

Published in final edited form as:

Diabetes Obes Metab. 2011 October ; 13(0 1): 144–150. doi:10.1111/j.1463-1326.2011.01447.x.

Novel insight into glucagon receptor action: lessons from knockout and transgenic mouse models

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Abstract

Using knockout and transgenic technology, genetically modified animal models allowed us to understand the role of glucagon signalling in metabolism. Mice with a global deletion of the glucagon receptor gene (*Gcgr*) were designed using gene targeting. The phenotype of *Gcgr*^{-/-} mouse provided important clues about the role of *Gcgr* in foetal growth, pancreatic development and glucose and lipid homeostasis. The lack of *Gcgr* activation was associated with: (i) hypoglycaemic pregnancies, poor foetal growth and increased foetal–neonatal demise; (ii) altered cytoarchitecture of pancreatic islets; (iii) altered glucose, lipid and hormonal milieu; (iv) reduced gastric emptying; (v) altered body composition and protection from diet-induced obesity; (vi) altered energy state; (vii) impaired hepatocyte survival; (viii) altered metabolic response to prolonged fasting and exercise and (ix) prevented development of diabetes in insulin-deficient mice. In contrast, mice overexpressing the *Gcgr* on pancreatic β -cells displayed an increase insulin secretion, pancreatic insulin content and β -cell mass, and partially protected against hyperglycaemia and impaired glucose tolerance when fed a high-fat diet. These findings suggest that glucagon signalling plays a significant role in the regulation of glucose and lipid homeostasis. Treatment options designed to block *Gcgr* activation may have negative implications in the treatment of diabetes.

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Conflict of Interests

The authors declare no conflicts of interests.

Keywords

diabetes; Gcgr knockout mouse model; glucagon; glucagon signalling; hypoglycaemia; islets; pancreas

Introduction

Glucagon is a 29-amino acid polypeptide secreted by the α -cell of the islet of Langerhans [1,2]. Initially synthesized as a larger precursor ‘proglucagon’, it is cleaved by a specific enzyme, proconvertase 2 to the active molecule ‘glucagon’ [3]. Glucagon is also produced by the central nervous system on which its actions may include the regulation of glucose production [1,2]. Glucagon secretion is stimulated by hypoglycaemia, arginine, gastric inhibitor polypeptide, gastrin and potassium chloride and inhibited by hyperglycaemia, insulin, zinc, GLP-1 (glucagon-like peptide 1) and somatostatin [4-12].

Glucagon action is transduced by a G-protein-coupled receptor (GCGR/Gcgr) that is a member of the class II GCGR superfamily of seven transmembrane spanning receptors that are coupled via GTP-binding proteins (G proteins) to adenylyl cyclase [13]. Glucagon also mediates an increase in intracellular calcium in a phospholipase-C-dependent manner and it activates AMPK and JNK [14,15]. Binding sites for glucagon have been identified in liver, kidney, intestinal smooth muscle, brain, adipose tissue, heart and pancreatic islet β -cells (figure 1). Controversy exists about the presence of the Gcgr in pancreatic islet α -cells [16,17]. Gcgr expression is positively regulated by glucose and negatively regulated by glucagon and agents that increase intracellular cAMP [18].

The mechanisms regulating the degradation and clearance of glucagon remain incompletely understood. Glucagon has been shown to be catabolized by the enzyme neutral endopeptidase 24.11 in pigs, as well as *in vitro* by dipeptidyl peptidase-4 at pharmacological levels [19,20]. Glucagon is cleared by the kidneys [21].

Glucagon generally functions as a counter-regulatory hormone, opposing the actions of insulin, and maintaining blood glucose levels by activating glycogenolysis and gluconeogenesis. Glucagon also elicits significant extrahepatic effects in tissues such as kidney, heart, adipose tissue, gastrointestinal tract, thyroid and central nervous system [1,2,16,22] (figure 1). Glucagon has potent hypolipemic actions and reduces triglyceride (TG) and very low-density lipoprotein release as well as the deposition of triacylglycerol in the liver, in addition to reducing cholesterol levels and stimulating fatty acid oxidation [23,24].

Studies Using the Gcgr Knockout Mouse Model (Gcgr^{-/-}) have Revealed Important Insight into the Role of the Glucagon in Glucose and Lipid Homeostasis

In order to investigate the role of glucagon in glucose and lipid homeostasis, mice with a targeted deletion within the glucagon receptor gene Gcgr^{-/-} were generated. The strategy

used involved use of the murine *Gcgr* gene and positive and negative selectable markers. Specifically, exons 3–6 were replaced with a phosphoglycerate kinase promoted neomycin resistance cassette and the negative selectable thymidine kinase gene was engineered into the 3' and 5' ends. Appropriate targeting of the mutant *Gcgr* allele in embryonic stem cells and germ line transmission was confirmed by Southern blot and PCR analysis as previously shown [25]. Additional studies showed that glucagon stimulated increases in cAMP were lacking in hepatocytes of *Gcgr*^{-/-} mice [25].

Lack of Glucagon Signal Alters Foetal Growth and Development

To determine whether glucagon signalling has an effect on foetal growth and development, *Gcgr*^{-/-}, *Gcgr*^{+/-} and WT pregnancies were sacrificed on embryonic day (e) e10.5, e15.5 and e18.5 of gestation and postnatal day 1. Embryos and pups were examined and weighed. *Gcgr*^{-/-} pregnancies were characterized by poor foetal growth and increased foetal demised during late gestation [26]. *Gcgr*^{-/-} females displayed significantly lower glucose levels throughout the pregnancy, leading to severe neonatal hypoglycaemia followed by an increase in neonatal death during the first 24 h of life [26]. Interestingly, *Gcgr*^{+/-} pregnancies were characterized by normal maternal glucose levels, litter size, foetal growth and neonatal survival [26]. These results indicate that lack of glucagon signalling in the pregnant dam alters the proper intra-uterine milieu, such as lowering glucose levels, during critical periods of development leading to poor foetal growth and increased foetal demise.

Lack of Glucagon Signal Alters Pancreatic Islet Development

To determine whether glucagon signalling has an effect on pancreatic islet development, foetal and adult *Gcgr*^{-/-} pancreata were studied as previously described by immunohistochemistry and gene expression analysis [26]. Deletion of the *Gcgr* delayed β -cell differentiation during foetal development [26]. Insulin-expressing cells were first observed in pancreata of *Gcgr*^{-/-} mice at e13.5 in contrast to e11.5 in wild type (WT) littermates, and at e15.5, pancreata of *Gcgr*^{-/-} embryos contained a similar number of insulin (β -) cells when compared to WT littermates. Insulin cell differentiation was not delayed because of alterations in the time line of expression of the peptide hormone processing enzymes which appeared at e10 in islet cell clusters [26]. Thus, ablation of *Gcgr* inhibited the differentiation of β -cells during the so-called protodifferentiated stage, which extends from early development until e day 14 in mice [27]. In addition, lack of glucagon signalling resulted in the presence of glucagon (α -cells) expressing embryonic markers [26] (figure 2). In WT islets, glucose transporter 2 (GLUT2) is usually found in the plasma membrane of β -cells and is absent from α -cells [28]; similarly, the transcription factor Pdx-1 is expressed by β -cells and by a subset of somatostatin (δ) cells throughout life and by α -cells of e10 but not of older embryos or adults [29-31]. Lack of glucagon signalling resulted in the expression of GLUT2 in the cytoplasm (figure 2g) and pancreatic duodenal homeobox-1 (PDX-1) in the nucleus and cytoplasm of α -cells (figure 2b-e) [26]. Similarly, cells co-expressing insulin and glucagon (GLU-IN cells), found only in the pancreas during the protodifferentiated stage of e10 to e15 WT mice, populated islets of adult *Gcgr*^{-/-} mice (figure 2b-d) [32,33]. Double- and triple immunostaining studies of early pancreatic buds (data not shown), as well as adult islets, showed that the vast majority of GLU-IN cells are negative for Pdx-1 (figure 2b-d). It is generally believed that, during embryonic

development, islet cells differentiate from precursors that first express Pdx-1, then initiate synthesis of GLUT2 and Neurogenin 3 (Ngn3) and is turned off by Ngn3 expression prior to the initiation of hormone expression [34]. Thus, the lack of PDX-1 expression in GLU-IN co-expressing cells suggests that those cells are generated by an independent set of precursor cells. In addition, β -cells of $Gcgr^{-/-}$ mice had low levels of Pdx-1, GLUT2 and mammalian homologues of avian A (MafA), which are molecules involved in the regulation of insulin expression [35-39]. These results indicated that the absence of glucagon signalling affects the initial stages of differentiation of islet precursor cells, may inhibit the progression of α -cells to maturity and alters the molecular profile of β -cells [26]. Thus, glucagon signalling during pancreatic development may have a local 'paracrine' role that either initiates or promotes the generation of other pancreatic endocrine progenitor cells.

Lack of Glucagon Signal Increases the Number of Islets, Alters Islet Morphology and Increases Circulating GLP-1 Levels

Loss of glucagon signalling resulted in an increase in the number of pancreatic islets [25,26]. $Gcgr^{-/-}$ islets were comprised of a thick mantle of α -cells and δ -cells surrounded by a core of β -cells (figure 3). In agreement with these findings, lack of glucagon signalling activated the expression of nestin-positive cells, which may represent endocrine precursor cells, in a subset of islets and exocrine cells of adult $Gcgr^{-/-}$ mice which suggested active islet neogenesis [40]. Morphometric analysis confirmed that the mean number of α -cells and somatostatin cells per section was more than twofold higher in $Gcgr^{-/-}$ than in WT littermates while the relative β -cell volume per section was similar in both lines. In addition to an increase in number, δ -cells in islets of $Gcgr^{-/-}$ mice exhibited an abnormal distribution as they were no longer localized to the mantle zone, but, rather, they were scattered in the core of the islet [26] (figure 3). The average polypeptide cell number per section was similar in islets of $Gcgr^{-/-}$ and WT mice [25,26]. In addition, pancreatic content of GLP-1 was significantly increased, and was accompanied by a 3- to 10-fold increase in circulating GLP-1 levels [25,41]. Interestingly, an animal model that lacks all the peptides derived from the glucagon gene (glucagon, GLP-1 and oxyntomodulin) displayed a similar phenotype of α -cell hyperplasia independent of improved glucose levels or absent GLP-1 levels [42].

These data suggest that lack of either glucagon signalling or glucagon and GLP-1 gene expression increased the number of islets and lead to non-insulin cell hyperplasia, suggesting that an 'autocrine/paracrine' stimulus independently of glucose or GLP-1 levels is present in the $Gcgr^{-/-}$ mice. The precise mechanism underlying the regulation of the number of the different islet cells per islet and of the number of islets per pancreas by glucagon remains to be elucidated.

Lack of Glucagon Signal Reduces Gastric Emptying

The effect of the lack of glucagon signalling on the oral (gastrointestinal) absorption of d-xylose was investigated using $Gcgr^{-/-}$ and WT littermates [41]. The time curve responses suggested that the lack of glucagon signalling reduced xylose absorption, indicating that $Gcgr^{-/-}$ mice may have a slower rate of gastric emptying. This observation could be the result of the increased GLP-1 levels [25,41,43], which has been shown to reduce gastric emptying [44].

Lack of Glucagon Signal Alters Glucose Homeostasis

To determine the effect of glucagon signalling on glucose homeostasis during the fed and fasting state, $Gcgr^{-/-}$ mice underwent glucose monitoring, intraperitoneal glucose tolerance test, glucagon challenge and insulin tolerance test [25]. One salient feature of the $Gcgr^{-/-}$ mice was the presence of lower blood glucose levels during the day and the development of hypoglycaemia during a prolonged fast [25]. Furthermore, despite similar insulin sensitivity during an insulin tolerance test, $Gcgr^{-/-}$ mice displayed a blunted blood glucose response to a glucose bolus when compared to WT littermates suggesting that glucose-stimulated insulin release was increased [25]. Ambient hepatic glycogen levels were significantly increased in $Gcgr^{-/-}$ compared to WT littermates but were not different during the fasting state [25]. Furthermore, lack of glucagon signalling led to a late-onset loss of retinal function, loss of visual acuity and eventual death of retinal cells [45]. Anatomical changes in the retina were seen at 10 months of age. Interestingly, the decreases in retinal function and visual acuity correlated directly with the degree of hypoglycaemia. When challenged with a high-fat diet (45% of energy from fat) $Gcgr^{-/-}$ did not develop hyperinsulinaemia, and displayed a blunted glucose excursion when challenged with a glucose bolus [41].

These data suggest that glucagon signalling is essential to maintain glucose homeostasis. Lack of glucagon signalling does not alter insulin action and may increase glucose-stimulated insulin secretion. Furthermore, lack of glucagon signalling may improve glycaemic control in models of high fat induced obesity.

Lack of Glucagon Signal Alters the Hormonal Milieu

To determine whether glucagon signal impact the hormonal milieu $Gcgr^{-/-}$ mice underwent a full metabolic profile in which several hormones and lipids were measured using standard methods [25]. $Gcgr^{-/-}$ mice display hyperglucagonaemia, similar insulin levels, fed and fasted serum free fatty acids (NEFA), lactate, cholesterol and high-density lipoprotein and increased plasma low-density lipoprotein and decreased leptin levels, when compared to a WT littermate [25]. In addition, female mice displayed decreased TG levels when compared to male $Gcgr^{-/-}$ littermates. $Gcgr^{-/-}$ mice have a small decrease in IGF-1 and a twofold increase in corticosterone during fasting, with low corticosterone levels in the afternoon, and an increased responsiveness to epinephrine [25]. In $Gcgr^{-/-}$ mice, high-fat diet does not alter plasma glucagon, GLP-1, TG, NEFA or corticosterone levels when compared to WT littermates [41]. Thus, lack of glucagon signalling does not alter insulin levels or corticosteroid release as well as circadian rhythm, increases counterregulatory hormone responsiveness and increases GLP-1 levels secondary to the overproduction of proglucagon. Furthermore, when challenged with a high-fat diet, $Gcgr^{-/-}$ mice displayed an improved hormonal milieu.

Lack of Glucagon Signal Alters Fat Deposition

To determine whether glucagon signal impacts growth or body composition, growth rates, food intake, indirect calorimetry and MRI analysis were performed using $Gcgr^{-/-}$ mice fed a control as well as a high-fat diet [25,41]. $Gcgr^{-/-}$ mice displayed similar growth rates, food intake and resting O_2 consumption and energy expenditure when compared to WT littermates [25,41]. MRI results revealed a significant decrease in total adipose mass that

was compensated for by an increase in lean body mass [25]. Interestingly, $Gcgr^{-/-}$ mice were resistant to a 12-week high fat feeding regime [41]. WT littermates consumed more fat and gained about 30% more weight compared to $Gcgr^{-/-}$. In addition, $Gcgr^{-/-}$ mice were resistant to the high-fat diet-induced hepatic steatosis as determined by Oil Red O staining of liver sections and by measuring hepatic lipid content [41]. These data suggest that the lack of glucagon signalling leads to a lean phenotype, independent of the diet, by either a direct action of the $Gcgr$ or by the increased GLP-1 levels in the central nervous system or the periphery.

Lack of Glucagon Signal Prevents the Development of Diabetes in Insulin-Deficient Mice

To determine the effect of glucagon signalling in murine models of insulin deficiency, $Gcgr^{-/-}$ mice have been treated with streptozotocin (STZ) [41,46]. Interestingly, a single dose of 125 mg/kg STZ (Sigma Chemical, St Louis, MO, USA) did not induce hyperglycaemia and pancreatic β -cell destruction when the mice were followed for 20 days after the treatment [41]. In contrast, it has been subsequently shown that two intravenous injections of STZ (100 mg/kg body weight followed 7 days later by 80 mg/kg), in $Gcgr^{-/-}$ mice results in a significant reduction in the islet β -cell mass (morphometric analysis and insulin levels followed a glucose challenge) with no clear metabolic or biochemical signs of diabetes in mice that were followed for 6 weeks [46]. These results suggest that blocking glucagon action in insulin-deficient diabetics will lead to an asymptomatic, benign, non-catabolic state.

Lack of Glucagon Signal Alters Hepatic Energy State

Based on the findings that $Gcgr^{-/-}$ mice have an attenuated energy response to fasting and lacked an exercise-induced energy change (hepatic AMP/ATP ratios) when compared to littermate controls, it has been hypothesized that glucagon signalling plays a major role in controlling hepatic energy state [47]. To test this hypothesis experiments were designed using conscious mouse models that display increased glucagon levels under stressful conditions (prolonged fasting, hypoglycaemia, diabetes, exercise, high-fat diet) [47]. In these models, PEPCK expression was found to be essential to reduce the hepatic energy changes during fasting. Similarly, increased glucagon levels decreased the hepatic energy state under euglycaemic conditions and resulted in increased AMPK activation [47]. These results suggest that $Gcgr$ activation stimulates gluconeogenic fluxes through PEPCK which lead to a low hepatic energy state that is sufficient to activate AMPK.

Lack of Glucagon Signal Alters Hepatocyte Survival

The role of the $Gcgr$ in hepatocyte and cell survival was studied in $Gcgr^{-/-}$ mice *in vivo* (susceptibility to hepatocyte apoptosis by Jo2 injection or diets deficient in methionine and choline) and *in vitro* (murine hepatocyte cell culture) [48]. Lack of glucagon signalling increased the susceptibility to experimental hepatocellular injury. Injured hepatocytes displayed signs of apoptosis, such as blebbing, chromatin condensation and cell lysis. These morphological alterations were accompanied by an increase of serum levels of transaminases. When the $Gcgr^{-/-}$ mice were challenged with a methionine and choline-deficient diet the liver developed morphological changes characteristic of non-alcoholic

steatohepatitis. The cytoprotective effects of glucagon were independent of the activation of protein kinase A (PKA), phosphatidylinositol-3 kinase (PI3K) and MAPK, but appeared dependent upon cAMP-mediated activation of adenylate cyclase [48]. These data suggest that glucagon signalling may have a critical role in the regulation of hepatocyte cell survival and challenges the idea of modulating glucagon action in patients with type 2 diabetes mellitus as a treatment option.

Lack of Glucagon Signal Alters the Metabolic Response to Fasting and Exercise

To determine the role Gcgr signalling in lipid metabolism (synthesis, secretion and oxidation) during short and long-term (5 or 16 h) fasting, Gcgr^{-/-} mice were studied *in vivo* (hepatic TG secretion) and *in vitro* (lipid synthesis, secretion and fatty acid oxidation in liver homogenates and in primary murine hepatocyte culture) [24]. After a prolonged fast, the lack of Gcgr signalling is associated with an increase in free fatty acid (FFA) and TG secretion independent of insulin and glucose levels and a decrease in FFA oxidation. Glucagon was shown to have no effect in the synthesis of newly synthesized FFA, but it significantly reduced TG synthesis by increasing FFA oxidation, and secretion as a result of a decreased availability of FFA and TG. Glucagon stimulates FFA oxidation by activating PPAR α in an AMPK- and p38 MAPK-dependent pathway [24]. To further understand the role of Gcgr signalling in the regulation of PPAR α and its subsequent effects on fibroblast growth factor 21 (FGF21), the hypothesis that the glucagon effects on AMPK and PPAR α signalling are amplified by an increased availability of lipids was tested [15]. Gcgr^{-/-} mice that underwent an 18-h fast, exhaustive treadmill exercise, conditions that have previously shown to increase glucagon action [49,50]; and a hyperglucagonaemic–euglycemic clamp with or without a simultaneous lipid infusion [15] were evaluated. Gcgr activation stimulated the expression of PPAR α and FGF21, which was further amplified by an elevation in FFAs. These data suggest that glucagon, in addition to playing a major role in the regulation of glucose metabolism in the fasted state (glycogenolysis and gluconeogenesis), plays a central role in fatty acid oxidization during prolonged fasting and in response to exercise.

Studies Using the RiP-Gcgr Transgenic Mice have Revealed Important Insights into the Role of the Gcgr in β -Cell Function

To study the functional role of Gcgr in β -cell function transgenic mice were engineered that over-express the Gcgr in insulin cells using rat insulin II promoter (RiP-Gcgr) [51]. To examine whether the RiP-Gcgr transgene increased glucagon-stimulated insulin release, islets were isolated from RiP-Gcgr mice and incubated in glucose in the presence or absence of glucagon concentrations that were subthreshold for insulin secretion in control islets. RiP-Gcgr islets displayed a significant increase in insulin secretion in response to glucagon compared to that of controls suggesting that the regulatory role of glucagon on insulin release may be associated with the number of glucagon binding sites on insulin cells. Furthermore, RiP-Gcgr mice displayed enhanced glucose tolerance compared to controls. Morphological studies of pancreata did not indicate any gross differences in the islet morphology of RiP-Gcgr mice when compared to controls. However, quantification of the islet cell population by stereology indicated a small, but significant increase (approximately

20%) in insulin cell volume compared to littermate controls. Given the enhanced glucose homeostasis displayed by RiP-Gcgr mice, it was hypothesized that insulin cell overexpression of the Gcgr may protect against the induction of diabetes when the mice were fed a highfat diet. When challenged with insulin, both RiP-Gcgr and littermate controls fed the high-fat diet displayed impaired glucose tolerance compared to low-fat-fed control and RiP-Gcgr mice. However, pancreatic islet overexpression of Gcgr resulted in a significant decrease in the glucose excursion in RiP-Gcgr mice fed the high-fat diet compared to controls fed the same diet [51]. These data suggest that increased glucagon action at the level of the insulin cell, similar to GLP-1 and GIP both in regulating insulin cell mass and in potentiating glucose-stimulated insulin secretion, by increasing insulin cell competency. These data were strengthened by the findings of low levels of Pdx-1, GLUT2 and MafA, which are molecules involved in the regulation of insulin expression, in insulin cells of Gcgr^{-/-} mice [26,35-39].

Conclusion

It has been postulated that in patients with diabetes, excess glucagon secretion plays a primary role in the metabolic perturbations associated with diabetes, such as hyperglycaemia and ketonuria [52]. Here we present evidence that glucagon signalling plays important roles in pancreatic development, insulin cell function and metabolic response to prolonged fasting, exercise, lipid metabolism, hepatic energy state and hepatocyte survival. Thus, the present findings further suggest that antagonizing glucagon action as a possible therapy for diabetes may have several unintended effects that may further compromise the regulatory response to an altered glucose metabolic state such as that seen in patients with diabetes.

Acknowledgements

We thank Drs R. Gelling, R. Burcelin, S. Ouhilal, all our collaborators and members of Charron and Vuguin laboratories who have contributed to this project over the years. This work was supported by the National Institutes of Health (Grants DK47425 and HL58119 to M. J. C. and KO8 HD-042172 to P. V.), the American Diabetes Association, Albert Einstein College of Medicine (AECOM) Comprehensive Cancer Center, the AECOM Diabetes Center and Danish Medical Research Council.

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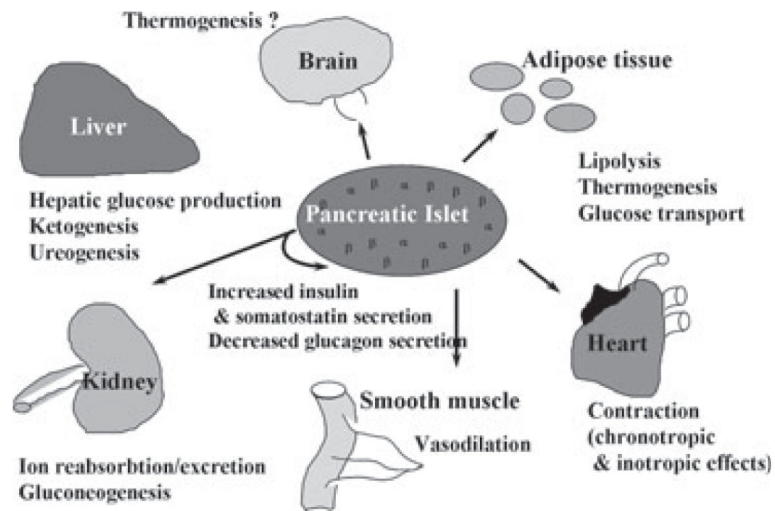


Figure 1. Glucagon action. Glucagon functions as a counterregulatory hormone, opposing the actions of insulin and maintaining blood glucose levels. Glucagon also elicits significant extrahepatic effects in tissues such as kidney, heart, adipose tissue, gastrointestinal tract (smooth muscle) and the central nervous system.

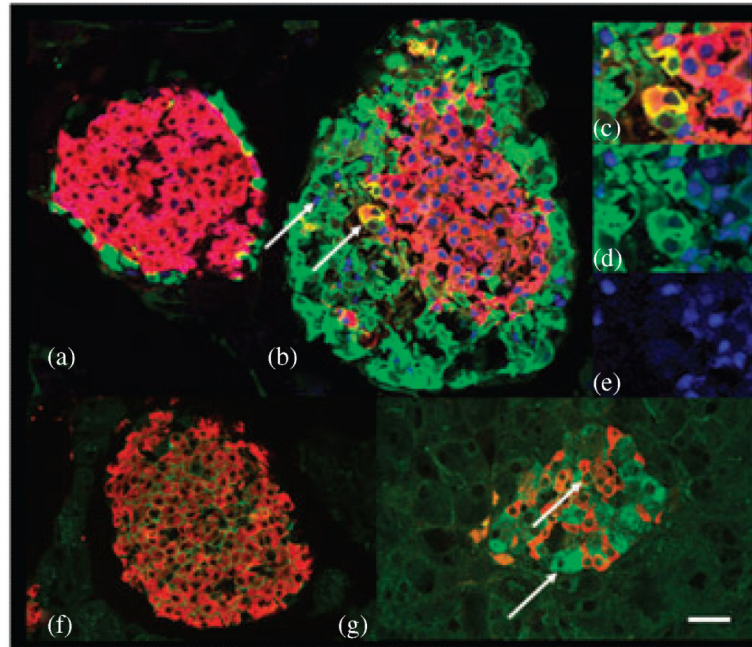


Figure 2.

α -Cells of $Gcgr^{-/-}$ mice express immature traits. Photomicrographs of islets of 3-month-old wild type mice (a and f) immunostained for visualization of (a) glucagon (green), PDX-1 (blue), insulin (red) and (f) GLUT2 (green) and insulin (red). Note that α -cells of WT mice lack GLUT2 and PDX-1 expression. Photomicrographs of islets of 3-month-old $Gcgr^{-/-}$ mice (b-e and g) immunostained for visualization of (b-e) glucagon (green), insulin (red), PDX-1 (blue) and (g) GLUT2 (green) and insulin (red). (b-e) In $Gcgr^{-/-}$ mice, a subset of α -cells coexpress Pdx-1 as indicated with arrowheads. (c-e) Similarly, coexpressing GLU-INS cells do not express PDX-1. (g) Most α -cells of $Gcgr^{-/-}$ mice express the GLUT2. Bars: 20 μ m.

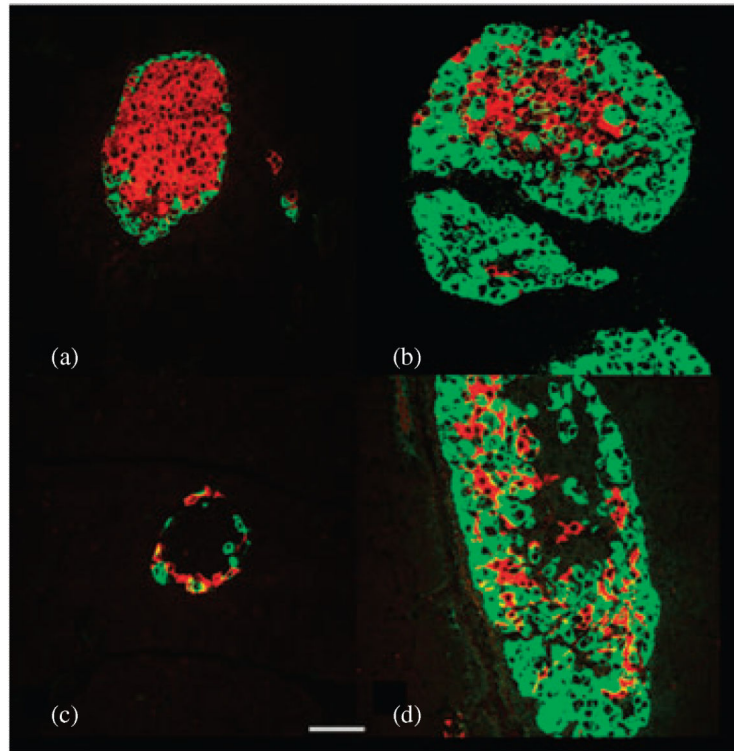


Figure 3.

Islets of adult $Gcgr^{-/-}$ mice display non β -cell hyperplasia. Photomicrographs of islets of 3-month-old wild type mice immunostained for visualization of (a) insulin (red) and glucagon (green), (c) somatostatin (red) and glucagon (green). Photomicrographs of islets of 3-month-old $Gcgr^{-/-}$ mice immunostained for visualization of (b) insulin (red) and glucagon (green), (d) somatostatin (red) and glucagon (green). Note that the number of α -cells and δ -cells in islets of $Gcgr^{-/-}$ mice is higher than in WT littermates. Bar: 20 μ m.