Glucagon Acting at the GLP-1 Receptor Contributes to β-Cell Regeneration Induced by Glucagon Receptor Antagonism in Diabetic Mice

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Dysfunction of glucagon-secreting α-cells participates in the progression of diabetes, and glucagon receptor (GCGR) antagonism is regarded as a novel strategy for diabetes therapy. GCGR antagonism upregulates glucagon and glucagon-like peptide 1 (GLP-1) secretion and, notably, promotes β-cell regeneration in diabetic mice. Here, we aimed to clarify the role of GLP-1 receptor (GLP-1R) activated by glucagon and/or GLP-1 in the GCGR antagonism–induced β-cell regeneration. We showed that in *db/db* mice and type 1 diabetic wild-type or Flox/cre mice, GCGR monoclonal antibody (mAb) improved glucose control, upregulated plasma insulin level, and increased β-cell area. Notably, blockage of systemic or pancreatic GLP-1R signaling by exendin 9-39 (Ex9) or *Glp1r* knockout diminished the above effects of GCGR mAb. Furthermore, glucagon-neutralizing antibody (nAb), which prevents activation of GLP-1R by glucagon, also attenuated the GCGR mAb–induced insulinotropic effect and β-cell regeneration. In cultured primary mouse islets isolated from normal mice and *db/db* mice, GCGR mAb action to increase insulin release and to upregulate β-cell–specific marker expression was reduced by a glucagon nAb, by the GLP-1R antagonist Ex9, or by a pancreas-specific *Glp1r* knockout. These findings suggest that activation of GLP-1R by glucagon participates in β-cell regeneration induced by GCGR antagonism in diabetic mice.

Loss of functional β-cell mass contributes to the development of diabetes (1). Glucagon, which is secreted from α-cells and mainly acts on the glucagon receptor (GCGR), has gained increasing attention owing to its counterregulatory function

ARTICLE HIGHLIGHTS

- Glucagon receptor (GCGR) antagonism promotes β-cell regeneration in type 1 and type 2 diabetic mice and in euglycemic nonhuman primates. Glucagon and glucagon-like peptide 1 (GLP-1) can activate the GLP-1 receptor (GLP-1R), and their levels are upregulated following GCGR antagonism.
- We investigated whether GLP-1R activated by glucagon and/or GLP-1 contributed to β-cell regeneration induced by GCGR antagonism.
- We found that blockage of glucagon–GLP-1R signaling attenuated the GCGR monoclonal antibody–induced insulinotropic effect and β-cell regeneration in diabetic mice.
- Our study reveals a novel mechanism of β-cell regeneration and uncovers the communication between α-cells and β-cells in regulating β-cell mass.

on glucose metabolism to insulin (2). Excessive glucagon secretion is common in patients with type 1 and type 2 diabetes (3). Hence, GCGR has been selected as a potential target for diabetes therapy. GCGR antagonism by distinct methods, including gene deletion (4), antisense oligonucleotides (5), small molecule antagonists (6), and antibody (7,8), improves glucose control in animals and humans with type 1 diabetes (T1D) or type 2 diabetes (T2D).

Although liver is the main target of glucagon action, glucagon also acts on β-cells and is responsible for insulin

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secretion (9–13). GCGR antagonism promotes β-cell regeneration (characterized by the increased β-cell mass through β-cell proliferation, β-cell redifferentiation, conversion of α- to β-cells, and β-cell neogenesis from progenitors) in T1D and T2D mice (7,14–16) and in euglycemic nonhuman primates (17). The beneficial effect might be mediated via glucagon-like peptide 1 (GLP-1), because plasma GLP-1 level and intestinal GLP-1 content were greatly elevated by GCGR antagonism (18). However, GLP-1 is immediately inactivated by local gut and systemic ubiquitous expression of dipeptidyl peptidase 4 and is rapidly cleared from circulation by the kidney (19,20). Therefore, it is somewhat difficult for intestinal L-cell–derived GLP-1 to exert effects on β-cells. α-Cells also own the ability to secrete GLP-1 (21,22). Whether α-cell–derived GLP-1 secretion is enhanced by GCGR antagonism and whether it participates in β-cell regeneration remains to be clarified.

GLP-1 exerts its protective effects on β-cells via activation of the GLP-1 receptor (GLP-1R) (20). Notably, glucagon also activates GLP-1R (9,13,23,24). Hyperglucagonemia and an elevated GLP-1 level are common when the glucagon–GCGR signaling pathway is blocked (25,26), making it complicated to clarify which hormone activates GLP-1R and whether the activated GLP-1R is responsible for β-cell regeneration.

In this study, we used REMD 2.59, a human GCGR monoclonal antibody (mAb), to specifically antagonize GCGR and detected its effect on β-cell regeneration in *db/db* mice (a T2D model) and streptozotocin (STZ)-induced T1D mice. First, we verified that glucagon and GLP-1 levels in the circulation and pancreas were elevated by GCGR mAb. Then, we determined whether GLP-1R signaling participated in GCGR mAb–induced β-cell regeneration by using the GLP-1R antagonist exendin 9-39 (Ex9) or global and pancreas-specific *Glp1r*-knockout mice. Next, whether glucagon–GLP-1R signaling took part in the process was investigated by application of the glucagon-neutralizing antibody (nAb) GLU-001, which could bind to glucagon and prevent it binding with GLP-1R under the GCGR mAb treatment condition. In addition, we clarified whether islet-derived GLP-1 was enhanced by GCGR mAb and determined the involvement of glucagon–GLP-1R signaling in GCGR mAb–mediated regulation of β-cell function and phenotype in primary normal and diabetic mouse islets. Our study provides a novel insight into understanding of glucagon–GLP-1R signaling and reveals a new mechanism of GCGR antagonism–induced β-cell regeneration.

RESEARCH DESIGN AND METHODS
Animal Experiments
All animal experiments were approved by the Peking University Animal Care and Use Committee. Male *db/db* mice (8 weeks old) were treated for 4 weeks with IgG, GCGR mAb, Ex9, or GCGR mAb combined with Ex9. GCGR mAb REMD 2.59 (5 mg/kg; REMD Biotherapeutics, Camarillo, CA) and IgG (5 mg/kg, as control) were intraperitoneally injected weekly. Ex9 (50 nmol/kg/day; Bachem, Bubendorf,

Switzerland) or saline was administered by micro-osmotic pumps (ALZET, Cupertino, CA) (27).

To induce the T1D model, male and female global *Glp1r*-knockout ($Glp1r^{-/-}$) mice (Supplementary Fig. 1*A* and *B*) and wild-type (WT) $Glp1r^{+/+}$ littermates, and male pancreas-specific *Glp1r*-knockout ($Glp1r^{pan-/-}$) mice and *Glp1r*-flox or Pdx1-Cre (Flox/cre) littermates (Supplementary Fig. 1*C* and *D*) were injected with STZ (125 mg/kg; MilliporeSigma, St. Louis, MO) at the age of 8–12 weeks. Diabetic mice were treated with 5 mg/kg GCGR mAb or IgG for 4 weeks.

STZ-induced T1D male C57BL/6J mice (12 weeks old) were treated with 5 mg/kg IgG or GCGR mAb with 4 mg/kg A-TNP (as control of glucagon nAb) or glucagon nAb GLU-001 (Novo Nordisk A/S, Bagsvaerd, Denmark) for 4 weeks. GLU-001 and A-TNP were injected intraperitoneally daily (28).

Glucose Monitoring and Glucose Tolerance Test
Blood glucose level in samples from a tail vein was monitored by OneTouch Ultra glucometer (LifeScan, Milpitas, CA). Glucose tolerance tests were performed after overnight fasting. Blood glucose levels were measured at baseline, 30, 60, and 120 min after intraperitoneal injection of 2 g/kg glucose.

Immunofluorescent Staining and Analysis
Pancreatic tissues were fixed with 10% (v/v) neutral-buffered formalin and embedded in paraffin. Sections (5-μm-thick) were dewaxed, hydrated, blocked with goat serum, and incubated with primary antibodies at 4°C overnight and with secondary antibodies for 1 h at room temperature, followed by nuclear staining with DAPI. Fluorescence was imaged using a Leica TCS SP8 confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany) or automatic digital slide scanner (Pannoramic MIDI, 3DHISTECH, Budapest, Hungary). The antibodies are summarized in Supplementary Table 1.

For cell quantification, three to four sections (which covered the whole pancreas) per pancreas from three mice per group were imaged. The area of positive-staining cells was analyzed by Fiji software (National Institutes of Health, Bethesda, MD) (29). Islet clusters with ≤10 cells are defined as small islets, indicating islet neogenesis (30).

Primary Mouse Islet Intervention
Primary mouse islets were isolated as previously reported (31). Islets from 8-week-old male normal C57BL/6J and *db/db* mice were treated with 1,000 nmol/L GCGR mAb or IgG in the absence or presence of Ex9 (200 nmol/L) or glucagon nAb (10 mg/L) for 24 h in a high glucose (30 mmol/L) condition. Islets from 8-week-old male $Glp1r^{pan-/-}$ mice were treated with 1,000 nmol/L GCGR mAb or IgG for 24 h.

Hormone Measurements
ELISA kits specific for insulin, C-peptide, glucagon, and active GLP-1 are summarized in Supplementary Table 1. The hormone levels in pancreatic lysates and islet culture
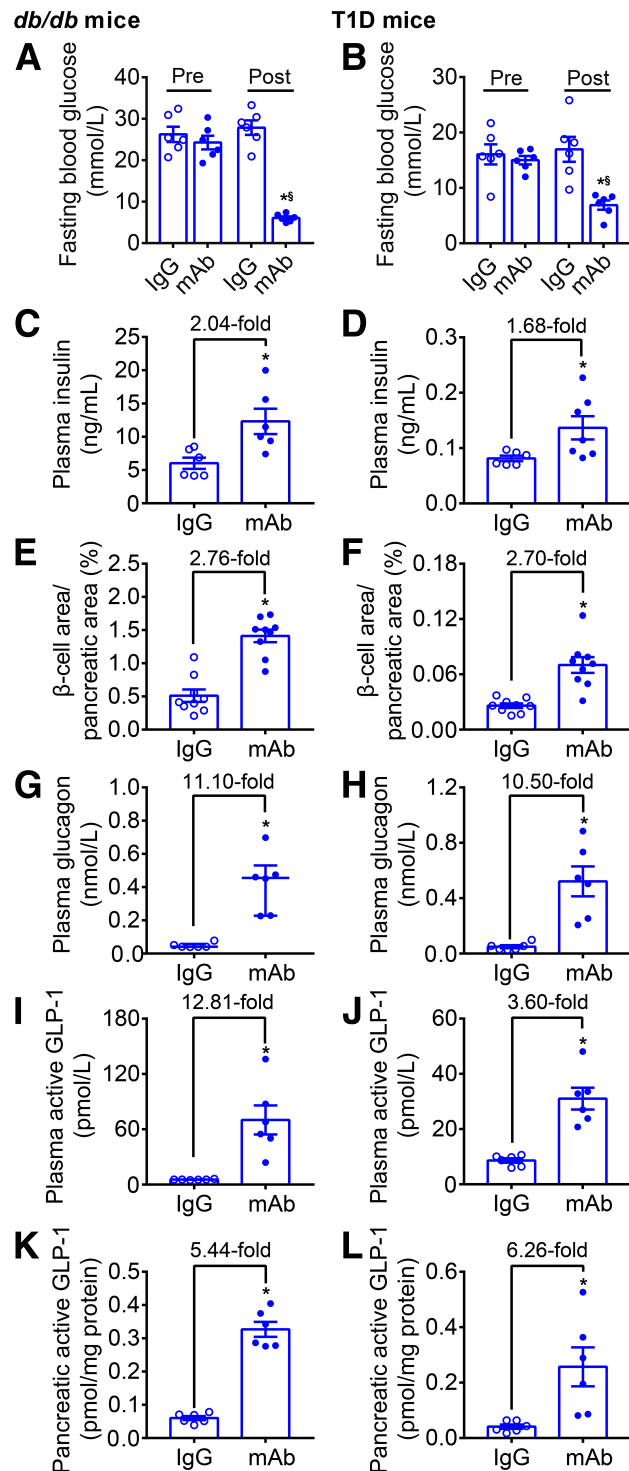
Figure 1—GCGR mAb promotes β-cell regeneration and upregulates plasma and pancreatic glucagon and GLP-1 levels in T2D and T1D mice. Male *db/db* mice (8 weeks) were used as a T2D model. Male C57BL/6J mice (12 weeks) were injected with STZ to induce a T1D model. Mice were treated weekly with IgG (5 mg/kg, as control) or GCGR mAb (5 mg/kg) for 4 weeks. *A, C, E, G, I,* and *K*: Parameters in *db/db* mice. *B, D, F, H, J,* and *L*: Parameters in T1D mice. *A* and *B*: Fasting blood glucose. *C* and *D*: Plasma insulin (*n* = 6 mice/group). *E* and *F*: Quantification of the β-cell area per pancreatic section (*n* = 3 sections/mouse × 3 mice/group). *G* and *H*: Plasma glucagon. *I* and *J*: Plasma active GLP-1. *K* and *L*: Pancreatic active GLP-1 (*n* = 6 mice/group). Data are expressed as the mean ± SEM or median (interquartile range). Statistical analysis was performed by two-way ANOVA, followed by the Bonferroni multiple comparisons test in *A* and *B*, by unpaired Student *t* test in *C*−*F* and *H*−*L*, or by Mann-Whitney test in *G*. *$P < 0.05$ vs. IgG control; §$P < 0.05$ vs. pretreatment in the same group.

supernatants were normalized to the protein content of pancreatic lysates or cultured islets, respectively.

RNA Extraction, Reverse Transcription, and Quantitative PCR
Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific, Waltham, MA), and cDNA was synthesized with a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). Quantitative PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). Relative gene expression was normalized to *Actb* and calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are summarized in Supplementary Table 2.

Statistical Analysis
If data were Gaussian distributed, they are presented as mean ± SEM and were analyzed by one-way or two-way ANOVA, followed by the Dunnett T3 multiple comparisons test, Tukey multiple comparisons test, or Bonferroni multiple comparisons test among three or more groups, or unpaired Student *t* test (two-tailed) between two groups, as appropriate. If data were not Gaussian distributed, they are presented as median (interquartile range) and were analyzed by Kruskal-Wallis test, followed by the Dunn multiple comparisons test among three or more groups, or the Mann-Whitney test (two-tailed) between two groups, as appropriate. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA).

Data and Resource Availability
The data sets and resources generated and analyzed during the current study are available from the corresponding authors upon reasonable request.

RESULTS

GCGR Antagonism Promotes β-Cell Regeneration and Upregulates Plasma and Pancreatic Glucagon and GLP-1 Levels in T2D and T1D Mice
In *db/db* mice and T1D mice, no significant difference was found between GCGR mAb and IgG groups in body weight during the 4-week treatment (Supplementary Fig. 2*A* and *B*). Compared with baseline or IgG control, GCGR mAb significantly lowered the fasting and random blood glucose levels in two diabetic mice (Fig. 1*A* and *B* and Supplementary Fig. 2*C* and *D*). Plasma insulin level was increased 2.04- and 1.68-fold by GCGR mAb in *db/db* mice and T1D mice, respectively (Fig. 1*C* and *D*). An approximately threefold increase of β-cell area was observed in the GCGR mAb group
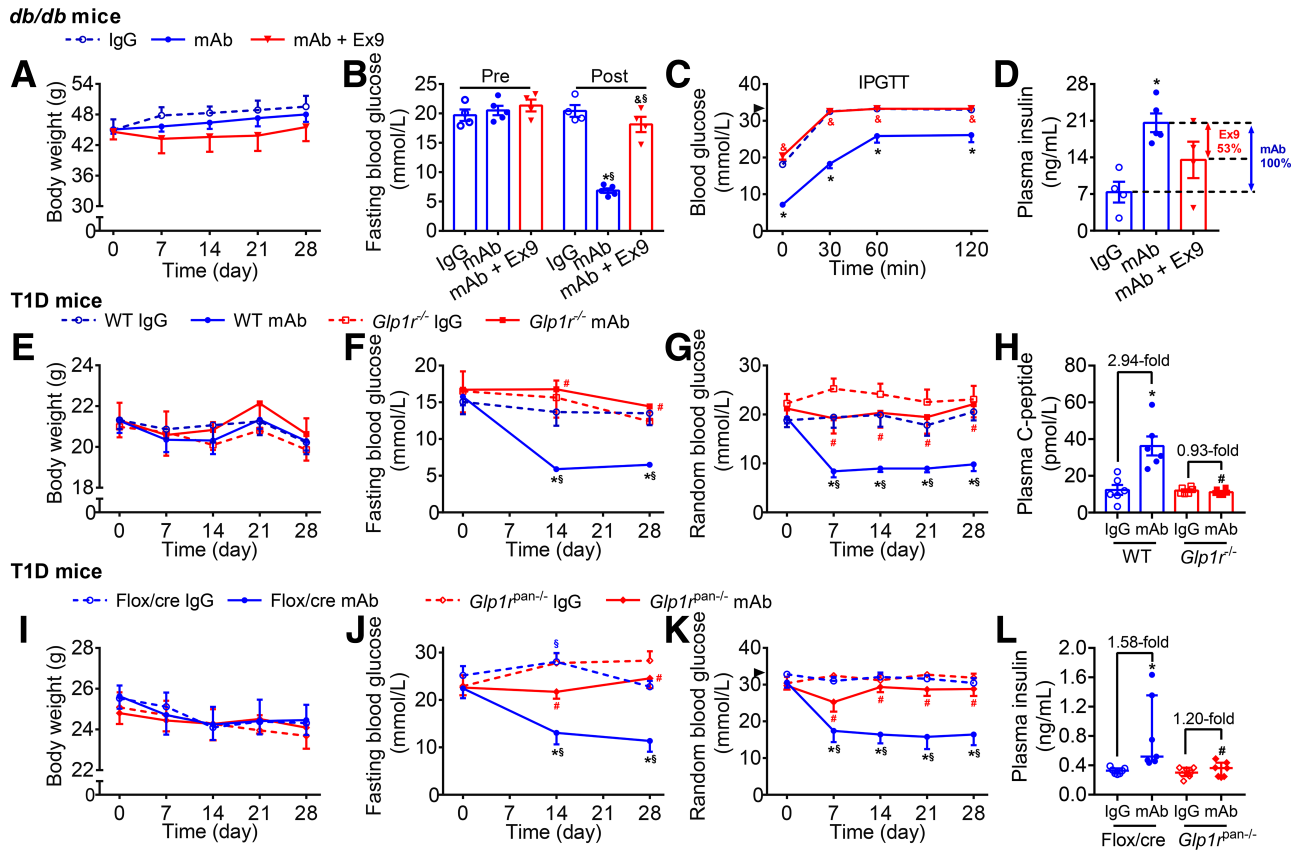
Figure 2—Systemic or pancreatic GLP-1R signaling participates in glucose-lowering and insulinotropic effects induced by GCGR antagonism in T2D and T1D mice. *A–D*: Parameters in *db/db* mice. Male *db/db* mice (8 weeks) were treated with IgG (5 mg/kg/week, *n* = 4), GCGR mAb (5 mg/kg/week, *n* = 5), or GCGR mAb combined with Ex9 (50 nmol/kg/day, *n* = 4) for 4 weeks. *E–H*: Parameters in T1D $Glp1r^{-/-}$ mice and WT $Glp1r^{+/+}$ littermates. Male and female $Glp1r^{-/-}$ mice and WT littermates were injected with STZ to induce T1D models at the age of 8–12 weeks and treated weekly with IgG (5 mg/kg) or GCGR mAb (5 mg/kg) for 4 weeks (*n* = 6 mice/group). *I–L*: Parameters in T1D $Glp1r^{pan-/-}$ mice and Flox/cre littermates. Male $Glp1r^{pan-/-}$ mice and Flox/cre littermates were injected with STZ to induce T1D models at the age of 8–12 weeks and treated weekly with IgG (5 mg/kg, as control) or GCGR mAb (5 mg/kg) for 4 weeks (*n* = 7 mice/group). *A*, *E*, and *I*: Body weight. *B*, *F*, and *J*: Fasting blood glucose. Blood glucose during intraperitoneal glucose tolerance test (IPGTT) (*C*) or random blood glucose (*G* and *K*). The arrowheads in *C* and *K* indicate the upper detection limit (33.3 mmol/L) of the glucometer. Plasma insulin (*D* and *L*) or C-peptide (*H*). Data are expressed as the mean ± SEM or median (interquartile range). Statistical analysis was performed by two-way ANOVA, followed by the Bonferroni multiple comparisons test in *A–C*, the Tukey multiple comparisons test in *E–G* and *I–K*, or by one-way ANOVA, followed by the Bonferroni multiple comparisons test in *D*, the Tukey multiple comparisons test in *H*, or by the Kruskal-Wallis test, followed by the Dunn multiple comparisons test in *L*. *$P < 0.05$ vs. IgG control in the same genotype of mice; §$P < 0.05$ vs. pretreatment in the same group; &$P < 0.05$ vs. GCGR mAb in *db/db* mice; #$P < 0.05$ vs. WT or Flox/cre littermates on the same treatment.

in two diabetic mice (Fig. 1*E* and *F* and Supplementary Fig. 3), suggestive of β-cell regeneration. GCGR mAb expanded α-cell area (Supplementary Figs. 2*E* and *F* and 3), and increased plasma glucagon (~11-fold), plasma GLP-1 (*db/db*: 12.81-fold; T1D: 3.60-fold), and pancreatic GLP-1 (~5- to 6-fold) levels in two diabetic models (Fig. 1*G–L*), indicative of changes of α-cells in number and function. These results suggested that elevated glucagon and GLP-1 levels might be involved in GCGR mAb–induced β-cell regeneration.

Systemic or Pancreatic GLP-1R Signaling Participates in Glucose-Lowering and Insulinotropic Effects Induced by GCGR Antagonism in T2D and T1D Mice

We used Ex9, a specific GLP-1R antagonist without affecting GCGR (9), to investigate the involvement of GLP-1R

in GCGR mAb–mediated metabolic effects. Since *db/db* mice treated with Ex9 alone died rapidly owing to uncontrollable hyperglycemia, this group was excluded. Body weight was comparable among IgG, GCGR mAb, and GCGR mAb + Ex9 groups (Fig. 2*A*). Four-week treatment of GCGR mAb significantly lowered fasting glucose and improved glucose tolerance compared with IgG treatment, and Ex9 addition abolished these effects (Fig. 2*B* and *C*). Plasma insulin level was higher in the GCGR mAb group than in the IgG group and decreased by 53% in the GCGR mAb + Ex9 group versus the GCGR mAb group (Fig. 2*D*).

STZ-induced T1D models of $Glp1r^{-/-}$ mice and WT mice were treated with GCGR mAb or IgG. Mice in the four groups had similar body weight (Fig. 2*E*). Compared with IgG control, GCGR mAb significantly decreased the

fasting and random blood glucose levels in WT mice but not in $Glp1r^{-/-}$ mice (Fig. 2F and G). GCGR mAb remarkably increased the plasma C-peptide level in WT mice, but this effect disappeared in $Glp1r^{-/-}$ mice (Fig. 2H).

GLP-1R is expressed not only in pancreata but also in other tissues, such as brain, where GLP-1R signaling is involved in control of food intake and thus influences glucose metabolism (20). Therefore, we constructed $Glp1r^{pan-/-}$ mice to detect the effect of pancreatic GLP-1R. STZ-induced T1D models of $Glp1r^{pan-/-}$ mice and Flox/cre littermates were treated with GCGR mAb or IgG. Results showed that there was no difference in body weight among the four groups (Fig. 2I). Compared with IgG control, GCGR mAb remarkably lowered the fasting and random blood glucose levels in Flox/cre mice but not in $Glp1r^{pan-/-}$ mice (Fig. 2J and K). GCGR mAb significantly increased plasma insulin level in Flox/cre mice, and this effect disappeared in $Glp1r^{pan-/-}$ mice (Fig. 2L).

Collectively, these results suggested that systemic and pancreas-specific GLP-1R blockage diminished the GCGR antagonism–induced glucose-lowering and insulinotropic effects.

Systemic or Pancreatic GLP-1R Signaling Contributes to β-Cell Mass Expansion Induced by GCGR Antagonism in T2D and T1D Mice

In *db/db* mice, GCGR mAb remarkably increased the number of islets, including small islets (≤10 cells) and large islets, islet area, α-cell area, and β-cell area, and Ex9 addition attenuated these effects, with 94% decrease in β-cell area (Fig. 3A–E and Supplementary Fig. 4), indicating that pharmacological blockage of systemic GLP-1R signaling diminished GCGR antagonism—induced islet regeneration.

Similarly, GCGR mAb significantly increased the number of small islets and large islets, islet area, α-cell area, and β-cell area in T1D WT mice (Fig. 3F–J and Supplementary Fig. 5). In T1D $Glp1r^{-/-}$ mice, GCGR mAb still increased islet number, islet area, and α-cell area (Fig. 3F–H). However, islet area was smaller in $Glp1r^{-/-}$ mice than in WT mice after GCGR mAb treatment (Fig. 3G). Notably, GCGR mAb no longer increased β-cell area in T1D $Glp1r^{-/-}$ mice (Fig. 3I). These results suggested that genetic elimination of systemic GLP-1R signaling abolished GCGR antagonism—induced β-cell regeneration but had little effect on α-cell hyperplasia.

In T1D Flox/cre mice and $Glp1r^{pan-/-}$ mice, GCGR mAb remarkably increased the number of small islets and large islets, islet area, and α-cell area (Fig. 3K–M and O and Supplementary Fig. 6). However, the small islet number was lower in $Glp1r^{pan-/-}$ mice than in Flox/cre mice after GCGR mAb treatment (Fig. 3K). Again, GCGR mAb could not enlarge β-cell area in T1D $Glp1r^{pan-/-}$ mice (Fig. 3N). These results indicated that genetic elimination of pancreatic GLP-1R signaling abolished GCGR antagonism—induced β-cell regeneration, while it did not affect α-cell hyperplasia.

Systemic or Pancreatic GLP-1R Signaling Is Involved in β-Cell Self-Replication, α- to β-Cell Transdifferentiation, and β-Cell Neogenesis Triggered by GCGR Antagonism in T1D Mice

GCGR mAb promoted β-cell regeneration through β-cell self-replication, α- to β-cell transdifferentiation, and β-cell neogenesis in diabetic mice, as indicated by our previous lineage-tracing studies (14,15,32). Here, we detected the effect of GLP-1R signaling on these regeneration paths. In T1D WT or Flox/cre littermates, GCGR mAb significantly increased the ratio of $BrdU^+insulin^+$ cells (the proliferating β-cells) and $glucagon^+insulin^+$ cells (indicating β-cells transdifferentiated from α-cells), and β-cell number in small islets (representing β-cell neogenesis [30]) compared with IgG control. In T1D $Glp1r^{-/-}$ or $Glp1r^{pan-/-}$ mice, GCGR mAb had no such effects (Supplementary Figs. 7 and 8). These results suggested that GCGR antagonism boosted β-cell regeneration through β-cell self-replication, α- to β-cell transdifferentiation, and β-cell neogenesis in T1D mice, which was mediated via GLP-1R signaling.

Activation of GLP-1R by Glucagon Is Involved in β-Cell Regeneration Induced by GCGR Antagonism in T1D Mice

In addition to GLP-1, glucagon has been recognized as another endogenous ligand of GLP-1R (9,23,24). Since GCGR mAb upregulated the levels of glucagon and GLP-1 in the circulation and pancreas (Fig. 1G–L), we tried to clarify the relative contribution of glucagon and GLP-1 to GLP-1R activation. A specific glucagon nAb was used to prevent glucagon binding to GCGR and GLP-1R (33,34). In the context of GCGR mAb treatment, glucagon nAb could only block the binding of glucagon with GLP-1R since GCGR was already occupied by GCGR mAb, a more potent competitor than glucagon (35) (Fig. 4A).

In T1D C57BL/6J mice, there was no significant difference in body weight among the four groups (Fig. 4B). The fasting and random blood glucose levels in the GCGR mAb group and the GCGR mAb + glucagon nAb group significantly decreased from baseline and were lower than those in the control group and glucagon nAb group after treatment (Fig. 4C and D). The glucose levels were comparable between GCGR mAb and GCGR mAb + glucagon nAb groups (Fig. 4C and D), suggesting that glucagon–GLP-1R signaling did not affect the glucose-lowing effect of GCGR mAb.

Plasma insulin level in the GCGR mAb group was higher than that in the control group, and this effect was attenuated by 67% when adding glucagon nAb on GCGR mAb treatment (Fig. 4E). Plasma and pancreatic GLP-1 levels were higher in the GCGR mAb group than in the control group, while they were comparable between the GCGR mAb and GCGR mAb + glucagon nAb groups (Fig. 4F and G), thereby excluding the possibility of compensation of GLP-1 production.

Compared with the control group, islet number, islet area, and α-cell area were significantly increased in the GCGR mAb group, and these parameters showed no difference between the GCGR mAb and GCGR mAb + glucagon
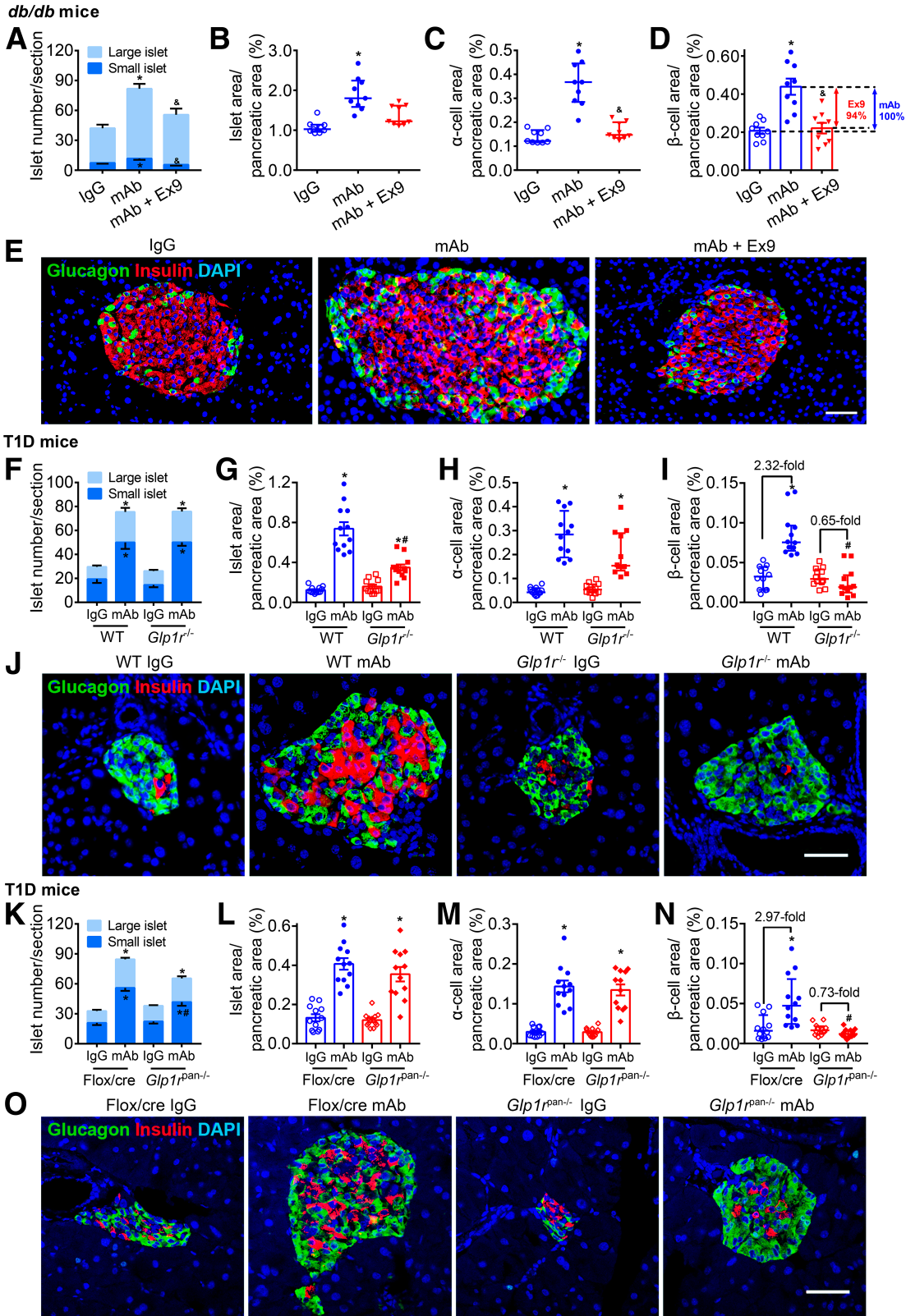
Figure 3—Systemic or pancreatic GLP-1R signaling contributes to β-cell mass expansion induced by GCGR antagonism in T2D and T1D mice. *A–E*: Parameters in *db/db* mice. Male *db/db* mice (8 weeks) were treated with IgG (5 mg/kg/week), GCGR mAb (5 mg/kg/week), or GCGR mAb combined with Ex9 (50 nmol/kg/day) for 4 weeks. *F–J*: Parameters in T1D *Glp1r*^{−/−} mice and WT *Glp1r*^{+/+} littermates. Male and female *Glp1r*^{−/−} mice and WT littermates were injected with STZ to induce T1D models at the age of 8–12 weeks and treated weekly with IgG (5 mg/kg) or GCGR mAb (5 mg/kg) for 4 weeks. *K–O*: Parameters in T1D *Glp1r*[pan−/−] mice and Flox/cre littermates. Male *Glp1r*[pan−/−] mice and Flox/cre littermates were injected with STZ to induce T1D models at the age of 8−12 weeks and treated weekly with

nAb groups (Fig. 4*H–J* and *L* and Supplementary Fig. 9). Notably, β-cell area was larger in the GCGR mAb group than in the control group, and this effect was diminished by 35% after adding glucagon nAb (Fig. 4*K* and *L*). Moreover, glucagon nAb alone appeared to increase α-cell area ($P = 0.075$), but showed little effect on islet number, islet area, and β-cell area (Fig. 4*H–L*). These results suggested that glucagon–GLP-1R signaling at least partly contributed to β-cell regeneration but not α-cell hyperplasia induced by GCGR antagonism.

Glucagon–GLP-1R Signaling Takes Part in Promotion of Insulin Secretion Evoked by GCGR Antagonism In Vitro

GLP-1 is mainly synthesized in and secreted from intestinal L cells (20). Recently, it has been demonstrated that α-cells–derived GLP-1 also plays an important role in glucose homeostasis, especially under metabolic stress (24,36–38). To determine the role of islet-derived GLP-1 in GCGR mAb–mediated regulation of β-cell identity, islets isolated from normal mice, *db/db* mice, and $Glp1r^{pan-/-}$ mice were used. Results showed that compared with IgG control, GCGR mAb significantly increased insulin, glucagon, and GLP-1 release in normal and diabetic mouse islets (Fig. 5). The above effects of GCGR mAb disappeared after blockage of GLP-1R by Ex9 or *Glp1r* knockout and after elimination of glucagon–GLP-1R signaling by glucagon nAb (Fig. 5 and Supplementary Fig. 10*A–C*). These results indicated that glucagon–GLP-1R signaling participated in the insulinotropic effect of GCGR mAb in vitro. By comparing insulin release levels among GCGR mAb groups, with or without other cotreatments, we inferred that glucagon and GLP-1 contributed 64% and 36% to the GCGR mAb–induced insulinotropic effect in normal mouse islets, respectively (Fig. 5*A*). In diabetic mouse islets, the relative contributions changed to 50% and 50%, respectively (Fig. 5*B*). These results suggested that both glucagon and GLP-1 activated GLP-1R, which contributed to the GCGR antagonism–induced insulinotropic effect in vitro.

Activation of GLP-1R by Glucagon Participates in Regulation of β-Cell Identity by GCGR Antagonism In Vitro

β-Cell identity was further evaluated by detecting the expression of genes related to insulin synthesis and processing (*Ins1*, *Ins2*, *Pcsk1*, and *Pcsk2*), glucose transporter (*Slc2a2*), key transcription factors (*Hnf4a*, *Nkx6-1*, *Neurod1*,

and *Pdx1*) and mature β-cell markers (*Mafa* and *Ucn3*). Results showed that GCGR mAb upregulated the mRNA levels of *Ins1*, *Pcsk1*, *Slc2a2*, *Nkx6-1*, and *Ucn3* in normal mouse islets, and *Ins1*, *Ins2*, *Pcsk1*, *Slc2a2*, and *Ucn3* in diabetic mouse islets (Fig. 6*A* and *B*). Blockage of GLP-1R by Ex9 or *Glp1r* knockout and elimination of glucagon–GLP-1R signaling by glucagon nAb attenuated the effects of GCGR mAb (Fig. 6*C–F* and Supplementary Fig. 10*D*). These results suggested that GCGR mAb influenced β-cell identity in primary mouse islets, which was mediated via glucagon–GLP-1R signaling.

DISCUSSION

In this study, we showed that antagonistic GCGR mAb increased β-cell area through β-cell proliferation, α- to β-cell transdifferentiation, and β-cell neogenesis in diabetic mice, and upregulated insulin secretion and the expression of β-cell specific markers in cultured mouse islets, suggesting that glucagon could influence β-cell mass and identity. Besides, the levels of glucagon and GLP-1 in circulation and the pancreas were greatly elevated by GCGR mAb. The GLP-1R antagonist Ex9 and global and pancreatic *Glp1r* knockout attenuated the effects of GCGR mAb on glucose metabolism and β-cell area and identity in T2D and T1D mice and in primary mouse islets. By using glucagon nAb to block the binding of glucagon with GLP-1R, we found that glucagon–GLP-1R signaling was responsible for the increased β-cell area induced by the GCGR mAb in T1D mice and involved in regulation of β-cell identity by GCGR antagonism in cultured mouse islets.

GCGR antagonism displays an efficacious glucose control in T1D or T2D animals and humans (4–8). Our series of studies showed that GCGR antagonism promoted β-cell regeneration in T1D and T2D mice (14–16,32), with GLP-1R activation as one of underlying mechanisms identified in this study. Thirteen-week treatment of GCGR mAb increased β-cell mass and the number of Nkx6.1$^+$/Pdx1$^+$ α-cells in cynomolgus monkeys (17), suggestive of β-cell regeneration in nonhuman primates. Although its effect on β-cell mass in humans is absent, GCGR antagonism was reported to increase plasma insulin and C-peptide levels in T2D patients and reduce daily insulin use in T1D patients (5,8). In T1D mice with transplanted human islets, GCGR mAb increased human insulin level even after a 2-week washout (7), indicating that GCGR antagonism might promote β-cell regeneration in humans. Importantly, compared with rodent islets, human islets contain more α-cells

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IgG (5 mg/kg, as control) or GCGR mAb (5 mg/kg) for 4 weeks. *A*, *F*, and *K*: Quantification of the islet number per pancreatic section. An islet with a cell number ≤10 is defined as a small islet and the others as a large islet. *B*, *G*, and *L*: Quantification of the islet area per pancreatic section. *C*, *H*, and *M*: Quantification of the α-cell area per pancreatic section. *D*, *I*, and *N*: Quantification of the β-cell area per pancreatic section (*n* = 3–4 sections/mouse × 3 mice/group). *E*, *J*, and *O*: Representative images of an islet immunostained for glucagon, insulin, and DAPI. Scale bar = 50 μm. Data are expressed as the mean ± SEM or median (interquartile range). Statistical analysis was performed by one-way ANOVA, followed by the Tukey multiple comparisons test in *A* and *K*, the Dunnett T3 multiple comparisons test in *D*, *F*, *G*, *L*, and *M*, or by the Kruskal-Wallis test, followed by the Dunn multiple comparisons test in *B*, *C*, *H*, *I*, and *N*. *$P < 0.05$ vs. IgG control in the same genotype of mice; &$P < 0.05$ vs. GCGR mAb in *db/db* mice; #$P < 0.05$ vs. WT or Flox/cre littermates on the same treatment.
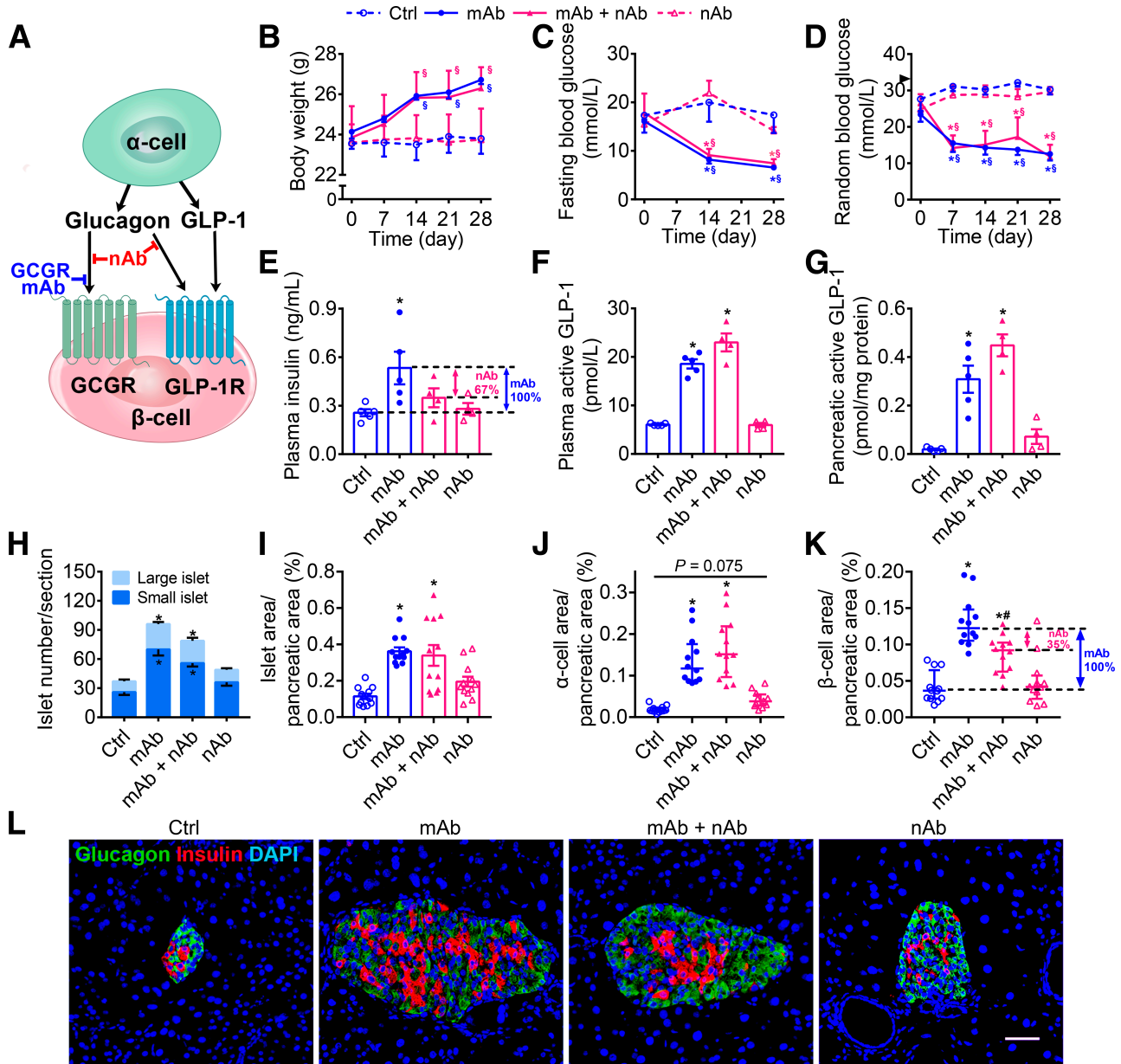
Figure 4—Activation of GLP-1R by glucagon is involved in β-cell regeneration induced by GCGR antagonism in T1D mice. Male C57BL/6J mice (12 weeks) were injected with STZ to induce a T1D model. The diabetic mice were divided into four groups and given the 4-week treatments as follows: *1*) control (Ctrl) group (*n* = 5), injected with IgG (5 mg/kg/week, as control of GCGR mAb) and A-TNP (4 mg/kg/day, as control of glucagon nAb); *2*) GCGR mAb group (*n* = 5), received injection of GCGR mAb (5 mg/kg/week) and A-TNP; *3*) GCGR mAb + glucagon nAb group (*n* = 4), injected with GCGR mAb and glucagon nAb (4 mg/kg/day); *4*) glucagon nAb group (*n* = 4), received injection of IgG and glucagon nAb. *A*: Schematic diagram about the action of GCGR mAb and glucagon nAb. GCGR mAb could bind to GCGR and act as a competitive antagonist against glucagon, while glucagon nAb could prevent glucagon binding to GCGR and GLP-1R. In the context of GCGR mAb treatment, glucagon nAb could only block the binding of glucagon with GLP-1R since GCGR was already occupied by GCGR mAb, a more potent competitor than glucagon. *B*: Body weight. *C*: Fasting blood glucose. *D*: Random blood glucose. The arrowhead indicates the upper detection limit (33.3 mmol/L) of the glucometer. *E*: Plasma insulin. *F*: Plasma active GLP-1. *G*: Pancreatic active GLP-1. *H*: Quantification of the islet number per pancreatic section. An islet containing a cell number ≤10 is defined as a small islet and the others as a large islet. Quantification of the islet area (*I*), α-cell area (*J*), and β-cell area (*K*) per pancreatic section (*n* = 4 section/mouse × 3 mice/group). *L*: Representative images of an islet immunostained for glucagon, insulin, and DAPI. Scale bar = 50 μm. Data are expressed as the mean ± SEM or median (interquartile range). Statistical analysis was performed by two-way ANOVA, followed by the Bonferroni multiple comparisons test in *B*–*D*, by one-way ANOVA, followed by the Bonferroni multiple comparisons test in *E*, the Dunnett T3 multiple comparisons test in *F*–*I*, or by the Kruskal-Wallis test, followed by the Dunn multiple comparisons test in *J* and *K*. *$P < 0.05$ vs. Ctrl group; §$P < 0.05$ vs. pretreatment in the same group; #$P < 0.05$ vs. GCGR mAb group.
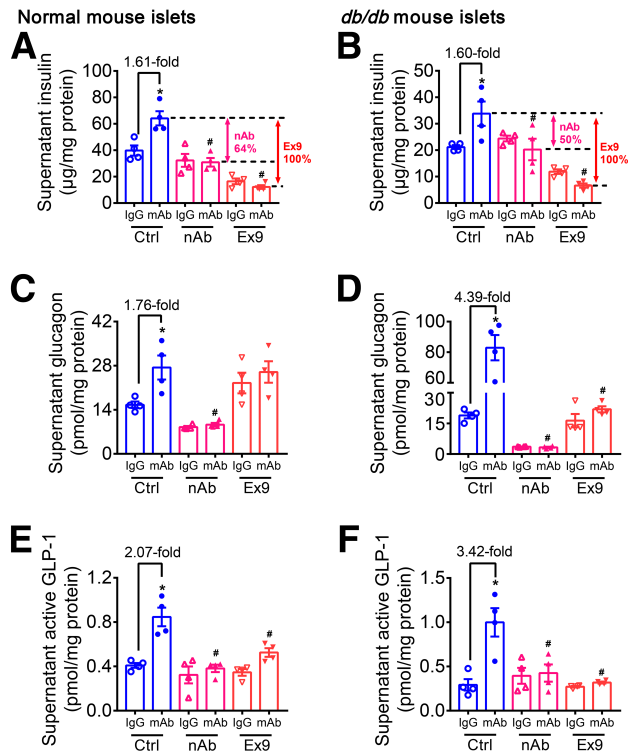
Figure 5—Glucagon–GLP-1R signaling takes part in promotion of insulin secretion evoked by GCGR antagonism in vitro. Primary mouse islets were isolated from 8-week-old male normal mice and *db/db* mice and cultured with 1,000 nmol/L GCGR mAb or IgG in the absence or presence of Ex9 (200 nmol/L) or glucagon nAb (10 mg/L) for 24 h in a high glucose (30 mmol/L) condition. *A*, *C*, and *E*: Parameters in normal mouse islets. *B*, *D*, and *F*: Parameters in *db/db* mouse islets. Ctrl, PBS control. *A* and *B*: Supernatant insulin level. *C* and *D*: Supernatant glucagon level. *E* and *F*: Supernatant active GLP-1 level (*n* = 4). Data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by the Tukey multiple comparisons test. *$P < 0.05$ vs. IgG control on the same cotreatment; #$P < 0.05$ vs. GCGR mAb on the control cotreatment.

(especially GLP-1–secreting α-cells), which are adjoining to β-cells instead of surrounding β-cells, making communication of α-cells with β-cells more convenient (22,39). Moreover, high-dose GGGR antibody upregulated serum glucagon and GLP-1 levels in healthy people (40). These observations suggest that α-cells may play more important roles in regulation of β-cell mass and function by GCGR antagonism in humans.

Glucagon secreted from α-cells might have a direct effect on β-cell mass and identity. Multiple studies have shown that exogenous glucagon input increases insulin secretion from rodent and human β-cells (9,41). Similarly, intraislet glucagon is needed for adequate insulin secretion in vivo (9,10,42). However, whether glucagon can affect β-cell regeneration remains unclear. Coculture of α-TC1.9 cells (an α-cell line) with Min6 cells (a β-cell line) promoted β-cell proliferation (43). Impaired β-cell formation induced by *Gcg* knockdown could be restored by infusion of glucagon or GLP-1 analog in zebrafish, suggesting

that the peptides encoded by *Gcg* were required for β-cell regeneration (44). In this study, we found that glucagon and GLP-1 levels in plasma and pancreas were greatly elevated after GCGR mAb treatment. Therefore, we supposed that elevated levels of glucagon and GLP-1 participated in GCGR antagonism–induced β-cell regeneration.

Multiple lines of evidence have proved that GLP-1R activation enhances insulin secretion and improves glucose control (20). Notably, GLP-1R activation promotes β-cell regeneration in diabetic mice and in cultured human islets (45,46). GCGR antagonist– or *Gcgr* knockout–mediated improvement in glucose control is dependent on functional pancreatic GLP-1R (26,47). However, the effect of GLP-1R on β-cell regeneration has not yet been investigated. Our recent report showed that the combination of GCGR mAb and liraglutide (a GLP-1R agonist) could not further increase β-cell area compared with GCGR mAb alone (48), suggesting that GCGR mAb itself already activated pancreatic GLP-1R sufficiently. By using the GLP-1R antagonist Ex9 and global and pancreatic *Glp1r*-knockout mice, our studies demonstrated that GLP-1R signaling contributed to GCGR antagonism–induced β-cell regeneration.

Classically, GLP-1R is thought to be activated by GLP-1. Recently, glucagon has been identified as a GCGR and GLP-1R dual agonist (9,23,24). In isolated human islets or perfused rodent pancreata, blockage of either GCGR or GLP-1R impaired exogenous glucagon-stimulated insulin secretion, and simultaneous antagonism of these two receptors abolished insulin output (9,24), albeit Ex9 only had a tendency to reduce glucagon-stimulated insulin secretion in some reports (49). Similarly, exogenous glucagon given under fed conditions robustly stimulated insulin secretion. Glucagon failed to induce meaningful insulin secretion in β-cell–specific *Glp1r*-knockout mice, while it promoted insulin secretion in β-cell–specific *Gcgr*-knockout mice, demonstrating that β-cell GLP-1R and GCGR are indispensable and dispensable for glucagon-induced insulin secretion, respectively (10). Consistently, we found that plasma insulin level and β-cell area were increased under the hyperglucagonemic state induced by GCGR mAb and that the insulinotropic effect of GCGR mAb was attenuated or even disappeared when glucagon–GLP-1R signaling was blocked in diabetic mice and in cultured mouse islets.

Since GLP-1R can be activated by glucagon and GLP-1 (9,23,24) and these two hormones were upregulated by GCGR antagonism (25,26), figuring out which one is responsible for GLP-1R activation is difficult. Traditional hormone gene deletion is not suitable owing to the same encoding gene *Gcg*. GLP-1R antagonist Ex9 is unavailable because it antagonizes activation of GLP-1R by both glucagon and GLP-1 (9). Fortunately, we got a specific glucagon nAb that prevented glucagon binding with GLP-1R and thus distinguished glucagon–GLP-1R signaling and GLP-1–GLP-1R signaling. We found that glucagon–GLP-1R signaling at least partly participated in the insulinotropic effect and β-cell regeneration induced by GCGR mAb. Of
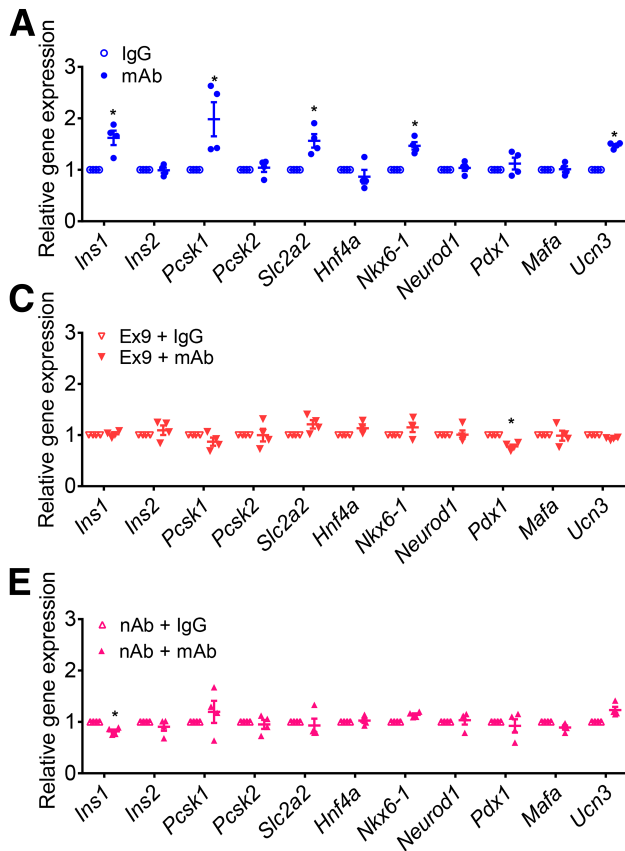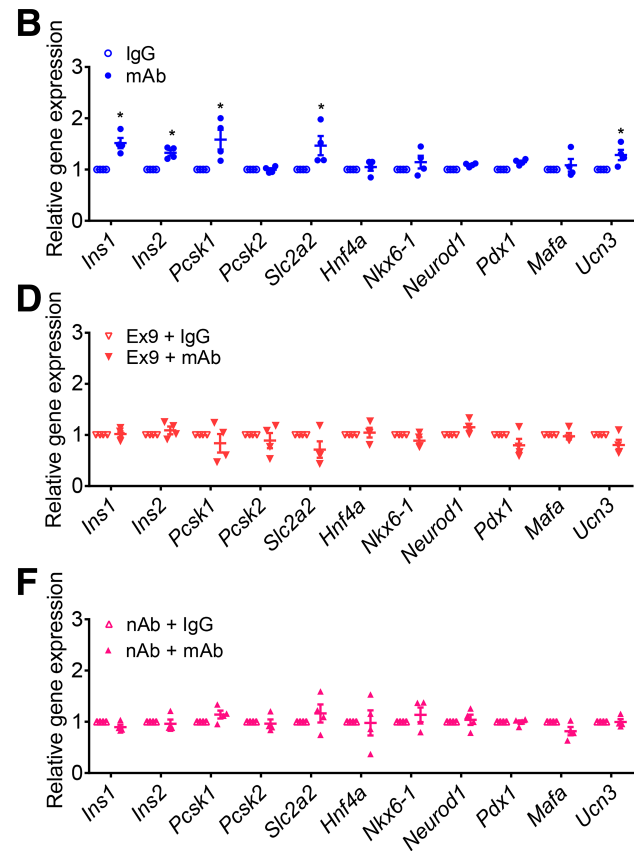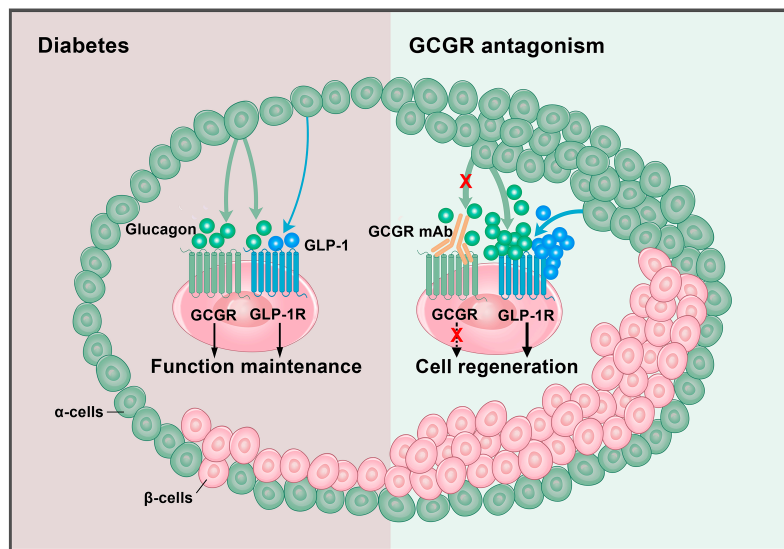
Figure 6—Activation of GLP-1R by glucagon participates in regulation of β-cell identity by GCGR antagonism in vitro and the working model for this study. Primary mouse islets were isolated from 8-week-old male normal mice and *db/db* mice and cultured with 1,000 nmol/L GCGR mAb or IgG in the absence or presence of Ex9 (200 nmol/L) or glucagon nAb (10 mg/L) for 24 h in a high glucose (30 mmol/L) condition. *A*, *C*, and *E*: Relative mRNA levels in normal mouse islets. *B*, *D*, and *F*: Relative mRNA levels in *db/db* mouse islets ($n = 4$). Data are expressed as the mean ± SEM. Statistical analysis was performed by unpaired Student t test. *$P < 0.05$ vs. IgG control. *G*: Working model for the involvement of glucagon–GLP-1R signaling in β-cell regeneration induced by GCGR antagonism. In diabetic mice, α-cell–derived glucagon and GLP-1 act on β-cell GCGR and GLP-1R to maintain β-cell function. GCGR antagonism upregulates α-cell–derived glucagon and GLP-1 production. High concentration of glucagon, together with elevated GLP-1 level, can activate GLP-1R on islet cells (especially β-cells) and thus promote β-cell regeneration.

course, involvement of GLP-1–GLP-1R signaling could not be excluded because the GCGR mAb–mediated increment of the β-cell area was only partially reversed by glucagon nAb. Therefore, both glucagon and GLP-1 are involved in GCGR mAb–induced β-cell regeneration. To our knowledge, this is the first study to explore the contribution of glucagon–GLP-1R signaling to β-cell regeneration.

Our study has some limitations. First, involvement of GLP-1–GLP-1R signaling was not studied directly due to absence of available tools. Maybe using the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) system to accurately target the exon encoding GLP-1 instead of deleting full-length *Gcg* gene is feasible (50).

Second, expression of *Glp1r* was eliminated not only in β-cells but also in other pancreatic cells in *Glp1r*^pan−/− mice, and therefore, β-cell–specific *Glp1r*-knockout mice may be needed to clarify the direct effect of GLP-1R on β-cells. However, GCGR antagonism or GLP-1R activation promotes β-cell regeneration from several sources, including β-cell proliferation, α- to β- transdifferentiation, and β-cell neogenesis from progenitors (7,14,15,17,32). In this regard, pancreas-specific knockout (Pdx1-Cre) was more suitable than β-cell–specific knockout (Ins1/Ins2-Cre); the latter could only block the regenerated β-cells from themselves.

Third, inducible knockout mice are more suitable for gene function research in adults. However, we used GLP-1R antagonist Ex9 as a pharmacological blockage to determine the function of GLP-1R in adult mice.

Finally, supraphysiological concentration of glucagon or glucagon analog to activate GLP-1R may be a more direct way to study glucagon function on β-cell regeneration.

In summary, our study reveals the mechanism of β-cell regeneration induced by GCGR antagonism, uncovers the communication between α-cells and β-cells in regulating β-cell mass and identity, and provides a new insight for GCGR antagonism–induced β-cell regeneration through activation of glucagon–GLP-1R signaling (Fig. 6*G*).

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