hyperglycemia.

Our current developmental KO models and previously published developmental KO models with deletion of both the GLP-1R and \textit{GcgR} all have some, but not full, protection against STZ-induced diabetes. These data suggest other protective factors at play. Along with GLP-1, FGF21 signaling has also been implicated as protective (10). Although we cannot rule out an impact of FGF21 in our mouse models, it is an interesting avenue for future investigation.
Our Gcg\textsuperscript{Null} and Gcg\textsuperscript{ΔIntest} mice have increased β-cell mass with and without STZ treatment, and we speculate that this increase provides some protection from STZ-induced diabetes.

We have previously shown that pancreatic Gcg expression was sufficient for a GLP-1R antagonist to impair glucose tolerance (2). At the time, we hypothesized that this was due to islet-produced GLP-1. This work was followed by multiple papers demonstrating that, at low levels, glucagon can act on the GLP-1R to stimulate nutrient-induced insulin secretion (1–3). Adding both exendin-4 or glucagon to the media during a static GSIS experiment of Gcg\textsuperscript{Null} islets normalized insulin secretion, suggesting that the lack of GLP-1R signaling is what impaired GSIS. Gcg\textsuperscript{ΔPanc} mice have normal in vivo and ex vivo GSIS and β-cell mass. Our previous work has shown that intestinal Gcg is not necessary (20) or sufficient (2) for nutrient-stimulated insulin secretion in vivo, yet these mice still have increased β-cell mass.

Interestingly, developmental but not adult reactivation of Gcg normalized KCl-stimulated insulin secretion, suggesting a developmental impact of Gcg that is important for normal islet morphology and function. Two previous studies with very acute activation of α-cells using chemogenetic activation (24) or Cre-driven viral intraislet reactivation of GCG in the same Gcg\textsuperscript{Null} (1) mouse model used here found that these acute manipulations of α-cells were sufficient to impair, or restore insulin secretion, respectively. Altogether, these data suggest a developmental contribution of intestinal Gcg to improve β-cell function in adulthood. Furthermore, there may be a dissociation between in vivo and ex vivo insulin responses that are due to physiological compensations (i.e., other gut peptides) and enhanced β-cell mass, respectively, in Gcg\textsuperscript{ΔNull} mice.

In conclusion, although GLP-1 and glucagon are critical glucoregulatory peptides, most of the mouse models demonstrating their protective or hyperglycemic impact, respectively, with STZ-induced diabetes, are in mouse models that have developmental compensations preventing a strong conclusion to be made about their individual roles. Regardless, the current data contribute to a growing body of literature indicating that it is likely not removal of glucagon alone, or the increase in GLP-1 alone, that reduces glucose levels in response to STZ but a combination of compensatory factors downstream of glucagon signaling ablation that mediates the protective response in the GcgR KO mice.

DATA AVAILABILITY
Data will be made available upon reasonable request.

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DISCLOSURES
D.A.S. has served as a consultant for Metis Therapeutics. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.
AUTHOR CONTRIBUTIONS

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REFERENCES


