

Liver-Specific Disruption of the Murine Glucagon Receptor Produces α -Cell Hyperplasia

Evidence for a Circulating α -Cell Growth Factor

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Glucagon is a critical regulator of glucose homeostasis; however, mechanisms regulating glucagon action and α -cell function and number are incompletely understood. To elucidate the role of the hepatic glucagon receptor (Gcgr) in glucagon action, we generated mice with hepatocyte-specific deletion of the glucagon receptor. *Gcgr*^{Hep-/-} mice exhibited reductions in fasting blood glucose and improvements in insulin sensitivity and glucose tolerance compared with wild-type controls, similar in magnitude to changes observed in *Gcgr*^{-/-} mice. Despite preservation of islet Gcgr signaling, *Gcgr*^{Hep-/-} mice developed hyperglucagonemia and α -cell hyperplasia. To investigate mechanisms by which signaling through the Gcgr regulates α -cell mass, wild-type islets were transplanted into *Gcgr*^{-/-} or *Gcgr*^{Hep-/-} mice. Wild-type islets beneath the renal capsule of *Gcgr*^{-/-} or *Gcgr*^{Hep-/-} mice exhibited an increased rate of α -cell proliferation and expansion of α -cell area, consistent with changes exhibited by endogenous α -cells in *Gcgr*^{-/-} and *Gcgr*^{Hep-/-} pancreata. These results suggest that a circulating factor generated after disruption of hepatic Gcgr signaling can increase α -cell proliferation independent of direct pancreatic input. Identification of novel factors regulating α -cell proliferation and mass may facilitate the generation and expansion of α -cells for transdifferentiation into β -cells and the treatment of diabetes. *Diabetes* 62:1196–1205, 2013

The islets of Langerhans comprise distinct populations of differentiated endocrine cells whose functions are critical for maintenance of metabolic homeostasis. Considerable progress has been made in understanding the control of β -cell growth, function, and survival (1). Moreover, advances in understanding the developmental and adaptive control of β -

cell formation coupled with insights gleaned from studies of adult and embryonic pancreatic endocrine stem cells have yielded new information regarding the cellular origin and formation of differentiated adult β -cells.

In contrast, much less is known about mechanisms governing the development, growth, and survival of the glucagon-secreting α -cell (2). Glucagon secretion is stimulated by exercise or hypoglycemia; conversely, glucagon secretion is suppressed during conditions of fuel abundance. However, development of diabetes is often associated with failure to suppress glucagon secretion in the fed state (3,4); hence, therapeutic efforts to suppress α -cell function for the treatment of type 2 diabetes are ongoing (4). Moreover, α -cell mass appears dynamic in the context of diabetes, with expansion of α -cell mass noted in the diabetic primate (5) and human pancreas (6).

The observation that functionally differentiated β -cells can arise from α -cell precursors (7–9) has engendered additional interest in the control of α -cell growth. α -Cell hyperplasia is frequently observed in settings of partial or complete glucagon deficiency (10,11) or resistance to glucagon action (12). Mice with targeted disruption of *Pcsk2* exhibit impaired generation of bioactive glucagon, mild hypoglycemia, and marked α -cell proliferation, findings that are rapidly reversed by glucagon administration (13). Similarly, transgenic expression of Pax4 in pancreatic endocrine cells results in relative glucagon deficiency and compensatory α -islet cell proliferation; exogenous glucagon administration in this setting also reduces α -cell proliferation (7). Both transient genetic reduction of glucagon receptor (*Gcgr*) expression in normoglycemic or diabetic mice using antisense oligonucleotides or complete genetic germline disruption of GCGR signaling are associated with α -cell hyperplasia (10,14). Collectively, these findings raise the possibility that α -cell transdifferentiation toward a β -cell phenotype may represent an alternative strategy for replenishment of β -cell mass in vivo.

Despite evidence linking reduction in GCGR signaling to α -cell hyperplasia, the precise tissues and signals important for stimulation of α -cell proliferation remain unknown. Because levels of GLP-1, a potent stimulator of islet cell proliferation, are extremely high in mice with partial or complete attenuation of Gcgr signaling (10,14), we analyzed α -cell mass in *Gcgr*^{-/-}:*Glp1r*^{-/-} mice (15). Although elimination of the *Glp1r* in *Gcgr*^{-/-} mice reversed improvements in β -cell function, fasting glycemia, and inhibition of gastric emptying, *Gcgr*^{-/-}:*Glp1r*^{-/-} mice continued to exhibit marked islet and α -cell hyperplasia. Similarly, mice with liver-specific disruption of Gs α exhibit α -cell hyperplasia despite genetic elimination of the *Glp1r* (16). Hence, although GLP-1 controls

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α -cell function, the *Glp1r* is not required for development of α -cell hyperplasia in *Gcgr*^{-/-} mice.

To elucidate mechanisms and tissues through which reduction in GCGR signaling promotes α -cell hyperplasia, we have assessed the importance of the liver and the tissue environment. Surprisingly, selective elimination of the hepatic *Gcgr* in *Gcgr*^{Hep-/-} mice was sufficient to recapitulate the phenotype of α -cell hyperplasia in the endogenous pancreas, suggesting that reduction of GCGR signaling in liver originates one or more signals that promote α -cell proliferation. Remarkably, transplantation of *Gcgr*^{+/+} islets under the kidney capsule of *Gcgr*^{-/-} or *Gcgr*^{Hep-/-} mice resulted in stimulation of α -cell proliferation and hyperplasia in transplanted islets, implying the existence of one or more circulating factors capable of promoting α -cell hyperplasia independent of the normal pancreatic microenvironment.

RESEARCH DESIGN AND METHODS

Animals. *Gcgr*^{-/-} mice on a C57BL/6 background were maintained as previously reported and generated by heterozygous-heterozygous breeding (10). Albumin-*Cre* (stock 003574) (17) and FLPe (stock 005703) transgenic mice were from Jackson Laboratory. *Gcgr*^{Flox} chimeric mice carrying one loxP site between exons 5 and 6 and two loxP sites in a neomycin cassette inserted between exons 12 and 13 of the *Gcgr* gene were generated in the C57BL/6 background, and the neomycin cassette was removed using the FLPe-FRT system. *Gcgr*^{Hep-/-} mice were generated by breeding *Gcgr*^{Flox} mice and Albumin-*Cre* mice. All animals were maintained on a standard rodent chow under a normal 12-h light/12-h dark cycle. All wild-type *Gcgr*^{+/+} control groups used were littermates; 1% cholic acid diet was obtained from Harlan. Experiments were conducted according to protocols and guidelines approved by the Mount Sinai Hospital Animal Care Committee or the Vanderbilt University Institutional Animal Care and Use Committee. The metabolic phenotype observed in *Gcgr*^{Hep-/-} mice was similar in males and females.

Glucose challenge and measurement of plasma metabolites. Glucose tolerance tests were performed as described (18,19). For plasma insulin and glucagon determinations, blood samples (100 μ L) were drawn from the tail vein during the 0-min and 15-min time periods after glucose administration in a heparinized tube. Plasma insulin was measured using a mouse insulin ELISA kit (Alpco), and plasma levels of glucagon, GIP, GLP-1, and interleukin-6 were measured using a mouse Milliplex assay (Millipore). SDF-1 levels were measured using the mouse CXCL12/SDF-1 α Quantikine ELISA Kit from R&D system. Plasma levels of total bile acids were measured with a total bile acids test kit (Enzyme Cycling) from Diazyme.

Glucagon and insulin tolerance tests. For glucagon challenge, mice were fasted for 5 h and then injected intraperitoneally with glucagon (16 mg/kg body weight). To test the effect of insulin on plasma glucose (insulin tolerance test), mice fasted for 5 h were administered 0.7 units/kg of human insulin (Novolin GE; Novo Nordisk) by intraperitoneal injection.

Hyperinsulinemic euglycemic clamp. Hyperinsulinemic euglycemic clamps were performed in conscious unrestrained mice as described, 5–6 days after catheter implantation (21).

Histology. Pancreata were weighed, fixed in 10% neutral buffered formalin solution for 48 h, and then embedded in paraffin or 4% paraformaldehyde/0.1 mol/L PBS as described (20,22). For assessment of α -cell and β -cell mass, pancreatic sections were immunostained for insulin and/or glucagon, followed by scanning using the ScanScope CS system (Aperio Technologies) at $\times 20$ magnification. Digital images were analyzed with ScanScope software (Aperio Technologies). Immunofluorescent imaging was performed using an Olympus Epifluorescent Microscope or a ScanScope FL Digital Slide Scanner (Aperio, Vista, CA). Cell counts were performed using Meta Imaging Series 7.1 (Metamorph) software. The α -cell and β -cell mass analyses were performed with Spectrum Analysis software (Aperio) using mass analyses algorithm normalizing glucagon or insulin staining area to total amylose staining area as an approximation of total pancreas area.

RNA preparation and real-time PCR. RNA was extracted from liver using Trizol Reagent (Sigma-Aldrich, St. Louis, MO). Real-time quantitative PCR reactions were performed using the TaqMan Gene Expression Assay Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Values for mRNA transcripts were normalized to the levels of *Ppia*, which were not different between groups.

Islet transplantation experiments. Pancreatic islets were isolated from wild-type and *Gcgr*^{-/-} mice for transplantation beneath the renal capsule as

described (20,22). Isolated hand-picked islets were incubated overnight in Roswell Park Memorial Institute medium (RPMI) 5.6 mmol/L glucose with 10% FBS before transplantation. Pancreatic islets isolated from 14 week-old wild-type or *Gcgr*^{-/-} animals were transplanted under the renal capsule of 14-week-old recipients (wild-type, *Gcgr*^{-/-} *Gcgr*^{Flox}, and *Gcgr*^{Hep-/-} mice). After 1, 4, or 8 weeks, the kidneys containing the islet grafts were removed and processed as described. Thin sections (5 μ m) were stained for glucagon, insulin, and Ki67 as described (22).

Hormone content in pancreas, isolated islets, and transplanted islet grafts. Whole pancreata were dissected in 0.1 mol/L PBS and weighed. For isolated islets, islets were isolated and 60 size-matched islets were hand-picked. For graft measurements, islet grafts were dissected from the kidney parenchyma. Tissues were homogenized with a Tissue Tearor or small hand-held homogenizer in 0.14 N HCL/95% ethanol. Aliquots were measured for insulin, C-peptide, and glucagon using radioimmunoassay or for GLP-1 using a mouse Milliplex assay (all from Millipore) by the Vanderbilt Hormone and Analytical Core.

Statistical analysis. Statistical significance was assessed by one-way or two-way ANOVA using Bonferroni multiple comparison posttest and, when appropriate, by unpaired Student *t* test using GraphPad Prism 5 (GraphPad Software; San Diego, CA). A *P* < 0.05 was considered to be statistically significant.

RESULTS

Generation of mice with hepatocyte-specific deletion of the glucagon receptor. To generate *Gcgr*^{Hep-/-} mice, *Gcgr*^{Flox} mice were mated with Alb-*Cre* mice (Supplementary Fig. 1A). *Gcgr* expression in the liver was abundant and comparable in wild-type and *Gcgr*^{Flox} mice and was virtually absent in *Gcgr*^{Hep-/-} mice (Supplementary Fig. 1B). In contrast, *Gcgr* mRNA transcripts were expressed at normal levels in other tissues, including pancreas, kidney, and jejunum (Supplementary Fig. 1C–E). **Deletion of the *Gcgr* in hepatocytes leads to mild hypoglycemia and abrogates the hyperglycemic response to acute glucagon challenge.** *Gcgr*^{Hep-/-} mice exhibited normal body weight compared with littermate controls (Supplementary Fig. 2A) and reductions in fasting (Fig. 1A) and random glucose levels (Supplementary Fig. 2B), similar to *Gcgr*^{-/-} mice (11,16). Plasma glucose levels increased briskly in response to exogenous glucagon in control mice; however, *Gcgr*^{Hep-/-} mice failed to exhibit a glycemic response after acute glucagon challenge (Supplementary Fig. 2C), findings that also were reported for *Gcgr*^{-/-} mice (10).

Both *Gcgr*^{-/-} and *Gcgr*^{Hep-/-} mice display improved glucose tolerance, decreased fasting insulin, and increased plasma glucagon levels. Intraperitoneal (Fig. 1B) and oral (Fig. 1F) glucose tolerance were improved in *Gcgr*^{Hep-/-} mice compared with littermate controls, findings that are similar to those described for *Gcgr*^{-/-} mice (10). Plasma glucagon levels were markedly elevated in *Gcgr*^{Hep-/-} mice (Fig. 1C, G) and similar to levels reported for *Gcgr*^{-/-} mice (10,11). Furthermore, plasma insulin levels were reduced in the fasting state, but were not significantly different during glucose challenge in *Gcgr*^{Hep-/-} mice compared with wild-type and *Gcgr*^{Flox} littermate controls (Fig. 1D, H), as reported in *Gcgr*^{-/-} mice (10). Nevertheless, fasting glucose and insulin were lower, and insulin-to-glucose ratios were significantly increased in *Gcgr*^{Hep-/-} versus control mice after glucose challenge (Fig. 1B, D–F, H, I). The similarity of the metabolic phenotypes in *Gcgr*^{-/-} and *Gcgr*^{Hep-/-} mice reveals a critical role for the hepatic *Gcgr* in the phenotype of *Gcgr*^{-/-} mice.

***Gcgr*^{Hep-/-} mice exhibit increased insulin sensitivity.** Insulin challenge resulted in a greater initial decline in blood glucose in *Gcgr*^{Hep-/-} mice (Fig. 2A) but no difference in overall glucose excursion (Fig. 2B), as previously

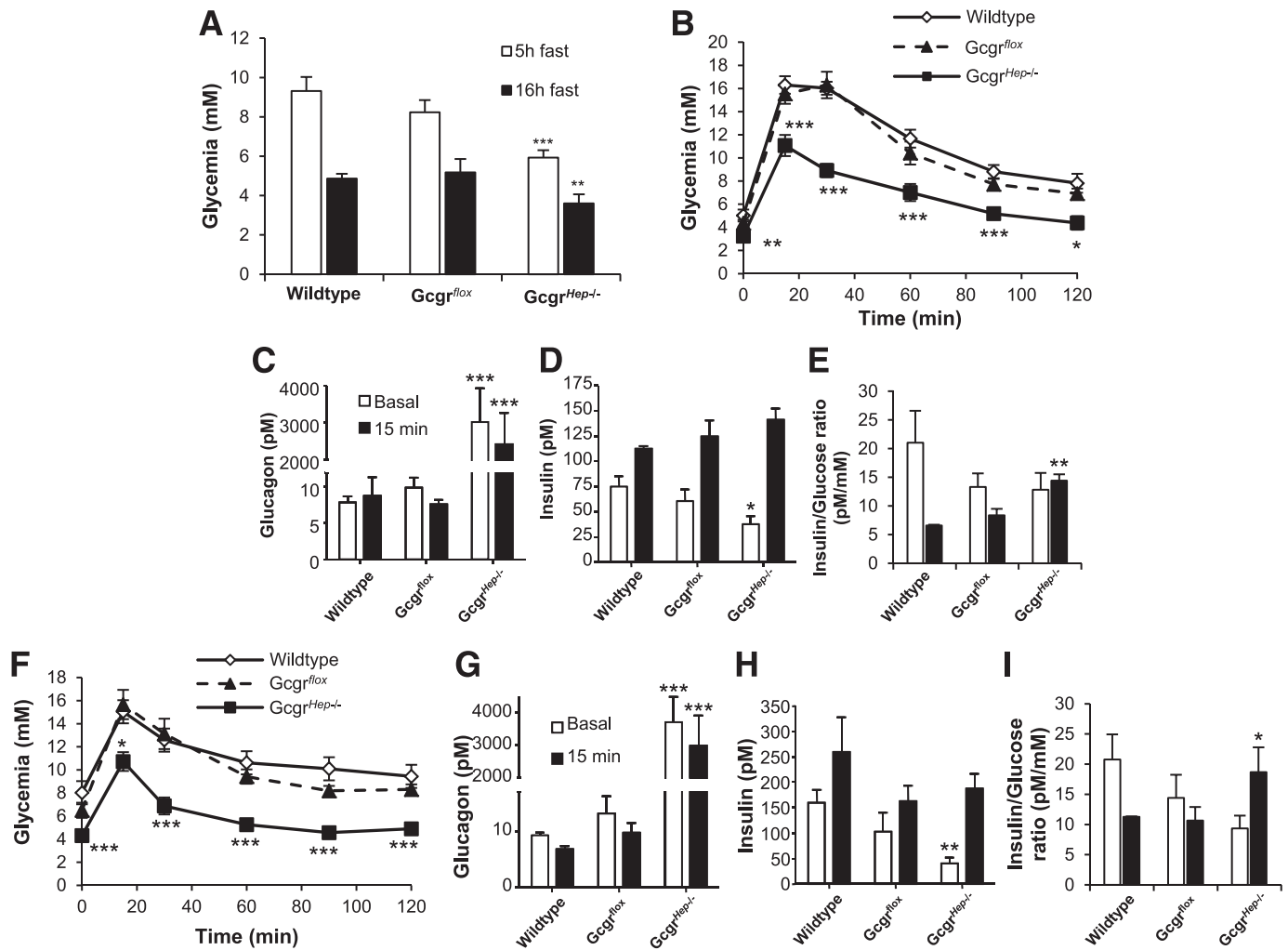


FIG. 1. Ablation of the hepatocyte *Gcgr* leads to hypoglycemia and improved glucose tolerance. **A:** Glycemia in 12-week-old male *Gcgr^{Hep-/-}* and littermate controls fasted for 5 h or 16 h ($n = 10-12$ mice per group). **B:** Intraperitoneal (IP) glucose challenge in 9-week-old male *Gcgr^{Hep-/-}* and littermate controls fasted for 16 h ($n = 8-10$ mice). Plasma glucagon (**C**) and insulin (**D**) levels measured before and 15 min after IP glucose injection ($n = 8-10$ mice per group). **E:** Insulin-to-glucose ratio before and 15 min after IP glucose injection. **F:** Oral glucose challenge in 8-week-old *Gcgr^{Hep-/-}* males and littermate controls fasted for 16 h ($n = 10-12$ mice per group). Plasma glucagon (**G**) and insulin (**H**) levels measured before and 15 min after oral glucose administration ($n = 10-12$ mice per group). **I:** Insulin-to-glucose ratio before and 15 min after oral glucose injection. Data are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. wild-type mice.

shown in *Gcgr^{-/-}* mice (10). Furthermore, *Gcgr^{-/-}* mice displayed increased insulin sensitivity compared with *Gcgr^{+/+}* littermate controls (Fig. 2C, D). Insulin sensitivity was similarly increased in *Gcgr^{Hep-/-}* mice (Fig. 2E, F). The comparable phenotypes of *Gcgr^{-/-}* versus *Gcgr^{Hep-/-}* mice indicate that the impact of glucagon on glucose tolerance and insulin sensitivity is largely mediated by hepatic glucagon receptors.

Disruption of GCGR signaling in the liver results in increased pancreas weight, α -cell proliferation, and α -cell hyperplasia. *Gcgr^{-/-}* mice exhibit markedly increased plasma glucagon levels, increased mass of the entire pancreas and islet, and α -cell hyperplasia (Fig. 3A, B) (10,11). Although total β -cell mass is increased in *Gcgr^{-/-}* and *Gcgr^{Hep-/-}* mice (Supplementary Fig. 3A, B), this increase is secondary to the enlargement of the pancreas itself, because β -cell mass per gram of pancreas is similar in 20-week-old *Gcgr^{-/-}* and wild-type littermate controls (Fig. 3C). Additionally, glucagon and GLP-1 content in the pancreas of *Gcgr^{-/-}* mice is markedly increased, whereas insulin and C-peptide content is unchanged when corrected

for changes in pancreatic mass (Supplementary Fig. 2D). The increase in α -cell mass (Fig. 3D) is accompanied by a significant increase in α -cell proliferation (Fig. 3E-G). Despite preservation of *Gcgr* expression in the pancreas (Supplementary Fig. 1C), *Gcgr^{Hep-/-}* mice exhibited markedly elevated plasma GLP-1 and glucagon levels (Supplementary Fig. 4A and Fig. 1C, G), increased pancreas weight (Fig. 4B), and absolute β -cell mass (Supplementary Fig. 3B), but normal β -cell mass per gram of pancreas (Fig. 4C), comparable with the phenotype of *Gcgr^{-/-}* mice (Fig. 3 and Supplementary Fig. 3A, B). Although GIP is produced in α -cells (23), plasma GIP levels were normal in *Gcgr^{-/-}* and *Gcgr^{Hep-/-}* mice (Supplementary Fig. 4C, D). Despite preservation of pancreatic *Gcgr* expression, *Gcgr^{Hep-/-}* mice exhibited increased α -cell proliferation with islet and α -cell hyperplasia (Fig. 4A, D-G), indicating that this process is mediated by loss of hepatic GCGR signaling.

Increased α -cell proliferation in islets transplanted into *Gcgr^{-/-}* mice. The findings of islet and α -cell hyperplasia arising in *Gcgr^{Hep-/-}* mice implies that

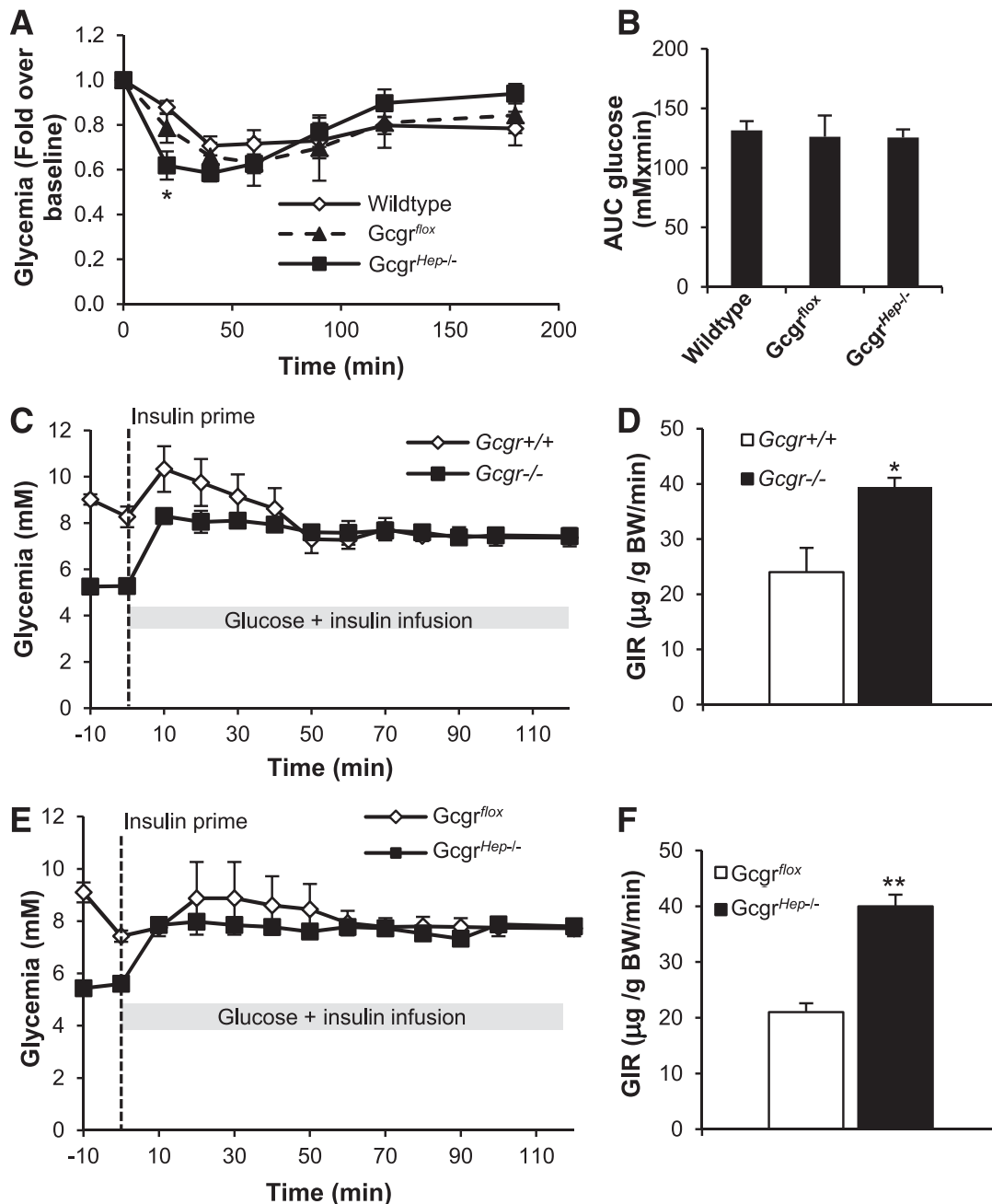


FIG. 2. Ablation of the *Gcgr* in hepatocytes increases insulin sensitivity. **A:** Intraperitoneal (IP) insulin tolerance test in 13-week-old male *Gcgr^{Hep-/-}* and littermate controls fasted for 5 h ($n = 8-10$ mice). **B:** Area under the curve (AUC) glucose from the IP insulin tolerance test shown in **A**. Hyperinsulinemic euglycemic clamp performed in conscious *Gcgr^{-/-}* (**C, E**) or *Gcgr^{Hep-/-}* (**E, F**) males and littermate controls fasted for 5 h ($n = 5-7$ mice). **C** and **E:** Glycemic excursion during stabilization and steady-state phase. **D** and **F:** Glucose infusion rates during steady state to maintain euglycemia. Data are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs. wild-type mice.

disruption of GCGR signaling in the liver initiates signals that are, in turn, communicated to the pancreas, perhaps through neural pathways or circulating factors, promoting α -cell proliferation. To determine if the proliferative signals are dependent on the pancreatic location, we transplanted 14-week-old *Gcgr^{+/+}* (wild-type) islets into 14-week-old *Gcgr^{+/+}* and *Gcgr^{-/-}* mice and allowed them to engraft for 8 weeks (Fig. 5A). We also transplanted *Gcgr^{-/-}* islets into the contralateral kidney of the same *Gcgr^{+/+}* and *Gcgr^{-/-}* mice. In this approach, the endogenous pancreatic islets in the transplant recipient serve as a control. Isolated islets from 14-week-old *Gcgr^{-/-}* mice before

transplantation had higher glucagon and GLP-1 content, but lower insulin and C-peptide content when compared with *Gcgr^{+/+}* islets (Fig. 5B). Remarkably, transplantation of *Gcgr^{+/+}* islets under the kidney capsule of *Gcgr^{-/-}* mice resulted in a striking increase in glucagon and GLP-1 content of the grafts (Fig. 5C). A small increase in insulin also was observed in *Gcgr^{+/+}* grafts in *Gcgr^{-/-}* recipient mice. The area of glucagon-positive cells in the transplanted *Gcgr^{+/+}* islets also was increased in *Gcgr^{-/-}* recipients (Fig. 5E, F, D). Furthermore, *Gcgr^{+/+}* islets transplanted into *Gcgr^{-/-}* mice exhibited significantly increased α -cell proliferation compared with *Gcgr^{+/+}*

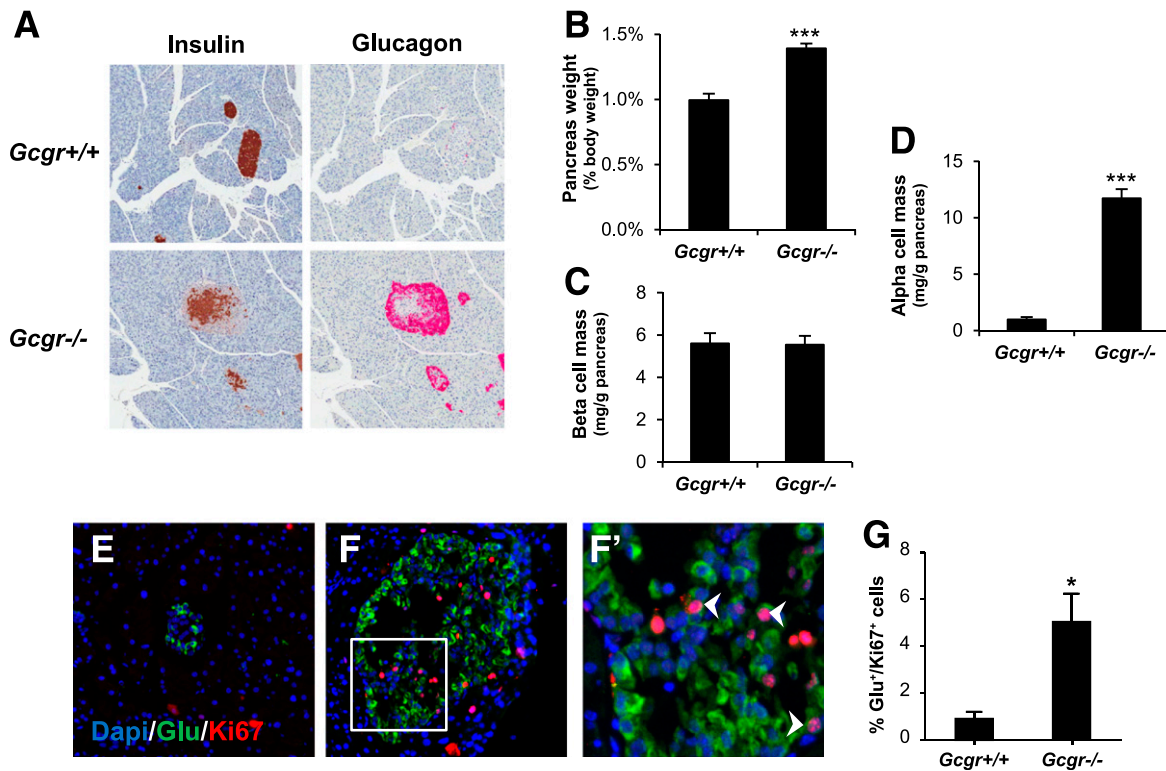


FIG. 3. Whole-body ablation of the *Gcgr* increases pancreatic weight, α -cell mass, and α -cell proliferation. **A:** Histological sections stained for insulin (left panels) or glucagon (right panels). **B:** Pancreas weight of 20-week-old *Gcgr*^{-/-} males and littermate controls corrected for body weight ($n = 8$ –10 mice). Pancreatic β -cell mass (**C**) and α -cell mass (**D**) per gram of pancreas. Data are mean \pm SEM. *** $P < 0.001$ vs. *Gcgr*^{+/+}. Representative immunofluorescent sections stained for dapi (blue), glucagon (green), and Ki67 (red) from 22-week-old *Gcgr*^{-/-} mice (**F**) and littermate controls (**E**). **F'**: Higher magnification of Ki67-positive nuclei from **F**. White arrows indicate Ki67-positive nuclei of α -cells. **G:** The α -cell proliferation rate (percentage of glucagon⁺/Ki67⁺ cells per total glucagon⁺ cells, $n = 4$ per group; 3 depths/pancreas). Data are mean \pm SEM. * $P < 0.05$ vs. *Gcgr*^{+/+}.

recipients, similar to the rate of α -cell proliferation in the *Gcgr*^{-/-} pancreas (Figs. 3G and 6B, E). In contrast, α -cell proliferation was extremely low after transplantation of *Gcgr*^{+/+} islets into *Gcgr*^{+/+} mice (Fig. 6A, E). Conversely, the grafts of *Gcgr*^{-/-} islets transplanted into *Gcgr*^{+/+} mice had reduced α -cell glucagon and GLP-1 content (Fig. 5D) and reduced α -cell proliferation (Fig. 6C, F) when compared with *Gcgr*^{-/-} recipients, whereas α -cell area was not significantly different (Fig. 5G, J).

To investigate how quickly α -cells respond to the proliferative stimulus and whether innervation is involved in the increased α -cell proliferation, 14-week-old wild-type islet grafts were removed 1 week after transplantation into *Gcgr*^{-/-} mice, a time point before reinnervation occurs or revascularization is complete (20,24). We observed a significant increase in α -cell proliferation in *Gcgr*^{+/+} islet grafts in *Gcgr*^{-/-} recipients 1 week after transplantation (Fig. 6G).

Because α -cell proliferation and hyperplasia occurs in the pancreas of both *Gcgr*^{-/-} and *Gcgr*^{Hep-/-} mice, we tested whether wild-type islets transplanted into *Gcgr*^{Hep-/-} recipients recapitulated the phenotype observed in *Gcgr*^{-/-} recipients. *Gcgr*^{+/+} islets transplanted into *Gcgr*^{Hep-/-} littermates had an increase in α -cell area and α -cell proliferation 1 month after transplantation compared with those transplanted into *Gcgr*^{Flox} mice (Fig. 7A–F). The magnitude of increase in glucagon area in *Gcgr*^{+/+} islets transplanted into *Gcgr*^{Hep-/-} recipients after 4 weeks was less than after transplantation of *Gcgr*^{+/+} islets into *Gcgr*^{-/-} mice after 8 weeks.

Analysis of potential mechanisms contributing to α -cell hyperplasia. Because β -cell hyperplasia arising as a result of liver insulin resistance has been linked to increased ERK1/2 phosphorylation through a liver–pancreas vagal signal, we assessed whether loss of hepatic glucagon signaling leads to enhanced ERK1/2 phosphorylation (25). Western blot analysis of hepatic extracts from wild-type, *Gcgr*^{Flox}, and *Gcgr*^{Hep-/-} mice revealed similar levels of ERK1/2 phosphorylation across genotypes (Supplementary Fig. 5).

Plasma levels of interleukin-6, associated with α -cell hyperplasia (26), were similar in control, *Gcgr*^{-/-}, and *Gcgr*^{Hep-/-} mice (Supplementary Fig. 6A, B). Plasma levels of SDF-1, known to induce expression of prohormone convertase-1, GLP-1, and islet cell proliferation (27), were similar in *Gcgr*^{-/-}, *Gcgr*^{Hep-/-}, and littermate controls (Supplementary Fig. 6C, D). Total bile acid levels were significantly increased in plasma from *Gcgr*^{Hep-/-} and *Gcgr*^{-/-} mice (Supplementary Fig. 6E, F), consistent with recent observations (28).

Because bile acids increase the secretion of proglucagon-derived peptides and promote cell proliferation, we fed wild-type mice a diet enriched with 1% cholic acid and assessed α -cell mass. Plasma bile acid levels were significantly increased after cholic acid feeding, in association with reduced levels of glucose and insulin (Supplementary Fig. 6G–I); however, pancreas weight (Supplementary Fig. 6J) and plasma glucagon levels (Supplementary Fig. 6K) were not increased. The α -cell mass was modestly increased, whereas β -cell mass was unchanged in cholic

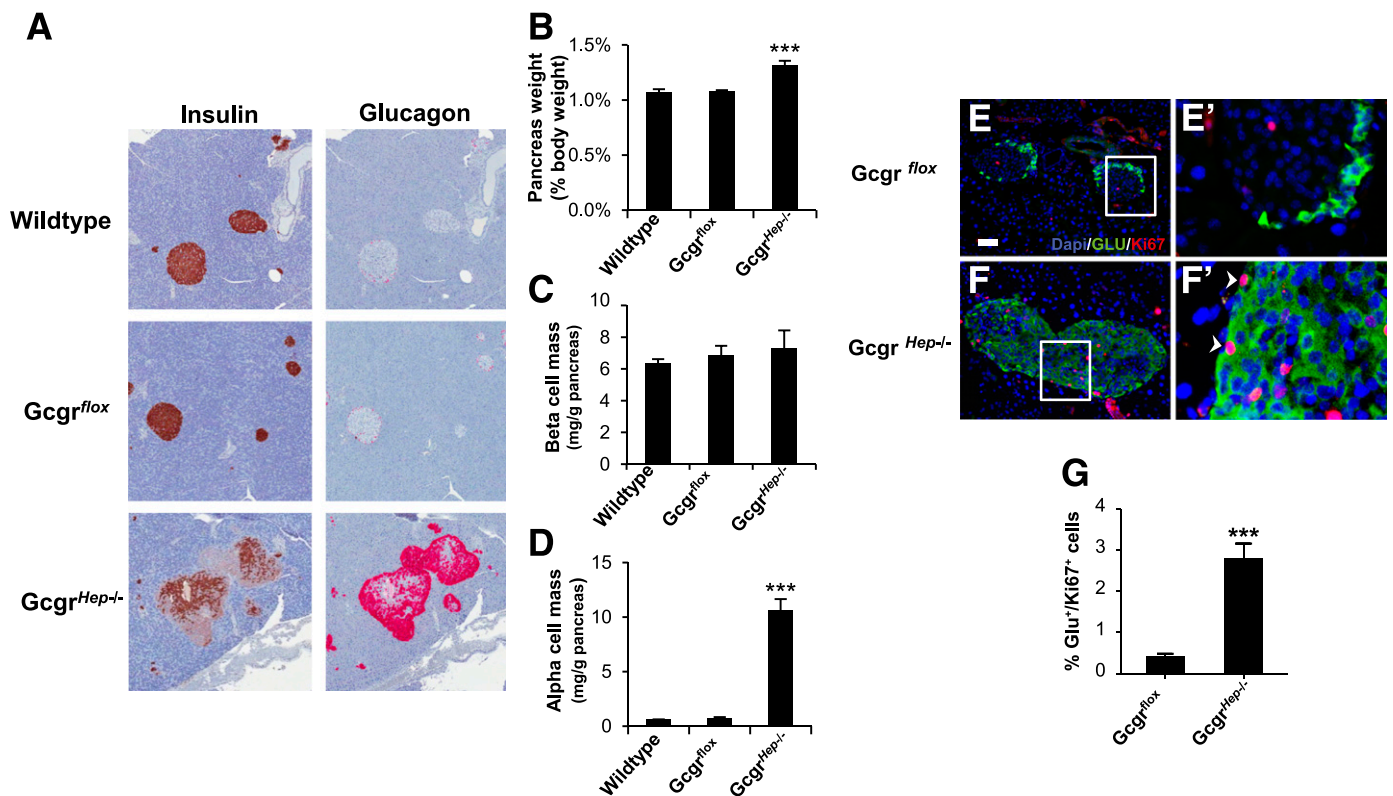


FIG. 4. Ablation of the *Gcgr* in hepatocytes results in increased pancreas weight and α -cell hyperplasia. **A:** Representative histological sections stained for insulin (left panels) or glucagon (right panels). **B:** Pancreas weight of 20-week-old *Gcgr^{Hep-/-}* males and littermate controls corrected for body weight ($n = 8-10$ mice). Pancreatic β -cell mass (**C**) and α -cell mass (**D**) per gram of pancreas. Data are mean \pm SEM. *** $P < 0.001$ vs. wild type. Representative immunofluorescent sections stained for dapi (blue), glucagon (green), and Ki67 (red) from 22-week-old *Gcgr^{Hep-/-}* mice (**F**) and littermate controls (**E**). **E'** and **F'**: Higher magnification of sections containing Ki67-positive nuclei, denoted by white arrows, in α -cells. Insets are magnifications of selected Ki67-positive nuclei of α -cells (white box). White scale bar represents 50 μ m. **G:** The α -cell proliferation rate (percentage of glucagon⁺/Ki67⁺ cells per total glucagon⁺ cells, $n = 4$ per group; 3 depths/pancreas). Data are mean \pm SEM. *** $P < 0.001$ vs. *Gcgr^{flox}*.

acid-fed mice (Supplementary Fig. 6L, M). Therefore, it seems unlikely that increased bile acids produce the phenotypes of hyperglucagonemia and increased α -cell mass in *Gcgr^{-/-}* mice. Taken together, the findings from *Gcgr^{Hep-/-}* mice and transplantation experiments support the existence of one or more circulating factors that arise after elimination of liver GCGR signaling, which promote α -cell proliferation independent of the normal pancreatic location.

DISCUSSION

By comparing mice with whole-body and hepatocyte-specific inactivation of the *Gcgr*, we found that the predominant phenotypes arising in *Gcgr^{-/-}* mice are recapitulated by selective loss of the *Gcgr* in hepatocytes. Both *Gcgr^{-/-}* mice and *Gcgr^{Hep-/-}* mice had similar reductions in fasting glucose and improvements in oral and intraperitoneal glucose tolerance and insulin sensitivity. *Gcgr^{-/-}* mice and *Gcgr^{Hep-/-}* mice also had development of similar increases in pancreatic and α -cell mass. Notably, normal islets transplanted into either *Gcgr^{-/-}* or *Gcgr^{Hep-/-}* mice displayed a marked increase in α -cell proliferation. This increased rate of α -cell proliferation at an extrapancreatic site establishes the liver as a critical organ for initiation of signals promoting the proliferation of islet α -cells after reduction or elimination of GCGR signaling.

Reduced or absent glucagon receptor signaling produced by several complementary approaches (inactivation or a reduction in glucagon and/or its receptor by genetic manipulation, immunoblockade, or antisense oligonucleotides)

leads to α -cell expansion (10,14,29). Conversely, increased glucagon receptor signaling by administration of glucagon several times daily for up to 20 days produces marked atrophy of α -cells in the endocrine pancreas (30). Experimental hyperglucagonemia in rodents with transplantable glucagonomas also produces rapid diminution of islet and α -cell mass, apoptosis of α -cells, and depletion of proglucagon mRNA expression and immunoreactivity of glucagon in the pancreas (31,32). In the transplantation studies presented here, α -cell proliferation and glucagon content are reduced when *Gcgr^{-/-}* islets are transplanted into *Gcgr^{+/+}* recipients versus *Gcgr^{-/-}* recipients (Figs. 5 and 6). This is similar to the reversal of α -cell hyperplasia observed in *Pcsk2^{-/-}* mice rescued with glucagon supplementation and GCGR antibody-treated mice on removal of treatment and restoration of glucagon signaling.

Our findings of robust α -cell hyperplasia in *Gcgr^{Hep-/-}* mice are consistent with reports of islet and α -cell hyperplasia arising in LGsKO mice with liver-specific inactivation of the gene encoding the Gs α subunit (12,16). In contrast, disruption of Gs α in the entire pancreas (33) or selectively in β -cells (34) does not produce α -cell hyperplasia. Furthermore, direct attenuation of GCGR signaling in α -cells does not result in increased rates of cell proliferation (35). In agreement with findings from LGsKO mice, our data establish the liver as critical for generation of signals promoting α -cell hyperplasia pursuant to disruption of GCGR-dependent signal transduction pathways.

An increase in α -cell proliferation has been reported in the pancreas of *Gcgr^{-/-}* embryos, raising the possibility

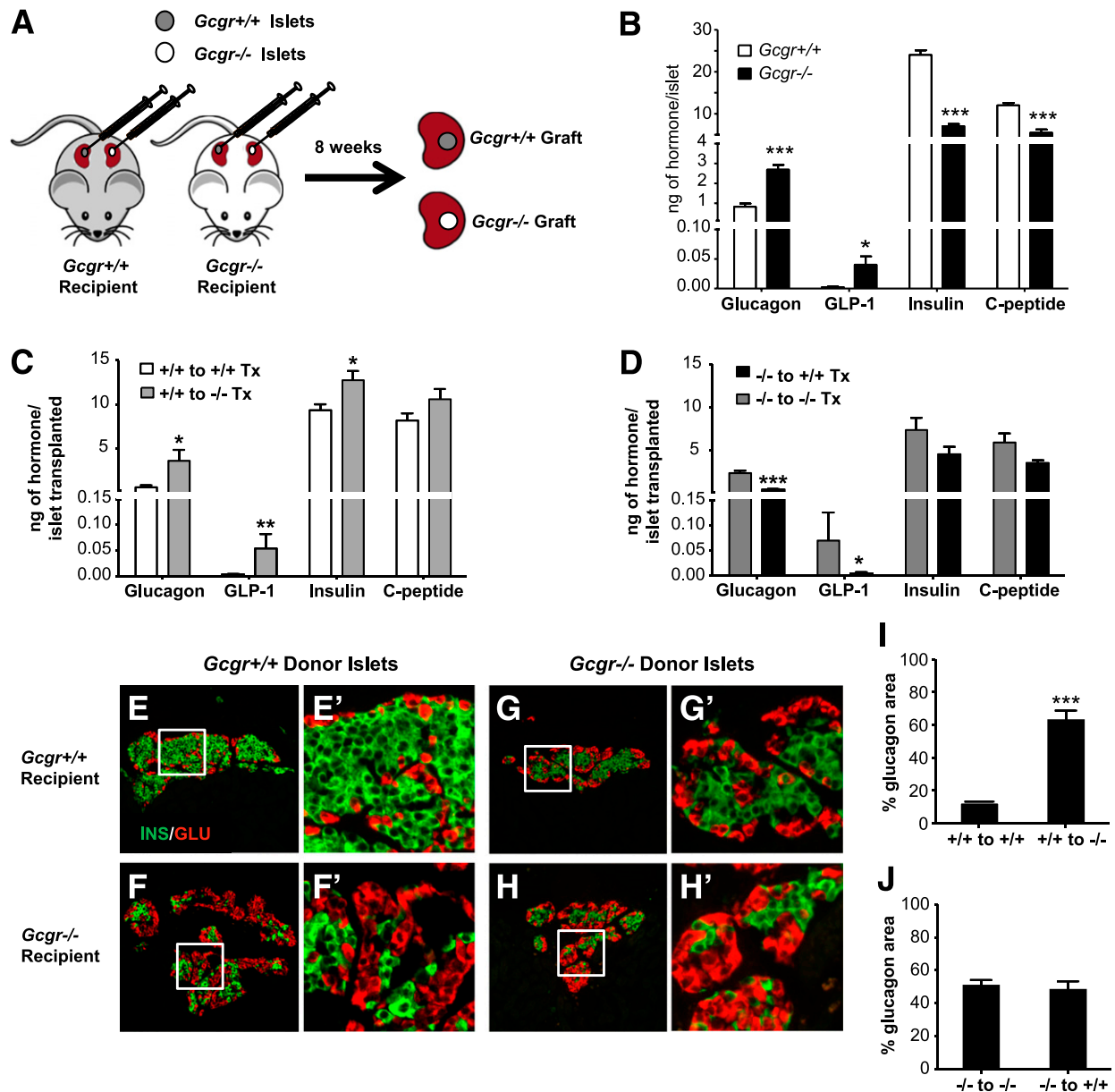


FIG. 5. *Gcgr*^{+/+} islets transplanted into *Gcgr*^{-/-} mice have development of α -cell hyperplasia. **A:** Experimental schematic showing islets from 14-week-old *Gcgr*^{+/+} or *Gcgr*^{-/-} mice transplanted beneath the right and left kidney capsule of 14-week-old *Gcgr*^{+/+} or *Gcgr*^{-/-} mice, respectively. The kidneys containing the islet graft were removed for analysis 8 weeks later. **B:** Hormone content of freshly isolated islets of 14-week-old *Gcgr*^{+/+} (open bars) and *Gcgr*^{-/-} (closed bars) mice. Data are mean \pm SEM. * $P < 0.05$, *** $P < 0.001$ vs. *Gcgr*^{+/+} islets. **C:** Hormone content of *Gcgr*^{+/+} grafts removed from 22-week-old mice *Gcgr*^{+/+} (open bars) and *Gcgr*^{-/-} (light grey bars) recipient mice 8 weeks after transplant. Data are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs. *Gcgr*^{+/+} recipients. **D:** Hormone content of *Gcgr*^{-/-} grafts removed from 22-week-old mice *Gcgr*^{+/+} (closed bars) and *Gcgr*^{-/-} (dark grey bars) recipient mice 8 weeks after transplantation. Data are mean \pm SEM. * $P < 0.05$, *** $P < 0.001$ vs. *Gcgr*^{-/-} recipients. Representative islet graft sections from *Gcgr*^{+/+} (donor) to *Gcgr*^{+/+} (recipient; **E**) and *Gcgr*^{+/+} (donor) to *Gcgr*^{-/-} (recipient; **F**) mice stained for insulin (green) and glucagon (red). **E'** and **F'**: Insets of **E** and **F** (white box). Representative islet graft sections from *Gcgr*^{-/-} (donor) to *Gcgr*^{+/+} (recipient; **G**) and *Gcgr*^{-/-} (donor) to *Gcgr*^{-/-} (recipient; **H**) mice stained for insulin (green) and glucagon (red). **G'** and **H'**: Insets of **G** and **H** (white box). White scale bar represents 50 μ m. Percentage of islet grafts that stained positive for glucagon for *Gcgr*^{+/+} donor islets (**I**) and *Gcgr*^{-/-} donor islets (**J**) into *Gcgr*^{+/+} and *Gcgr*^{-/-} mice ($n = 3$ /mice, 3 sections/islet graft). Percent glucagon area is defined as the percentage of glucagon area/total insulin + glucagon area. Data are mean \pm SEM. *** $P < 0.001$ vs. *Gcgr*^{+/+} recipient grafts.

that the signal triggering expansion of α -cell mass is attributable to the developmental loss of GCGR signaling (11). However, even partial reduction of GCGR signaling in adult mice (14,29,36) induces α -cell hyperplasia, and adult islets rapidly exhibit increased α -cell proliferation after transplantation into *Gcgr*^{-/-} recipients. Because albumin-*Cre*-mediated recombination in hepatocytes occurs largely postnatally (17,37), *Gcgr*^{Hep-/-} mice would not likely undergo significant elimination of *Gcgr* expression during

embryonic development. Hence, the available evidence strongly suggests that signals triggering α -cell expansion do not require disruption of GCGR-regulated signaling pathways during liver or islet development.

Possible pathways by which reduced or absent hepatic glucagon receptor signaling leads to α -cell hyperplasia include, among others, a stimulus transmitted from liver via intraislet nerves; however, 1-week transplantation data suggest that islet innervation is not necessary for the α -cell

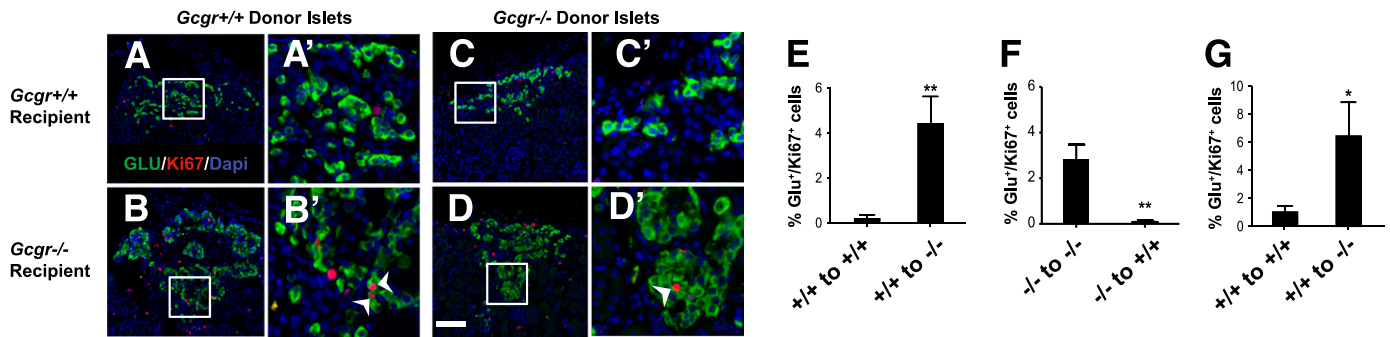


FIG. 6. *Gcgr*^{+/+} islets transplanted into *Gcgr*^{-/-} recipients exhibit increased α -cell proliferation. Islet graft sections from *Gcgr*^{+/+} (donor) to *Gcgr*^{+/+} (recipient; A) and *Gcgr*^{+/+} (donor) to *Gcgr*^{-/-} (recipient; B) mice stained for Ki67 (red), glucagon (green), and dapi (blue). A' and B': Insets of A and B (white box from A, B). White arrows indicate Ki67-positive α -cells. Islet graft sections from *Gcgr*^{+/+} (donor) to *Gcgr*^{+/+} (recipient; C) and *Gcgr*^{-/-} (donor) to *Gcgr*^{-/-} (recipient; D) mice stained for Ki67 (red), glucagon (green), and dapi (blue). C' and D': Insets of C and D (white box from C, D). White arrows indicate Ki67-positive α -cells. White scale bar represents 50 μ m. Proliferation rate of α -cells in grafts of islets from 14-week-old *Gcgr*^{+/+} (E) or *Gcgr*^{-/-} (F) mice (donors) transplanted beneath the right and left kidney capsule, respectively, of 14-week-old *Gcgr*^{+/+} (recipient) or *Gcgr*^{-/-} (recipient) mice. The kidneys containing the islet graft were removed for analysis 8 weeks later ($n = 3$ /mice, 3 sections/islet graft). Data are mean \pm SEM. ** $P < 0.01$ vs. control grafts (donor and recipient of same genotype). G: Proliferation rate of α -cells in grafts of islets from 14-week-old *Gcgr*^{+/+} mice transplanted beneath the right and left kidney capsule of 14-week-old *Gcgr*^{+/+} or *Gcgr*^{-/-} mice. The kidney containing the islet graft was removed for analysis 1 week later ($n = 4$ –6/mice, 3 sections/graft). Data are mean \pm SEM. * $P < 0.05$ vs. *Gcgr*^{+/+} recipient grafts.

proliferation. Slightly lower blood glucose could stimulate α -cell hyperplasia; however, previous studies show that mild hypoglycemia arising in some murine models of disrupted glucagon action is not required or sufficient to stimulate α -cell hyperplasia (14,29,31,38–40). Impaired glucagon signaling in β -cells could stimulate α -cell hyperplasia by intraislet communication; however, *Gcgr*^{Hep-/-} mice retain intact GCGR function in β -cells yet have development of severe hyperglucagonemia. Excessive insulin signaling in α -cells in the absence of adequate glucagon signaling promotes α -cell hyperplasia; a recent report suggests that insulin can induce α -cell proliferation, and Kawamori et al. (41) have shown that interruption of insulin receptor signaling in α -cells alone can reduce α -cell mass. However, interruption of insulin receptor signaling in α -cells is not sufficient to avert α -cell hyperplasia observed in mice with global interruption of GCGR signaling (41). A circulating factor released from the liver or released from other tissues after loss of liver GCGR signaling could be responsible for regulating α -cell mass. The current results strongly support the latter possibility, namely

the existence of one or more circulating factors regulating α -cell mass. Because α -cell proliferation increased to a greater extent in islets transplanted into *Gcgr*^{-/-} recipients for 8 weeks, relative to findings in *Gcgr*^{+/+} islets engrafted in *Gcgr*^{Hep-/-} mice for 4 weeks, interpretation of quantitative differences in the magnitude of changes observed in islets transplanted into *Gcgr*^{-/-} versus *Gcgr*^{Hep-/-} mice will require additional experimentation.

Imai et al. (25) demonstrated that increased activity of a hepatic extracellular regulated kinase triggered β -cell proliferation in murine models of insulin resistance or deficiency, and pancreatic afferent vagotomy abolished this stimulation of β -cell proliferation and increased ERK1/2 phosphorylation through a liver–pancreas vagal signal. We did not find a change in ERK1/2 phosphorylation or in plasma levels of interleukin-6, a circulating factor that promotes α -cell proliferation in experimental models of diabetes (26). Similarly, plasma levels of SDF-1, a chemokine known to promote islet GLP-1 production and islet cell proliferation, were normal. Although plasma levels of bile acids are markedly elevated in *Gcgr*^{-/-} mice, α -cell

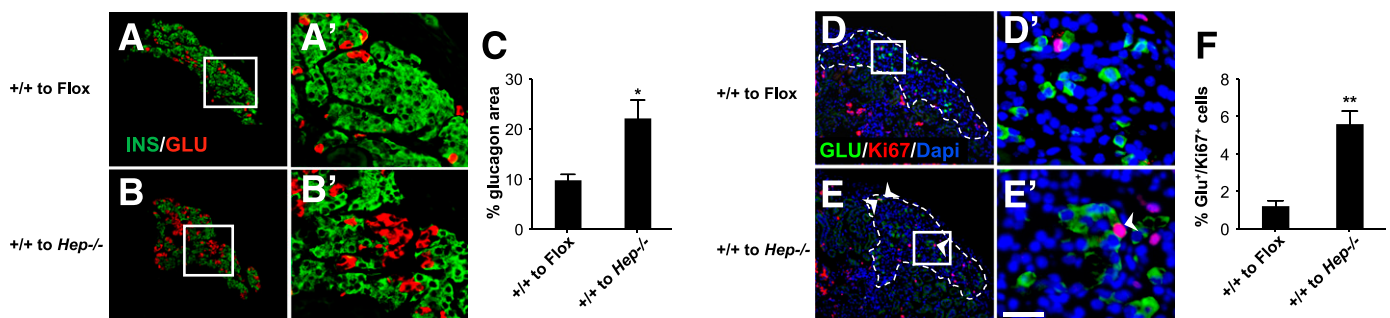


FIG. 7. Wild-type islets transplanted into *Gcgr*^{Hep-/-} recipients for 4 weeks have increased α -cell area and proliferation. Representative islet graft sections from *Gcgr*^{+/+} (donor) to *Gcgr*^{Flox} (recipient; A) and *Gcgr*^{+/+} (donor) to *Gcgr*^{Hep-/-} (recipient; B) mice stained for insulin (green) and glucagon (red). A' and B': Insets of A and B (white box). C: Percentage of islet grafts that stained positive for glucagon for *Gcgr*^{+/+} donor islets into *Gcgr*^{Flox} (recipient, open bars) and *Gcgr*^{Hep-/-} (recipient, closed bars) mice ($n = 4$ mice, 3 sections/islet graft). Percent glucagon area is defined as the percentage of glucagon area/total insulin + glucagon area. Data are mean \pm SEM. * $P < 0.05$ vs. *Gcgr*^{Flox} recipient grafts. Islet graft sections from *Gcgr*^{+/+} (donor) to *Gcgr*^{Flox} (recipient; D) and *Gcgr*^{+/+} (donor) to *Gcgr*^{Hep-/-} (recipient; E) mice stained for Ki67 (red), glucagon (green), and dapi (blue). D' and E': Insets of D and E (white box). White arrows indicate the Ki67-positive α -cells. White scale bar represents 25 μ m. F: Proliferation rate of α -cells in *Gcgr*^{+/+} islet graft (donor) transplanted into *Gcgr*^{Flox} (recipient, open bars) or *Gcgr*^{Hep-/-} (recipient, closed bars) mice ($n = 4$ mice, 3 sections/islet graft). Data are mean \pm SEM. ** $P < 0.01$ vs. *Gcgr*^{Flox} recipient grafts.

mass appears only modestly increased and glucagon levels are not increased in cholic acid-fed mice. Hence, the mechanisms responsible for induction of α -cell proliferation remain elusive and require further investigation.

Insulin-resistant states also have been shown to trigger β -cell proliferation in transplanted islets. Transplantation of murine islets under the kidney capsule of lean or obese normoglycemic insulin-resistant mice resulted in stimulation of β -cell proliferation and expansion of islet mass in transplanted islets (42). The findings in the current report are similar in that islet cell proliferation at an extrapancreatic site appears to be induced by a circulating factor. Gu et al. (43) transplanted human islets underneath the kidney capsule of streptozotocin-treated mice, and then administered a single course of a monoclonal antibody directed against the glucagon receptor. Although the GCGR antibody improved glucose tolerance and increased plasma levels of glucagon and GLP-1, the extent, if any, of α -cell proliferation in the islet transplant was not examined (43). Because of efforts to develop therapies targeting the GCGR for the treatment of type 2 diabetes, it is important to establish whether α -cell proliferation and hyperplasia can occur in adult human islets in response to acute interruption of GCGR signaling.

The α -cell hyperplasia arising pursuant to chronic reduction or elimination of GCGR signaling also has been reported in humans. Zhou et al. (44) reported a 60-year-old woman presenting with a pancreatic mass, extremely elevated levels of glucagon, and persistent hyperglucagonemia; marked α -cell hyperplasia was detected after surgical resection of the pancreatic mass, and sequencing of genomic DNA revealed a homozygous missense mutation in the *Gcgr* gene, resulting in markedly reduced receptor affinity. Furthermore α -cell hyperplasia, adenomatosis, and hyperglucagonemia have been reported in patients without histological or clinical evidence of malignancy, some of whom may harbor loss-of-function mutations in the *Gcgr* (45).

Recent evidence for enhanced proliferation of α -cells in the pancreas from patients with newly diagnosed type 1 diabetes (46), coupled with reports demonstrating conversion or transdifferentiation of α -cells to β -cells (7–9), have rekindled therapeutic interest in understanding the signals and mechanisms regulating α -cell proliferation. Our data combining disruption of the *Gcgr* and islet transplantation establish a new model for analysis of the control of α -cell hyperplasia. Together with recent efforts directed at promoting generation of functional β -cells from α -cells (7,8), identification of this circulating factor may provide new opportunities for regeneration of functional β -cell mass for the treatment of diabetes.

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C.L., A.M.R., E.D.D., C.D., S.A., V.d.C., and I.M. designed and carried out experiments and wrote and reviewed the manuscript. P.M.V., M.J.C., A.C.P., and D.J.D. designed experiments, reviewed results, and wrote the manuscript. A.C.P. and D.J.D. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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