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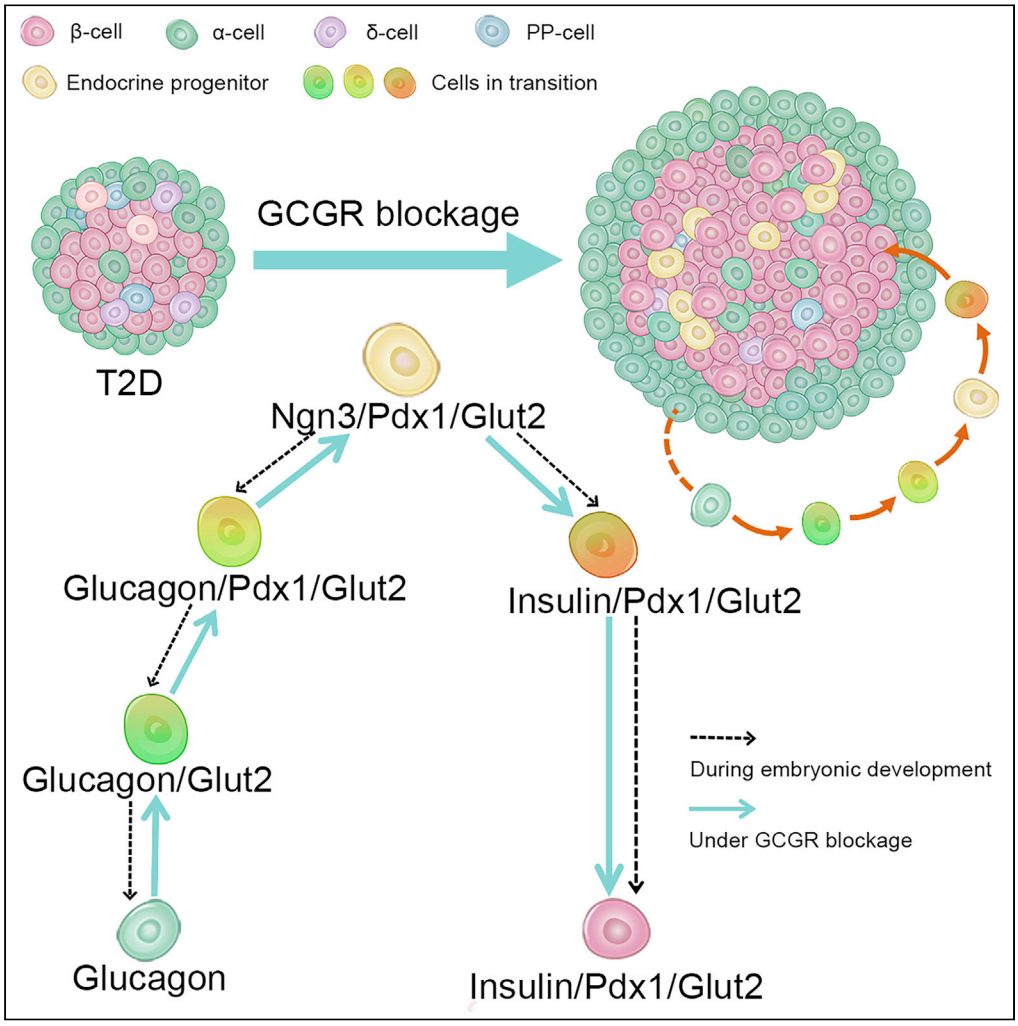

Xiaona Cui, Jin Feng, Tianjiao Wei, ..., Hai Yan, Rui Wei, Tianpei Hong

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Highlights

Blockage of α-cell-derived glucagon promotes β-cell regeneration *in situ* in type 2 diabetic (T2D) mice

Glucagon receptor (GCGR) mAb induces the trans-differentiation of α-cells to β-cells

GCGR mAb promotes α-cell regression to pancreatic endocrine progenitors

GCGR mAb induces Ngn3^{+} progenitor reactivation and differentiation toward β-cells

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Pro-α-cell-derived β-cells contribute to β-cell neogenesis induced by antagonistic glucagon receptor antibody in type 2 diabetic mice

Xiaona Cui,[1,2,5] Jin Feng,[1,5] Tianjiao Wei,[1,2] Liangbiao Gu,[1,2] Dandan Wang,[1] Shan Lang,[1] Kun Yang,[1,2] Jin Yang,[1,2] Hai Yan,[3,4] Rui Wei,[1,2,*] and Tianpei Hong[1,2,6,*]

SUMMARY

The deficiency of pancreatic β-cells is the key pathogenesis of diabetes, while glucagon-secreting α-cells are another player in the development of diabetes. Here, we aimed to investigate the effects of glucagon receptor (GCGR) antagonism on β-cell neogenesis in type 2 diabetic (T2D) mice and explore the origins of the neogenic β-cells. We showed that GCGR monoclonal antibody (mAb) elevated plasma insulin level and increased β-cell mass in T2D mice. By using α-cell lineage-tracing (*glucagon-cre-β-gal*) mice and inducible Ngn3^{+} pancreatic endocrine progenitor lineage-tracing (*Ngn3-CreERT2-tdTomato*) mice, we found that GCGR mAb treatment promoted α-cell regression to progenitors, and induced Ngn3^{+} progenitor reactivation and differentiation toward β-cells. Besides, GCGR mAb upregulated the expression levels of β-cell regeneration-associated genes and promoted insulin secretion in primary mouse islets, indicative of a direct effect on β-cell identity. Our findings suggest that GCGR antagonism not only increases insulin secretion but also promotes pro-α-cell-derived β-cell neogenesis in T2D mice.

INTRODUCTION

Global prevalence of diabetes has been rising in adults, and 90-95% of diabetes is type 2 diabetes (Li et al., 2020). The deficiency of pancreatic insulin-producing β-cells is an essential factor for the pathophysiology of type 2 diabetes (Kahn, 2003; Weyer et al., 1999). Restoration of functional β-cell mass is a promising therapeutic strategy. So far, however, there is almost no clinically available anti-diabetic agent that can achieve β-cell recovery. Moreover, only a few agents have shown a clinical potential to achieve β-cell regeneration. In patients with type 2 diabetes, treatment with anti-diabetic agents is difficult to maintain the long-term glycemic control on target as they cannot prevent the progressive failure of β-cell function (Turner et al., 1999). Therefore, it is urgent to develop an anti-diabetic agent which can effectively promote β-cell regeneration.

Recently, the role of glucagon-producing α-cells in glucose homeostasis regulation and diabetes development has become increasingly emphasized (Lee et al., 2014). Blockage of glucagon receptor (GCGR) lowers blood glucose level and improves glucose tolerance in type 1 diabetic (T1D) and type 2 diabetic (T2D) animals and humans (Conarello et al., 2007; Lee et al., 2011; Okamoto et al., 2017), which indicates the therapeutic potential in developing GCGR antagonists. Our recent studies have shown that REMD 2.59, an antagonistic GCGR monoclonal antibody (mAb), has a strong hypoglycemic effect in T1D and T2D mice (Lang et al., 2020b; Wei et al., 2019). Interestingly, we and other groups found that GCGR mAb promoted β-cell regeneration by inducing the trans-differentiation of α-cells to β-cells in T1D mice (Wang et al., 2021b; Wei et al., 2019). Whether GCGR mAb also has such beneficial effects on β-cell regeneration in T2D animal models has not been revealed, and the potential mechanisms remain unclear.

In this study, we investigated the effects of GCGR mAb on β-cell mass and function in two mouse models of T2D and traced the source of β-cell regeneration by using several tracing methods. We also determined whether GCGR mAb had a direct effect on islet cell phenotype conversion and β-cell function in cultured primary mouse islets. Our research reveals a novel pharmacological function of GCGR mAb on diabetes

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control and suggests that blockage of α-cell-derived glucagon can promote pro-α-cell-derived β-cell neogenesis, a new approach to β-cell regeneration, in T2D mice.

RESULTS

Glucagon receptor monoclonal antibody lowers the blood glucose level and improves glucose tolerance in type 2 diabetic mice

During the 4-week treatment, no significant difference was identified between GCGR mAb and IgG control groups in terms of body weight in *db/db* mice and high-fat diet + streptozotocin (HFD + STZ)-induced T2D mice (Figures 1A and 1G). Compared with baseline or IgG treatment, blood glucose levels at random or fasting state were significantly declined after treatment with GCGR mAb in these two T2D mouse models (Figures 1B and 1H). GCGR mAb significantly decreased the post-load glucose levels during the intraperitoneal glucose tolerance test (IPGTT) in *db/db* mice (Figures 1C and 1D) and HFD + STZ-induced T2D mice (Figures 1I and 1J). After treatment with GCGR mAb, fasting plasma glucagon levels were significantly increased in these two T2D mouse models, compared with control groups (Figures 1E and 1K). Fasting plasma insulin level in GCGR mAb-treated *db/db* mice was higher than that in the control group (Figure 1F). Similarly, GCGR mAb had a tendency to increase fasting plasma insulin and C-peptide levels in HFD + STZ-induced T2D mice ($p = 0.204$ and 0.125, respectively) (Figures 1L and S1A). To further evaluate the β-cell function, we detected the insulin level during IPGTT. Results showed that GCGR mAb increased the glucose-challenged insulin levels, especially 30 min after the glucose loading (Figure S1B).

Glucagon receptor monoclonal antibody increases islet number and area, and α-cell and β-cell numbers in type 2 diabetic mice

The islet number and islet area were significantly increased by GCGR mAb in *db/db* mice and HFD + STZ-induced T2D mice as indicated by histological analysis of the entire pancreata (Figure S2). Notably, GCGR mAb induced a striking expansion in the number of glucagon$^+$ α-cells and insulin$^+$ β-cells in *db/db* mice compared with IgG control group and *db/m* mice (Figures 2A and 2B). Similarly, glucagon$^+$ α-cell and insulin$^+$ β-cell numbers were significantly increased in HFD + STZ-induced T2D mice after treatment with GCGR mAb (Figures 2C and 2D).

Glucagon receptor monoclonal antibody accelerates progenitor-derived β-cell neogenesis in type 2 diabetic mice

As GCGR mAb treatment increased insulin$^+$ β-cell number and plasma insulin level, we tried to explore the possible origin of the increased β-cells. Interestingly, histological analysis in the pancreata of *db/db* mice and HFD/STZ-induced T2D mice showed that there were some glucagon$^+$ cells or insulin$^+$ cells located in the ductal region in GCGR mAb treatment groups, but they were rare in IgG control groups (Figures 3A and 3B). These findings implied that GCGR mAb might induce progenitor-derived β-cell neogenesis in T2D mice.

Neurogenin 3 (Ngn3), a notch-regulated transcription factor that is required for pancreatic endocrine differentiation during the development, has been shown to be also required for adult endocrine cell neogenesis (Al-Hasani et al., 2013). To confirm that GCGR mAb promoted β-cell neogenesis from pancreatic endocrine progenitors, we used inducible Ngn3$^+$ cell lineage-tracing T2D mice, which labeled reactivated endocrine progenitors by tdTomato (RFP). As expected, there were much more RFP$^+$insulin$^+$ cells in GCGR mAb group than in control group ($4.48 \pm 0.44\%$ vs. $1.82 \pm 0.26\%$, $p < 0.0001$) (Figure 3C). These results provided strong evidence that GCGR mAb induced pancreatic endocrine progenitor reactivation and differentiation toward β-cells in T2D mice.

Glucagon receptor monoclonal antibody induced α-cell regression to the progenitor state in type 2 diabetic mice

Subsequently, we explored the source of progenitors which contributed to β-cell neogenesis. Progenitor harbored in pancreatic ducts is one source of β-cell regeneration (Bonner-Weir et al., 2008; Xu et al., 2008). In this study, we found that GCGR mAb induced the appearance of insulin$^+$ cells located in the ductal region (Figures 3A and 3B), but the insulin$^+$ cells were not co-immunostained with cytokeratin 19, a marker of mature duct cells (Figure S3). The progenitor lineage-tracing marker RFP$^+$ cells were rarely located within or near pancreatic ducts in the Ngn3$^+$ cell lineage-tracing T2D mice despite treatment with GCGR mAb or IgG

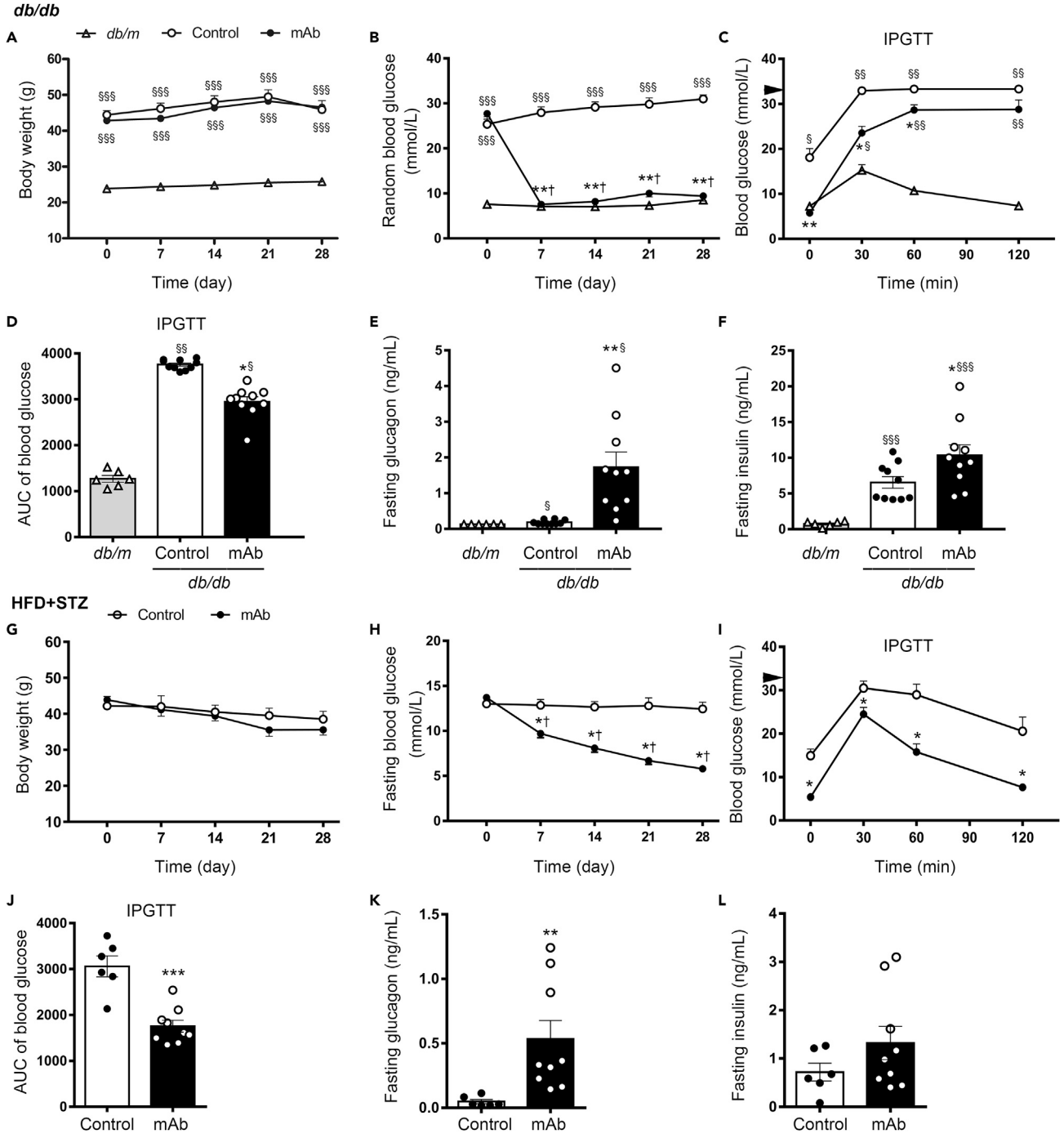


Figure 1. Metabolic parameters and hormone levels in two T2D mouse models treated with GCGR mAb or IgG control for 4 weeks

(A–F) Parameters in *db/db* mice. Age-matched *db/m* mice treated with IgG were included as a normal control.

(G–L) Parameters in HFD + STZ-induced T2D mice.

(A and G) Body weight. (B and H) Random or fasting blood glucose. (C, I) Blood glucose during the intraperitoneal glucose tolerance test (IPGTT). Black triangle indicated 33.3 mmol/L (the upper detection limit of the glucometer). (D, J) The areas under curve (AUC) of blood glucose during the IPGTT. (E, K) Fasting plasma glucagon. (F, L) Fasting plasma insulin.n = 6 in *db/m* mice and n = 9 per group in *db/db* mice. n = 6 in control group and n = 9 in GCGR mAb group in HFD + STZ-induced T2D mice. Data represent the mean ± SEM. Statistical analysis was conducted by ANOVA followed by the *post hoc* Tukey-Kramer test or by Student's *t*-test, as appropriate. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control; §p < 0.05, §§p < 0.01, §§§p < 0.001 vs. *db/m*; †p < 0.05 vs. pretreatment in the same group. See also Figure S1.

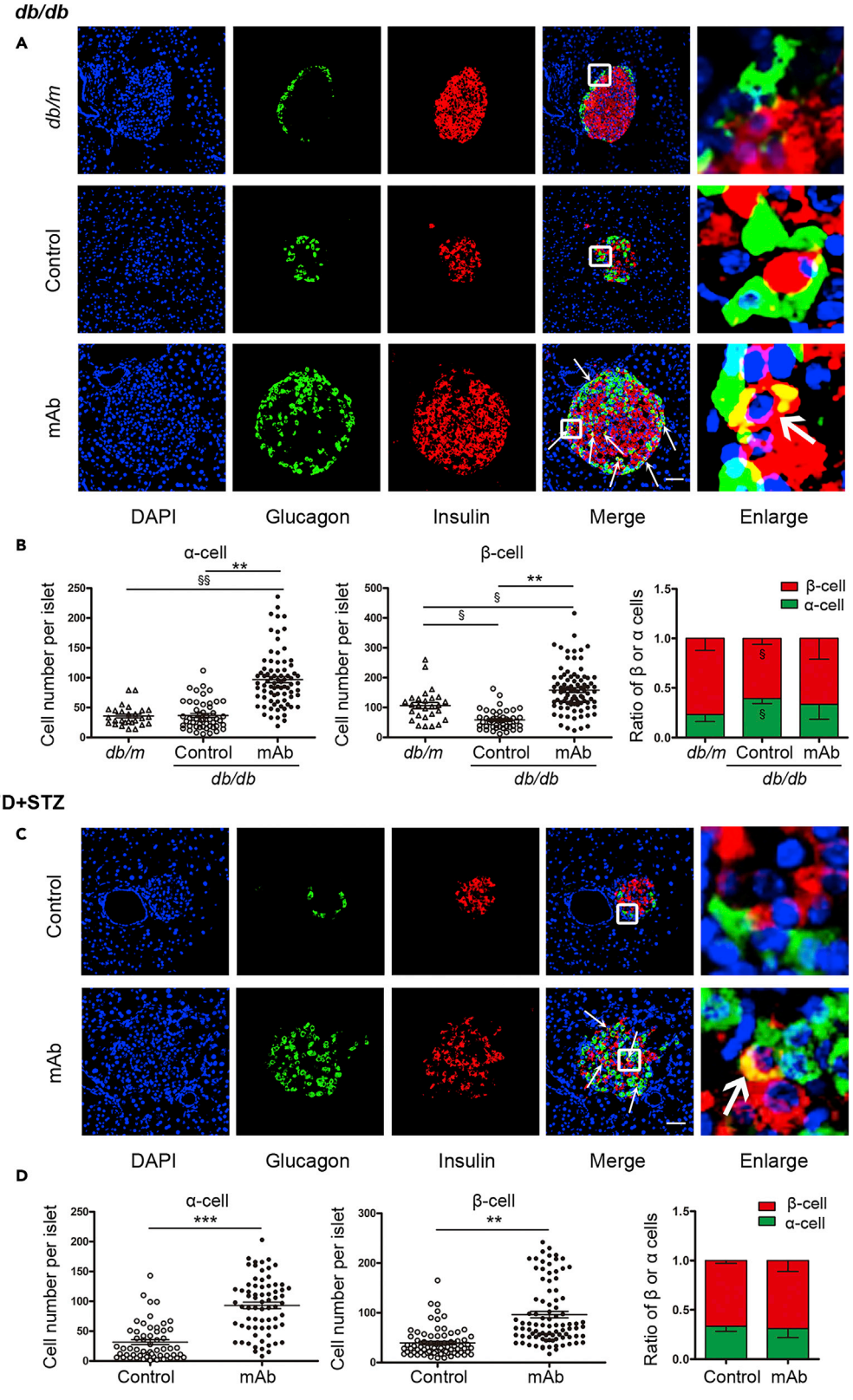

db/db
A
db/m
Control
mAb
DAPI
Glucagon
Insulin
Merge
Enlarge
B
α-cell
β-cell
Cell number per islet
Ratio of β or α cells
β-cell
α-cell
db/m
Control
mAb
db/db
HFD+STZ
C
D

Figure 2. Histological analysis of pancreatic α-cell number and β-cell number in the pancreatic tissues of two T2D mouse models treated with GCGR mAb or IgG control for 4 weeks

(A and C) Representative image of an islet immunostained for glucagon and insulin in *db/db* mice (A) and HFD + STZ-induced T2D mice (C) treated with GCGR mAb or IgG control. The arrows indicate co-labeled cells. The cells in the small box are enlarged at the right of the image. Scale bar = 50 μm.

(B and D) Quantification of the number of α-cells and β-cells per islet slice, and the ratio of β-cell number and α-cell number in *db/db* mice (B) and HFD + STZ-induced T2D mice (D).n = 3-5 sections/mouse multiplied by 6 mice/group in *db/db* mice, and n = 3-5 sections/mouse multiplied by 9 mice/group in HFD + STZ-induced T2D mice. Data are expressed as the median (interquartile range). Statistical analysis was conducted by the Mann-Whitney test. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ vs. *db/m*. See also Figure S2.

control (Figure S4). These results indicated that duct-derived progenitors might not be the main source of progenitors which were responsible for β-cell neogenesis in T2D mice.

As α-cells were significantly expanded in the pancreatic tissues of these two T2D mouse models after being treated with GCGR mAb (Figure 2), we inferred that α-cells might be the source of progenitors which contributed to β-cell neogenesis. It is generally believed that during embryonic development, islet cells differentiate from pancreatic endocrine progenitors in which pancreatic and duodenal homeobox 1 (Pdx1) expression is an early step to initiate the production of glucose transporter 2 (Glut2) and Ngn3, and is subsequently turned off by Ngn3 before the initiation of islet hormone expression (Serafimidis et al., 2008; Vuguin et al., 2006). In order to confirm our inferences, we analyzed the state of α-cells in the GCGR mAb-treated T2D mice by using pancreatic α-cell lineage-tracing, which labeled pre-existing α-cells by β-galactosidase (β-gal) reporter (Figure S5). As expected, the β-gal$^+$Ngn3$^+$ cells were increased by GCGR mAb treatment, which suggested that Ngn3$^+$ progenitors might derive from the α-cell regression (Figure 4A). In addition, both β-gal$^+$Glut2$^+$ cells and β-gal$^+$Pdx1$^+$ cells were also increased in the GCGR mAb group compared with IgG control group (Figures 4B and 4C). These results strongly indicated that after treatment with GCGR mAb, α-cells regressed to the immature state, and expressed the progenitor's markers (Ngn3, Glut2, and Pdx1). Therefore, the pro-α-cell-derived progenitors appeared to be the important source of progenitors which were responsible for β-cell neogenesis in T2D mice.

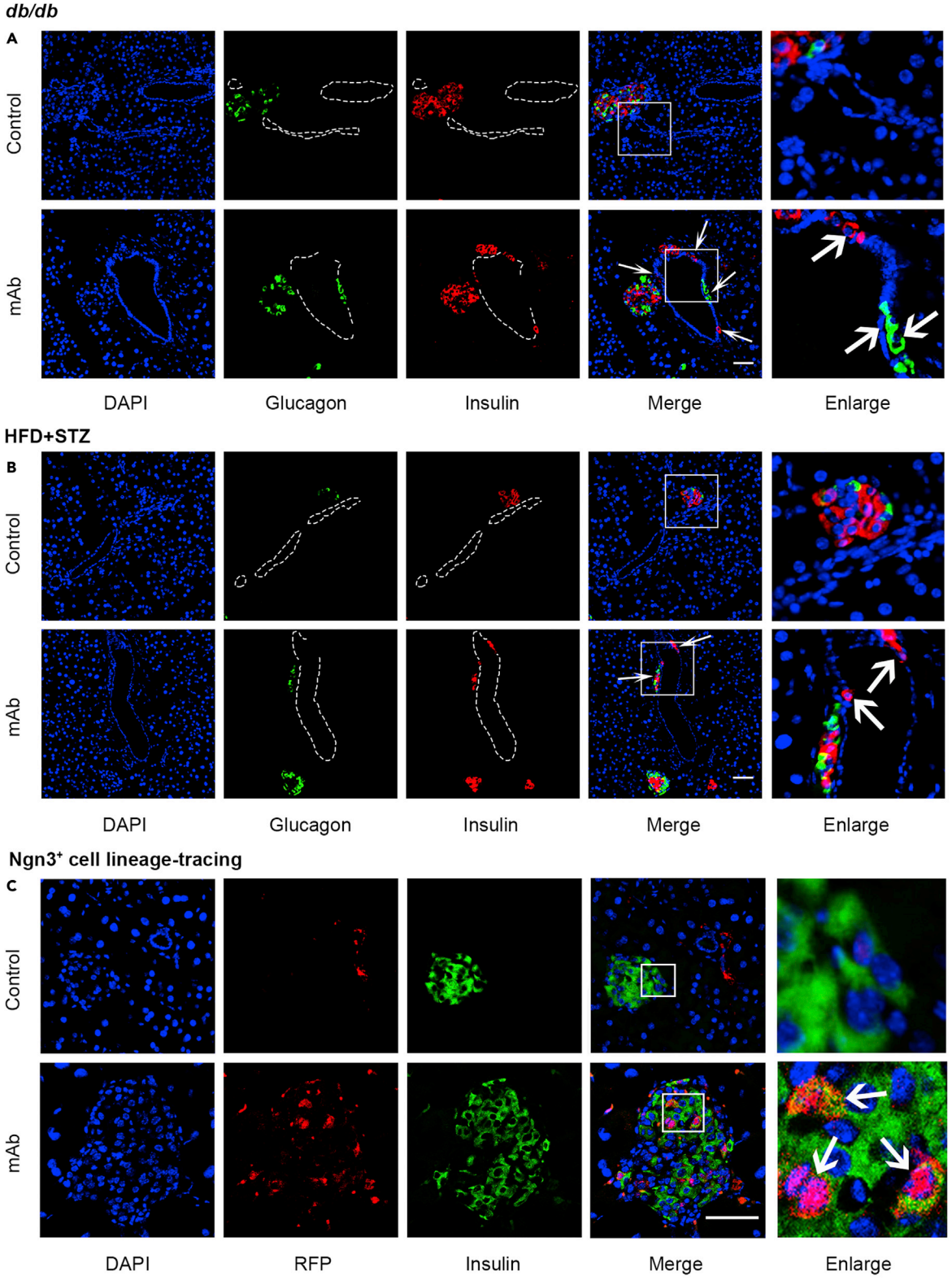
Glucagon receptor monoclonal antibody promotes the trans-differentiation of α-cells to β-cells in type 2 diabetic mice

We noticed that after treatment with GCGR mAb, α-cells were located not only in the islet mantle zone but also in the islet core, where β-cells are generally detected (Figures 2A and 2C). Furthermore, the numbers of glucagon and insulin co-localizing cells were much higher in GCGR mAb groups than in IgG control groups (Figures 2A, 2C, 5A, and 5B). We inferred that these glucagon$^+$insulin$^+$ cells were the intermediate state of α-to-β cell conversion. In support of this inference, we found that some glucagon$^+$ cells were co-immunostained with Nkx6.1 (a transcription factor for β-cell development and function), prohormone convertase 1/3 (PC1/3, an important enzyme for processing proinsulin to insulin in β-cells), or C-peptide in these two T2D mouse models treated with GCGR mAb rather than with IgG (Figures S6 and S7).

To confirm that α-cells were the origins of progenitors which contributed to β-cell neogenesis, we tracked the insulin expression of α-cells in pancreatic α-cell lineage-tracing mice. As expected, some β-gal$^+$ cells contained insulin in the islets of the GCGR mAb group. By contrast, the β-gal$^+$insulin$^+$ cells were scarce in the control group (Figures 5C and 5D). The results indicated that pro-α-cell-derived β-cell neogenesis or direct α-to-β cell conversion was an important origin of the regenerated β-cells induced by the GCGR antagonism in T2D mice.

Glucagon receptor monoclonal antibody enhances insulin secretion and regulates islet cell phenotype in cultured primary mouse islets

Subsequently, we tried to clarify whether GCGR mAb treatment could directly affect pancreatic β-cell function and islet cell phenotype. We first detected GCGR expression in β-cells. Results showed that GCGR was expressed in mouse β-cells at both the mRNA and protein levels (Figure S8A, S8B, and S8C), suggesting that GCGR mAb might have direct effects on β-cells. Besides, data from RNA Chip and RNA sequencing analyses indicated that *GCGR* expression in human and mouse β-cells were comparable between T2D and control individuals (Figures S8D and S8E). We then incubated primary mouse islets with GCGR mAb or IgG for 24 h in high glucose (25 mmol/L) medium. As expected, the intracellular glucagon content and supernatant glucagon level were higher in the islets treated with GCGR mAb than those with IgG


db/db
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Glucagon
Insulin
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HFD+STZ
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Glucagon
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Merge
Enlarge
Ngn3+ cell lineage-tracing
C
Control
mAb
DAPI
RFP
Insulin
Merge
Enlarge

Figure 3. Immunofluorescent analysis of progenitor-derived β-cell neogenesis in the pancreatic tissues of T2D mice treated with GCGR mAb or IgG control for 4 weeks

(A and B) Representative photograph showing glucagon$^+$ cells and insulin$^+$ cells located in the ductal region of *db/db* mice (A) and HFD + STZ-induced T2D mice (B). The arrows indicate glucagon$^+$ cells or insulin$^+$ cells in the ductal lining.

(C) Representative image of an islet immunostained with RFP (Ngn3$^+$ cell lineage-tracing marker) and insulin in Ngn3$^+$ cell lineage-tracing T2D mice that were induced by HFD + STZ. The arrows indicate co-labeled cells, and the ductal lumen is outlined with dashed lines. The cells in the small box are enlarged at the right of the image. Scale bar = 50 μm. See also Figures S3 and S4.

control (Figures 6A and 6B). Although the insulin content in the GCGR mAb-treated islets was unchanged (Figure 6C), the insulin level in supernatant was significantly increased by GCGR mAb (Figure 6D). We also detected the glucose-stimulated insulin secretion in primary mouse islets treated with GCGR mAb. Results showed that GCGR mAb enhanced glucose concentration-dependent insulin secretion (Figure 6E). Furthermore, the mRNA levels of *Gcg* (which encodes proglucagon), *Pcsk1* (which encodes PC1/3), *Ins1* (which encodes insulin), *Pdx1* (a master transcription factor that participates in pancreatic fate determination and β-cell maturation and function) and *Ngn3* (a specific marker of pancreatic endocrine progenitors) were upregulated by GCGR mAb. By contrast, the mRNA levels of *Pcsk2* (which encodes PC2, an important enzyme for processing proglucagon to glucagon) and *Ins2* (which encodes insulin) were unchanged by GCGR mAb (Figure 6F). These results suggested that GCGR mAb had a direct beneficial effect on β-cell function and islet cell phenotype conversion.

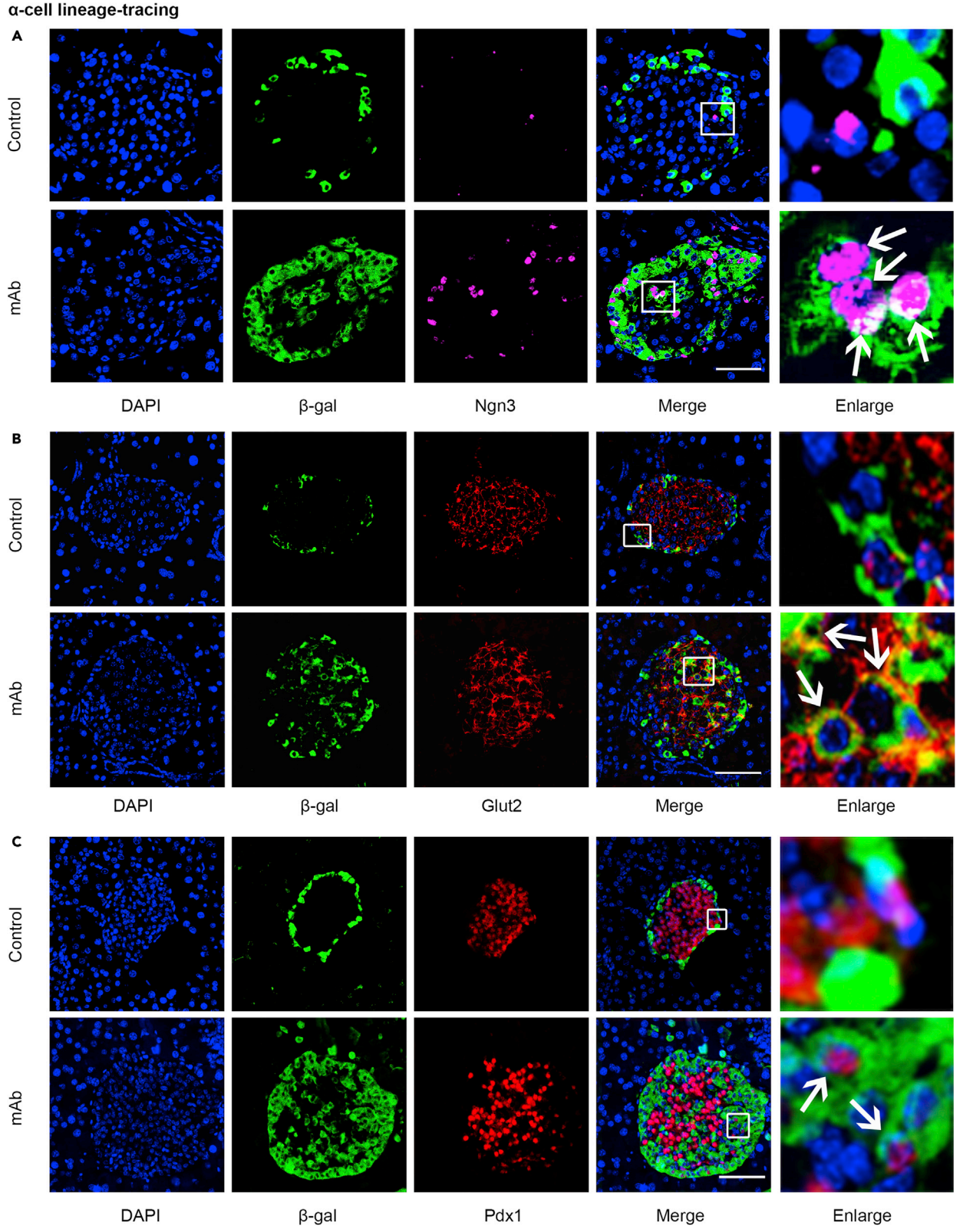
DISCUSSION

Loss of functional β-cell mass is the key pathogenesis of diabetes, while glucagon-secreting α-cells also play a role in the development of diabetes. Here, we showed that the inhibition of glucagon function by REMD 2.59, an antagonistic GCGR mAb, not only improved blood glucose control, but also promoted insulin secretion and increased β-cell mass in *db/db* mice and HFD + STZ-induced T2D mice. Notably, we further revealed that the GCGR mAb treatment promoted α-cell regression to the progenitor state, and induced the progenitor-derived β-cell neogenesis. Besides, the GCGR mAb enhanced insulin secretion and upregulated the expression of genes associated with β-cell regeneration in cultured primary mouse islets, indicative of a direct effect on islet cell phenotype conversion.

Restoration of functional β-cell mass is a promising therapeutic strategy. Blockage of α-cell-secreted glucagon has an effect on β-cells. In mice with severe insulin resistance induced by the insulin receptor antagonist S961, GCGR mAb not only increased the total amount of α-cells but also increased the total amount of β-cells (Okamoto et al., 2017). We and other groups demonstrated that GCGR antagonism could increase β-cell mass in T1D mice (Wang et al., 2021b; Wei et al., 2019). In this study, we showed that GCGR mAb also increased β-cell mass in T2D mice. There are several ways to increase β-cell mass, including the inhibition of β-cell apoptosis or dedifferentiation, promotion of β-cell proliferation, induction of α-to-β cell conversion, promotion of β-cell differentiation from pancreatic endocrine progenitors or stem cells (Nair et al., 2020; Path et al., 2019; Wang et al., 2021a; Wei and Hong, 2016). Here, we assessed the origins of increased β-cells induced by GCGR mAb in T2D mice and revealed a new approach to β-cell regeneration.

Ngn3$^+$ progenitors are considered to be required for β-cell neogenesis (Wang et al., 2009). Our previous study showed that GCGR mAb induced duct-derived neogenesis in T1D mice (Wei et al., 2019). Here, we conducted a more comprehensive exploration by using Ngn3$^+$ cell lineage-tracing mice to confirm progenitor-derived β-cell neogenesis in T2D mice. Moreover, we demonstrated that the main source of progenitors which contributed to β-cell neogenesis might be α-cell regression to the progenitor state. Notably, human islets could also regenerate from progenitors (Qadir et al., 2020; Yoneda et al., 2013). Therefore, the promoting effects of GCGR mAb on progenitor-derived β-cell neogenesis as shown in this study suggest the potential clinical application of GCGR mAb to regenerate human islets.

Cell trans-differentiation, also known as lineage reprogramming, is a new path for β-cell regeneration (Wei and Hong, 2016). Several strategies were reported to promote the trans-differentiation of α-cells to β-cells (Ben-Othman et al., 2017; Collombat et al., 2009; Courtney et al., 2013; Furuyama et al., 2019; Thorel et al., 2010). However, the extreme β-cell loss conditions and gene modification methods are difficult to be translated into clinical treatment. GABA and artemisinins were reported to induce


α-cell lineage-tracing
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Control
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Ngn3
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B
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Glut2
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C
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Pdx1
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Figure 4. Immunofluorescent analysis of α-cell regression to the progenitor state in the pancreatic tissues of T2D mice treated with GCGR mAb or IgG control for 4 weeks

(A–C) Representative photograph showing β-gal (α-cell lineage-tracing marker)$^+$Ngn3$^+$ cells (A), β-gal$^+$Glut2$^+$ cells (B), and β-gal$^+$Pdx1$^+$ cells (C) in α-cell lineage-tracing T2D mice that were induced by HFD + STZ. The arrows indicate co-labeled cells. The cells in the small box are enlarged at the right of the image. Scale bar = 50 μm. See also Figures S5.

α-to-β cell conversion (Ben-Othman et al., 2017; Li et al., 2017), albeit there were some controversial results (van der Meulen et al., 2018). Our previous studies indicated that GCGR mAb induced α-to-β cell conversion in two T1D mouse models (Wei et al., 2019). Similarly, this study showed that GCGR mAb also promoted the trans-differentiation of α-cells to β-cells in T2D mice. As α-to-β cell conversion can also occur in human islets (Ben-Othman et al., 2017), we believe that GCGR mAb may have a similar effect in T2D humans.

However, the exact process of α-to-β cell conversion is not yet clear. In this study, we investigated whether α-to-β cell conversion induced by GCGR mAb was direct or indirect trans-differentiation. We found that after treatment with GCGR mAb, α-cells could regress to the immature state, and express the progenitor's markers (Ngn3, Glut2, and Pdx1) in pancreatic α-cell lineage-tracing T2D mice. During embryonic development, pancreatic islet cells differentiate from endocrine progenitors in which Pdx1 expression is early step for initiating the synthesis of Glut2 and Ngn3 and then is turned off by Ngn3 before the initiation of islet hormone expression (Serafimidis et al., 2008; Vuguin and Charron, 2011). It has been shown that the global deletion of *Gcgr* inhibits the progression of α-cells to a mature state (Vuguin et al., 2006). In addition, we also revealed that GCGR mAb promoted progenitor-derived β-cell neogenesis in Ngn3$^+$ cell lineage-tracing T2D mice. Taken together, these results suggested that GCGR antagonism not only could directly promote α-to-β cell conversion but also induced α-cell regression to progenitors which were responsible for β-cell neogenesis, thereby contributing to β-cell regeneration in T2D mice.

In conclusion, our study demonstrates that treatment with GCGR mAb in T2D mice ameliorates hyperglycemia and increases functional β-cell mass via inducing pro-α-cell-mediated progenitor-derived β-cell neogenesis. Our study suggests that blockage of α-cell-derived glucagon promotes β-cell regeneration, and provides new insight into the clinical development of GCGR mAb in treating T2D.

Limitation of the study

It has been demonstrated that GCGR mAb displays an improved glycemic control with less need for exogenous insulin in T1D rodents and humans (Pettus et al., 2018; Wang et al., 2021b; Wei et al., 2019). This effect might be accounted for by the enhanced insulin action in both liver and skeletal muscle (Sharma et al., 2018), or by the upregulated circulating glucagon-like peptide-1 (GLP-1) level (Lang et al., 2020a, 2020b). In this study, we found that GCGR mAb increased β-cell mass and elevated plasma insulin levels in T2D mice. Whether this elevation in plasma insulin is owing to altered insulin clearance or other mediators induced by GCGR mAb needs to be clarified.

The levels of endogenous glucagon are highest in the venous drainage of pancreas and hepatic portal vein, and its principal target is liver. GCGR is predominantly expressed in liver, and also lowly expressed in other tissues, including pancreas, kidney, adipose tissue, and brain (Muller et al., 2017). Therefore, the possible mechanism of the GCGR mAb-induced β-cell regeneration may be driven by the loss of GCGR signaling in pancreas per se, or because of the upregulation of the proglucagon-related peptide production that results from loss of GCGR activity in hepatocytes. The exact mechanism deserves further in-depth investigation in the future.

The incretins GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are gut hormones that potentiate insulin secretion in a glucose-dependent manner. GLP-1 and GIP exert their insulinotropic actions through distinct G-protein-coupled receptors highly expressed on pancreatic β-cells (Campbell and Drucker, 2013). Moreover, it has been reported that GLP-1 and GIP improve β-cell survival in rodents (Drucker, 2013). In our previous studies, we demonstrated that treatment with GCGR mAb could significantly upregulate the GLP-1 production and its circulating level in T2D mice (Lang et al., 2020a, 2020b). Therefore, GLP-1 might participate in the process of β-cell regeneration induced by GCGR

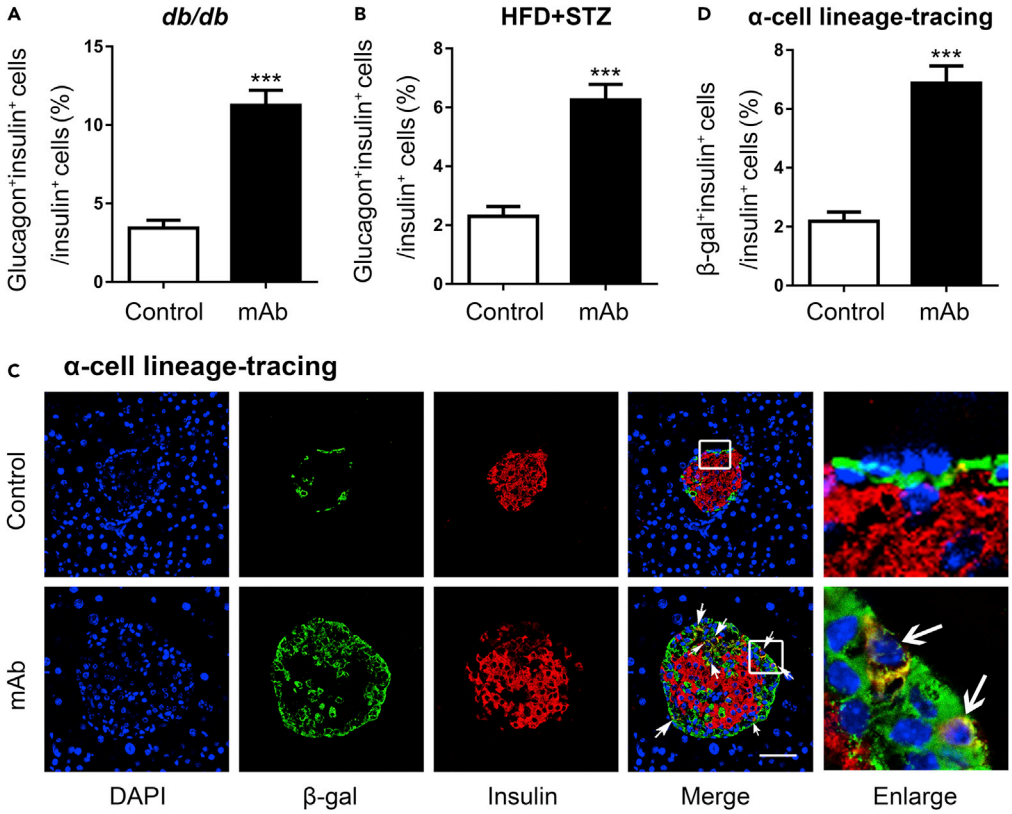

Figure 5. Immunofluorescent analysis of α-to-β cell conversion in the pancreatic tissues of T2D mouse models treated with GCGR mAb or IgG control for 4 weeks

(A and B) Quantification of glucagon$^+$insulin$^+$ cells in *db/db* mice (A) and HFD + STZ-induced T2D mice (B) as shown in (Figures 2A and 2C).

(C and D) Representative image of an islet immunostained with β-gal (α-cell lineage-tracing marker) and insulin (C), and quantification of β-gal$^+$insulin$^+$ cells (D) in α-cell lineage-tracing T2D mice that were induced by HFD + STZ. The arrows indicate co-labeled cells. The cells in the small box are enlarged at the right of the image. Scale bar = 50 μm. n = 5 sections/mouse multiplied by 6 mice/group in *db/db* mice, n = 3 sections/mouse multiplied by 9 mice/group in HFD + STZ-induced T2D mice, and n = 3 sections/mouse multiplied by 5 mice/group in the α-cell lineage-tracing T2D mice. Data represent the mean ± SEM. Statistical analysis was conducted by Student's *t*-test. ***$p < 0.001$ vs. control. See also Figures S5, S6, and S7.

mAb. Genetic and pharmacological blockage of the GLP-1 receptor would help to answer the question.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
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  - Animals
  - Genetically modified mice
  - Primary mouse islet isolation and culture
- METHOD DETAILS
  - Animals' intervention
  - Glucose monitoring

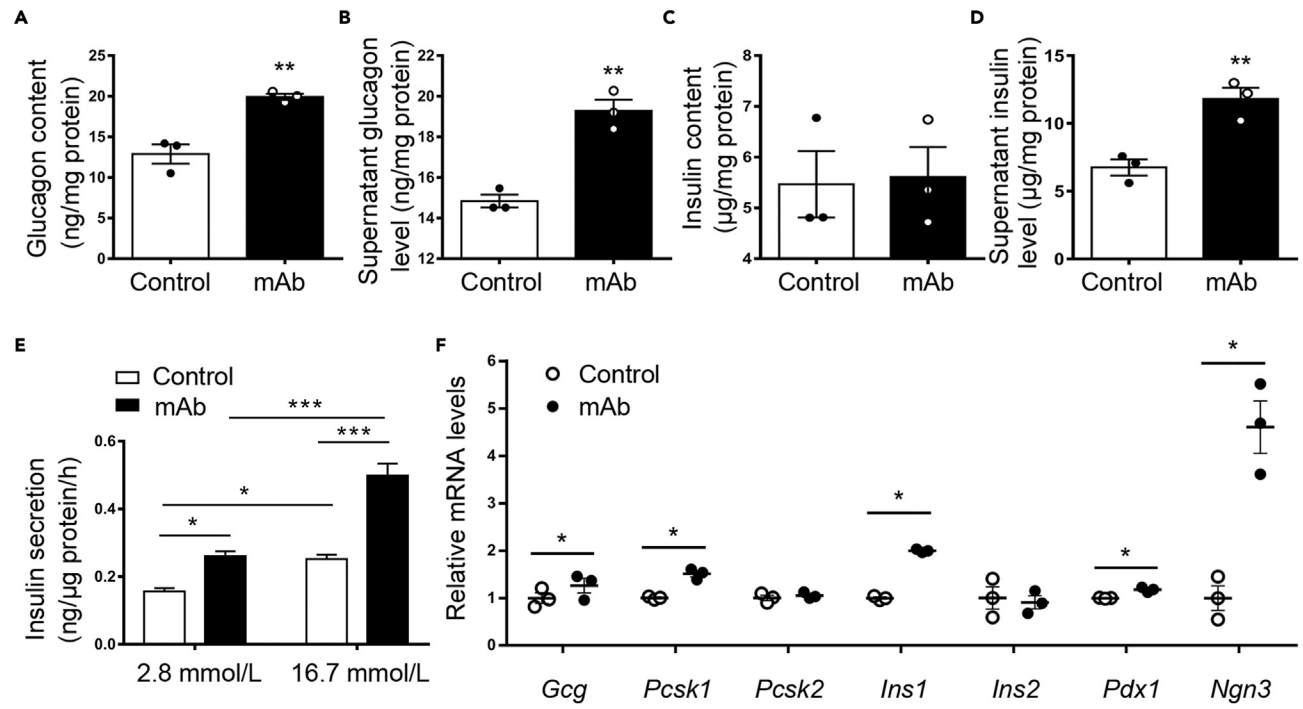

Figure 6. Hormone production and gene expression in primary mouse islets incubated with GCGR mAb (1,000 nmol/L) or IgG (as control) for 24 h in high glucose (25 mmol/L) medium

(A–D) Intracellular glucagon content (A), supernatant glucagon level (B), intracellular insulin content (C), and supernatant insulin level (D) were detected by ELISA. The hormone levels were normalized to total protein content.

(E) Glucose-stimulated insulin secretion assay in the islets after treated with GCGR mAb or IgG. The insulin levels were normalized to total protein content.

(F) Gene expression was determined by quantitative RT-PCR. The experiments were repeated 3 times. Data represent the mean ± SEM. Statistical analysis was conducted by Student's *t*-test or two-way ANOVA as appropriate. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control. See also Figure S8.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104567.

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AUTHOR CONTRIBUTIONS

Conceptualization: X.C., R.W., and T.H.; methodology: X.C., J.F., T.W., L.G., D.W., S.L., and K.Y.; software: T.W.; validation: J.F. and T.W.; formal analysis: X.C., J.F., R.W., and T.H.; investigation: X.C., J.F., D.W., S.L., K.Y., and J.Y.; resources: H.Y.; writing – original draft: X.C. and J.F.; writing – review & editing: R.W. and T.H.; visualization: X.C., J.F. and L.G.; supervision: J.Y., H.Y., R.W., and T.H.; project administration: R.W. and T.H.; funding acquisition: X.C., L.G., R.W., and T.H.

DECLARATION OF INTERESTS

Hai Yan is a shareholder of REMD Biotherapeutics. The authors have no additional financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-glucagon	Cell Signaling Technology	Cat#2760; RRID: AB_659831
Mouse monoclonal anti-glucagon	Sigma-Aldrich	Cat#G2654; RRID: AB_259852
Mouse monoclonal anti-insulin	Sigma-Aldrich	Cat#I2018; RRID: AB_260137
Rabbit monoclonal anti-cytokeratin 19	Abcam	Cat#ab52625; RRID: AB_2281020
Rabbit polyclonal anti-RFP	Abcam	Cat#ab62341; RRID: AB_945213
Rabbit polyclonal anti-β-gal	Abcam	Cat#ab203749; RRID: AB_2920785
Mouse monoclonal anti-Ngn3	Santa Cruz	Cat# sc-374442; RRID: AB_10988579
Mouse monoclonal anti-Glut2	Santa Cruz	Cat#sc-518022; RRID: AB_2890905
Rabbit polyclonal anti-Pdx1	Abcam	Cat#ab47267; RRID: AB_777179
Rabbit monoclonal anti-Nkx6.1	Abcam	Cat#ab221549; RRID: AB_2754979
Rabbit polyclonal anti-PC1/3	Millipore	Cat#AB10553; RRID: AB_1977441
Rabbit polyclonal anti-C-peptide	Cell Signaling Technology	Cat#4593; RRID: AB_10691857
Rabbit polyclonal anti-GCGR	Proteintech	Cat# 26784-1-AP; RRID: AB_2880634
Mouse monoclonal anti-GAPDH	Zhongshan Biotechnology	Cat#TA-08; RRID: AB_2107448
Alexa Fluor 488-conjugated AffiniPure goat polyclonal anti-rabbit IgG (H + L)	Jackson ImmunoResearch Laboratories	Cat#115-545-003; RRID: AB_2338046
Alexa Fluor 594-conjugated AffiniPure goat polyclonal anti-mouse IgG (H + L)	Jackson ImmunoResearch Laboratories	Cat#115-585-003; RRID: AB_2338871
Chemicals, peptides, and recombinant proteins		
REMD 2.59 (a human GCGR mAb and competitive antagonist)	REMD Biotherapeutics	N/A
High-fat diet (HFD)	Research Diets	Cat#D12492
Streptozocin (STZ)	Sigma-Aldrich	Cat#S0130
Tamoxifen	Sigma-Aldrich	Cat#T5648
Goat serum	Zhongshan Biotechnology	Cat#ZLI-9056
DAPI	Sigma-Aldrich	Cat#D9542
Trizol reagent	Thermo Fisher Scientific	Cat#15596018
SYBR qPCR Mix	TOYOBO	Cat#QPS-201
RIPA lysis buffer	Applygen Technologies Inc.	Cat#C1053
Protease inhibitor	Applygen Technologies Inc.	Cat#P1265
Phosphatase inhibitor	Applygen Technologies Inc.	Cat#P1260
Critical commercial assays		
Insulin ELISA kit	Millipore	Cat#EZRMI-13K
Glucagon ELISA kit	R&D Systems	Cat#DGCG0
C-peptide ELISA kit	Millipore	Cat#EZRMCP2-21K
Opal 7 color manual kit	Akoya Bioscience	Cat#NEL811001KT
RevertAid First Strand cDNA Synthesis kit	Thermo Fisher Scientific	Cat#K1622
Experimental models: Organisms/strains		
Mouse: C57BL/6N	Vital River Animal Center	Cat#213
Mouse: *db/db* (BKS.Cg-Dock7^{m}+/+Lepr^{db}/Nju) mice	Nanjing Biomedical Research Institution of Nanjing University	Cat#T001463

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: Tg(Ngn3-cre/ERT2)1Able/J	The Jackson Laboratory	JAX: 028,365
Mouse: B_6/JGpt-Rosa26$^{tm1(CAG-LSL-Cas9-tdTomato)}$/Gpt	GemPharmatech	Cat#T002249
Mouse: B_6. Cg-Tg (Gcg-cre)1Herr/Mmnc	Mutant Mouse Resource & Research Centers	MMRRC: 000358-UNC
Mouse: B_6; 129-Gt (ROSA)26Sor^{tm1Sho}/J	The Jackson Laboratory	JAX: 003,504
Software and algorithms		
ImagePro Plus 6.0	Media Cybernetics	N/A
GraphPad Prism v.7.0	GraphPad Software Inc.	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Tianpei Hong (tpho66@bjmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact Tianpei Hong (tpho66@bjmu.edu.cn) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All animal experimental procedures were conducted at Peking University Health Science Center and approved by the Institutional Animal Care and Use Committee. All the animals were group-housed conventionally and maintained on a 12-h light/dark cycle with free access to food and water. Eight-week-old male *db/db* (BKS.Cg-Dock7^{m}+/+Lepr^{db}/Nju) mice (Nanjing Biomedical Research Institution of Nanjing University, Nanjing, China) were used as a typical T2D model. The littermate male *db/m* mice served as normal controls. n = 10 per group in *db/db* mice, and n = 6 in *db/m* mice.

To generate high-fat diet + streptozotocin (HFD + STZ)-induced T2D model, 5-week-old male C57BL/6N (Vital River Animal Center, Beijing, China) or genetically modified mice were fed with HFD (fat 60%, carbohydrate 20% and protein 20%; Research Diets, New Brunswick, NJ, USA) for 3–4 months, and then were given 75 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally. Diabetic condition was confirmed if the fasting blood glucose level was $\geq$11.1 mmol/L. Mice were sorted into groups having similar distributions based on body weight and blood glucose levels.

Genetically modified mice

Tg(Ngn3-cre/ERT2)1Able/J mice (expressing a tamoxifen-inducible cre recombinase directed to Ngn3-expressing cells; the Jackson Laboratory, Barr Harbor, ME, USA) and B_6/JGpt-Rosa26$^{tm1(CAG-LSL-Cas9-tdTomato)}$/Gpt mice (when crossed to a cre recombinase-expressing strain, tdTomato expression is observed in the cre-expressing tissues; GemPharmatech, Nanjing, China) were crossed to generate pancreatic endocrine progenitor lineage-tracing mice, namely, *Ngn3-CreERT2-tdTomato* mice. Male mice were selected for subsequent experiments. Tamoxifen (Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich), and intraperitoneal injected (20 mg/day) for 5 days since 1 week before being sacrificed. n = three to four per group.

B_6.Cg-Tg(Gcg-cre)1Herr/Mmnc mice (cre expression in pancreatic α-cell lineage; Mutant Mouse Resource & Research Centers, Columbia, MO, USA) and B_6; 129-Gt(ROSA)26Sor^{tm1Sho}/J mice (when crossed to a cre recombinase-expressing strain, lacZ (which encodes β-gal) expression is observed in the cre-expressing tissues; the Jackson Laboratory) were crossed to generate pancreatic α-cell lineage-tracing mice, namely, *glucagon-cre-β-gal* mice. Male mice were selected for subsequent experiments. n = five to eight per group.

Primary mouse islet isolation and culture

Primary islets were isolated from male C57BL/6N mice aged 8–10 weeks as previously reported (Wang et al., 2014; Wei et al., 2020; Zmuda et al., 2011). Briefly, the pancreas was perfused by collagenase V (Sigma-Aldrich), and individual islets were handpicked to near 100% purity under a dissecting microscope. The islets were cultured for 24 h before treatment in the RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (*v/v*) fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mmol/L GlutMax, 1 mmol/L sodium pyruvate, and 1% (*v/v*) penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA).

METHOD DETAILS

Animals' intervention

All mice were treated for 4 weeks via weekly intraperitoneal administration of REMD 2.59 (5 mg/kg; REMD Biotherapeutics, Camarillo, CA, USA), a human antagonistic GCGR mAb, or IgG (5 mg/kg, as control).

Glucose monitoring

Blood samples for glucose detection were collected from the tail vein, and measured by the glucose oxidase method using a hand-held OneTouch Ultra glucometer (LifeScan, Milpitas, CA, USA).

Glucose tolerance test and blood sample collection

To perform IPGTT, basal blood glucose levels were first measured after overnight fasting. A 40% (*w/v*) of glucose solution was administrated by intraperitoneal injection at 2 g/kg, and blood glucose levels were measured at 30, 60 and 120 min after the glucose loading. If the glucose level was higher than 33.3 mmol/L (upper detection limit of the glucometer), the value of 33.3 mmol/L was recorded.

Blood samples for hormone detection were collected from the orbital vein. Aprotinin (1 μg/mL; Sigma-Aldrich) and heparin sodium (1,000 IU/mL; Qianhong Bio-pharma, Changzhou, China) were added to each blood sample.

Immunofluorescent staining and quantification

Pancreata were fixed with 10% (*v/v*) neutral-buffered formalin and embedded in paraffin, and 5-μm-thick sections were prepared. For immunofluorescence, sections were incubated with primary antibodies at 4 °C overnight and secondary antibodies for 1 h at room temperature, followed by staining with DAPI. Images were captured under Leica TCS SP8 confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany) or an automatic digital slide scanner (Pannoramic MIDI, 3D HISTECH, Budapest, Hungary).

The primary antibodies were as follows: rabbit polyclonal anti-glucagon (1:800; Cell Signaling Technology, Boston, MA, USA; RRID: AB_659831), mouse monoclonal anti-glucagon (1:400; Sigma-Aldrich; RRID: AB_259852), mouse monoclonal anti-insulin (1:800; Sigma-Aldrich; RRID: AB_260137), rabbit monoclonal anti-cytokeratin 19 (1:200; Abcam, Cambridge, UK; RRID: AB_2281020), rabbit polyclonal anti-RFP (1:200; Abcam; RRID: AB_945213), rabbit polyclonal anti-β-gal (1:100; Abcam; RRID: AB_2920785), mouse monoclonal anti-Ngn3 (1:50; Santa Cruz, CA, USA; RRID: AB_ 10988579), mouse monoclonal anti-Glut2 (1:100; Santa Cruz; RRID: AB_2890905), rabbit polyclonal anti-Pdx1 (1:200; Abcam; RRID: AB_777179), rabbit monoclonal anti-Nkx6.1 (1:400; Abcam; RRID: AB_2754979), rabbit polyclonal anti-PC1/3 (1:400; Millipore, Darmstadt, Germany; RRID: AB_1977441), rabbit polyclonal anti-C-peptide (1:400; Cell Signaling Technology; RRID: AB_10691857), rabbit anti-GCGR (Proteintech, Rosemont, IL, USA; RRID: AB_2880634). The secondary antibodies were as follows: Alexa Fluor 488-conjugated AffiniPure goat polyclonal anti-rabbit IgG (H + L) (RRID: AB_2338046) and Alexa Fluor 594-conjugated AffiniPure goat polyclonal anti-mouse IgG (H + L) (RRID: AB_2338871) (both at 1:800; Jackson ImmunoResearch Laboratories, Philadelphia, PA, USA).

Opal seven color manual kit (Akoya Bioscience, Marlborough, MA, USA) was used for co-immunostaining of Nkx6.1, PC1/3 and C-peptide with glucagon, which was detected by Vectra Polaris (Akoya Bioscience).

For cell quantification in the immunofluorescent staining, three to five equally spaced sections (which covered the entire pancreas) per pancreas were imaged, and the total numbers of positive staining cells from three to nine mice per group were counted manually.

Primary mouse islet culture and intervention

Primary mouse islets were incubated with 1,000 nmol/L REMD 2.59 or human IgG for 24 h in high glucose (25 mmol/L) condition. Islets and supernatants were collected for quantitative RT-PCR analysis and hormone measurement, respectively.

Glucose-stimulated insulin secretion test

After cultured with 1,000 nmol/L REMD 2.59 or IgG for 24 h, five similarly-sized islets were selected from each group. The islets were preincubated in Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 1 g/L BSA and 2.8 mmol/L glucose for 1 h at 37 °C, and then were transferred to another dish with the same buffer. After 1 h of incubation, the buffer was sampled for insulin measurement, and the islets were exposed to a 1-h 16.7 mmol/L glucose challenge followed by another sample for insulin measurement. The insulin secretion in each group was normalized to the total protein content.

Hormone measurement

Blood samples, culture supernatants and islet lysates, and KRBB buffer samples were evaluated with specific ELISA kits for detecting insulin (Millipore), C-peptide (Millipore) and glucagon (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

Protein extraction and Western blot analysis

The mouse pancreatic β-cell line Min6 cells, kindly gifted by Prof. Yiming Mu from the General Hospital of the People's Liberation Army (Beijing, China), were cultured in DMEM (25 mmol/L glucose; Invitrogen) supplemented with 15% (*v/v*) FBS, 2 mmol/L GlutMax and 55 μmol/L β-mercaptoethanol (Thermo Fisher Scientific). Total proteins from Min6 cells were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer, which contained protease inhibitor and phosphatase inhibitor. The denatured proteins (approximately 30 μg) were separated by 12% (*w/v*) SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4 °C with the primary antibodies (both at 1:1,000 dilution): rabbit anti-GCGR (Proteintech; RRID: AB_2880634) and mouse anti-GAPDH (Zhongshan Biotechnology, Beijing, China; RRID: AB_2107448). After three washes, the blots were incubated for 1 h with RDye 800CW-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (both at 1:10,000 dilutions; LICOR Biosciences, Lincoln, NE, USA). Protein bands were visualized with an Odyssey 290 infrared imaging system (LICOR Biosciences). GAPDH was used as a loading control.

RNA extraction and reverse transcription

Total RNA was extracted with Trizol reagent (Thermo Fisher Scientific) and reversely transcribed to cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific).

Conventional and quantitative RT-PCR

The cDNA was subjected to either the conventional RT-PCR analysis using Taq PCR Mastermix (Tiangen biotechnology, Beijing, China) on an Applied Biosystems Veriti 96 well Thermal Cycler PCR detection system (Thermo Fisher Scientific) or the quantitative RT-PCR analysis using iQ SYBR Green supermix (BioRad Laboratories, Hercules, CA, USA) on a QuantStudio five Real-Time PCR System (Thermo Fisher Scientific). Relative quantification for gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, which was normalized to the internal reference, *β-actin*. The primer sequences were summarized in Table S1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean ± SEM or median (interquartile range). All statistical analyses were performed using GraphPad Prism v.7.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as $p < 0.05$ and determined by Student's *t* tests, one-way or two-way ANOVA followed by the *post hoc* Tukey-Kramer test, or Mann-Whitney test, when appropriate.