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Fibroblast Growth Factor 21 (FGF21) and Glucagon Like-Peptide 1 Contribute to Diabetes Resistance in Glucagon Receptor Deficient Mice

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Abstract

Mice genetically deficient in the glucagon receptor (Gcgr$^{-/-}$) show improved glucose tolerance, insulin sensitivity and α-cell hyperplasia. In addition, Gcgr$^{-/-}$ mice do not develop diabetes after chemical destruction of β-cells. Since fibroblast growth factor 21 (FGF21) has insulin independent glucose lowering properties we investigated whether FGF21 was contributing to diabetes resistance in insulin deficient Gcgr$^{-/-}$ mice. Plasma FGF21 was 25 fold higher in Gcgr$^{-/-}$ mice than in wild type mice. FGF21 was found to be expressed in pancreatic β- and α-cells, with high expression in the hyperplastic α-cells of Gcgr$^{-/-}$ mice. FGF21 expression was also significantly increased in liver and adipose tissue of Gcgr$^{-/-}$ mice. To investigate the potential anti-diabetic actions of FGF21 in insulin deficient Gcgr$^{-/-}$ mice, an FGF21 neutralizing antibody was administered prior to oral glucose tolerance tests (OGTT). FGF21 neutralization caused a decline in glucose tolerance in insulin deficient Gcgr$^{-/-}$ mice during the OGTT. Despite this decline, insulin deficient Gcgr$^{-/-}$ mice did not develop hyperglycemia. Glucagon-like peptide (GLP-1) also has insulin independent glucose lowering properties and elevated circulating GLP-1 is a known characteristic of Gcgr$^{-/-}$ mice. Neutralization of FGF21 while concurrently blocking the GLP-1 receptor with the antagonist Exendin9-39 resulted in significant hyperglycemia in insulin deficient Gcgr$^{-/-}$ mice, while Exendin9-39 alone did not. In conclusion, FGF21 acts additively with GLP-1 to prevent insulinopenic diabetes in mice lacking glucagon action.

Glucagon is a 29 amino acid peptide hormone secreted by the α-cells in the pancreas, and acts as a counter-regulatory hormone to insulin, such that glucagon is secreted during hypoglycemia, in contrast to insulin which is secreted in response to hyperglycemia. Glucagon receptors are highly expressed on hepatocytes and stimulate hepatic glucose production in order to maintain euglycemia. Glucagon plays a role in the hyperglycemia observed in diabetes, as a deficiency of insulin leads to inadequate suppression of glucagon secretion from the α-cells (1). The excess glucagon secretion increases hepatic glucose production, exacerbating the existing hyperglycemia.

Glucagon receptor deficient ($Gcgr^{-/-}$) mice have multiple phenotypic characteristics of interest in the study of diabetes. These mice display lower blood glucose levels throughout the day and have improved glucose tolerance (2). The $Gcgr^{-/-}$ mice also have reduced adiposity, LDL cholesterol and leptin levels, but normal body weight, food intake, and energy expenditure (2). Enlargement of the pancreas and post-natal hyperplasia of islets is observed, predominantly due to hyperplasia of α-cells up to 12-fold greater than $Gcgr^{+/+}$ mice, possibly as a result of a liver-derived circulating α-cell growth factor (3). An increase in circulating GLP-1 is also observed, most likely derived from the processing of elevated α-cell produced pro-glucagon (2). The $Gcgr^{-/-}$ mice have also been shown to be resistant to diet-induced obesity (4). $Gcgr^{-/-}$ mice were also shown to be resistant to the development of insulin deficient diabetes, as high doses of the diabetogen streptozotocin (STZ), which nearly completely destroyed all β-cells, did not cause hyperglycemia (4; 5). The suggested mechanism behind the anti-diabetic effect is lack of glucagon action (6), but the finding that the $Gcgr^{-/-}$ mice not only do not develop diabetes upon treatment with STZ, but also still display rapid elimination of glucose, suggests that other factors besides insulin play a role in the postprandial regulation blood glucose in these mice. In fact, Lee et. al. suggested that a hormone with insulinomimetic properties might be increased and causing the insulin-like

responses to glucose in the insulin deficient Gcgr^{-/-} mice, however no increase in leptin or IGF-1 levels could be observed (5). Fibroblast growth factor 21 (FGF21) is a 181-amino acid circulating protein which is observed to increase in plasma upon long-term starvation (7; 8), but also during high fat feeding (9-11). FGF21 stimulates glucose uptake into adipocytes in an insulin independent manner (12) and FGF21 mediated glucose uptake in skeletal muscle has been demonstrated (13). Furthermore, FGF21 has been shown to suppress hepatic glucose output *in vivo* and *in vitro* (14; 15). Injection of recombinant FGF21 normalizes blood glucose, improves insulin sensitivity and corrects dyslipidemia in several rodents and non-rodent animal models of type 2 diabetes (12; 16; 17). It is highly expressed in the liver and whole pancreas extracts, with lower expression levels in muscle and adipose tissue (18; 19). Given the pleiotropic actions of FGF21 on whole body glucose regulation, we hypothesized that FGF21 may be a factor contributing to glucose regulation in insulin deficient Gcgr^{-/-} mice. We therefore determined the contribution of FGF21 action to the regulation of glucose disposal in insulin deficient Gcgr^{-/-} mice.

REASERCH DESIGN AND METHODS

Animals. Mice were housed with a 12 hour dark light cycle and fed standard rodent chow (R34 Lantmännen Stockholm, Sweden). All experimental protocols were approved by the regional animal ethical committee in Lund, Sweden. Induction of diabetes by streptozotocin: Fifteen week old female Gcgr^{-/-} and Gcgr^{+/+} were anesthetized with an intraperitoneal injection of midazolam (12.5 mg/kg) (Dormicum; Roche, Basel Switzerland) and a combination of fluanison (25 mg/kg) and fentanyl (0.78 mg/kg) (Hypnorm; Janssen, Beerse, Belgium). Streptozotocin (Sigma-Aldrich St. Louis MO), or vehicle, was injected into the tail vein at a dose of 150 mg/kg. The protocol was repeated 7 days later with a dose of 100 mg/kg

according to protocol described by Lee et al. (5). The dose response of streptozotocin on circulating FGF21 was determined in male C57BL6 mice given single i.v. injections of 0, 150 or 200 mg/kg. Blood sampling for FGF21 was done 7 days after streptozotocin treatment. For fasting plasma values, mice were fasted for five hours anesthesized and blood was collected from the retrobulbar intraorbital sinus plexus. For the oral glucose tolerance test mice were fasted for five hours prior to oral administration of D-glucose (75 mg/mouse) by gavage. Blood samples were collected before and at 15, 30, and 60 minutes after the administration of glucose. A neutralizing antibody to FGF21 or an IgG control (AIS, Hong Kong) (10 μg/mouse) was given to Gcgr$^{-/-}$ and STZ-treated Gcgr$^{-/-}$ mice in a single i.p injection at the start of the fasting period, 5 hours prior to the start of the OGTT. The use of the neutralizing antibody has been previously described (20). Exendin 9-39 (Sigma-Aldrich, St. Louis, MO) was injected i.p. at a dose of 30 nmol/kg 10 minutes prior to the start of the OGTT.

Immunohistochemistry. Pancreata were removed and fixed in neutral buffered formalin prior to embedding in paraffin. Five μm thick sections were mounted onto superfrost plus glass slides. Briefly, after de-paraffinization, sections were blocked in 1% H_2O_2 andsubsequently blocked with 0.5% tris-NaCl blocking reagent (Perkin-Elmer, Waltham, MA, USA). Sections were incubated with rabbit anti-FGF21 antibody (1:200; ab64857 Abcam Cambridge, MA, USA) overnight and developed with TSA-cy3 (tyramide signal amplification; Perkin-Elmer). On top of the primary antibodies, guinea pig anti-insulin (1:150; ab7842 Abcam) and mouse anti-glucagon antibodies were added: (1:100; Glu001 Novo Biolabs, Bagsvaerd, Denmark). Guinea pig antibody was visualized with donkey anti-guinea pig-cy5 and mouse antibody with donkey anti-mouse-cy2 (1:300; Jackson ImmunoResearch, West Grove, PA. USA). DAPI (0.2 μg/mL) was used to stain nuclei. Images were taken on an LSM510 LASER SCANNING confocal microscope (Carl Zeiss, Oberkochen, Germany).

False color images were generated with the LSM software. Absorption studies were conducted to verifying the specificity of the anti-FGF21 antiserum.

Biochemical measurements. Plasma glucose concentrations were determined using the glucose oxidase method as described previously (21). Plasma insulin was measured by ELISA (Mercodia, Uppsala, Sweden). Plasma FGF21 was measured using a mouse/rat specific FGF21 ELISA assay (Biovendor, Prague, Czech Republic). The detection limit was measured to be 7 pg/ml, and the intra assay CV was 4%. Plasma samples in which FGF21 was below the detection limit were set to 7 pg/ml, as this was the detection limit of the assay.

Isolation of proteins. liver and gonadal adipose tissue were excised and frozen for measurement of FGF21 protein levels. FGF21 expression in tissues was determined using a mouse/rat specific FGF21 ELISA (Biovendor). In brief, liver and fat tissues were homogonized and lysed in lysis buffer (Invitrogen, Carlsbad, CA, USA) with a protein inhibitor cocktail mix (Invitrogen). Total protein was determined using a BCA assay from Pierce using albumin as standard.

Quantitative real-time PCR analysis

Total RNA was extracted from liver and WAT using Trizol (Invitrogen) and RNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA was synthesized using iScript reverse transcription kit (BioRad). Quantitative real-time PCR was performed on an ABI 7900 Sequence Detection System (Applied Biosystems) using a LNA probe based system from Roche. Primers were designed using Primer3 software (bioinfo.ut.ee/primer3). All samples were run in triplicates and expression was calculated using the $\Delta\Delta C_T$ method. Samples were normalized to beta-actin expression.

Statistical analysis. Data are presented as mean ± s.e.m. Area under the curve was calculated using the trapezoidal rule and differences between groups was determined by one way ANOVA or Student's t-test. Krusal-Wallis test (1-way ANOVA) was performed on plasma FGF21 levels in mice given single dose injection of STZ. Differences between groups with regards to qPCR was determined by Student's t-test. Significant differences were assumed for p vales <0.05. All statistical analysis was performed using GraphPad Prism software, version 5.0 (SanDiego, CA USA).

RESULTS

FGF21 is highly expressed in pancreatic islet α- and β-cells. FGF21 is highly expressed in the liver and in the pancreas, however no data on the cellular expression or role of FGF21 in the pancreas have been published. In figure 1, an immunostaining of insulin, glucagon and FGF21 is shown in wild type litter mates and in Gcgr$^{-/-}$ mice. FGF21 is localized in both α- and β-cells in the control litter-mates (C57/BL6J) (Fig. 1), however in the islet sections from the Gcgr$^{-/-}$ mice FGF21 is highly expressed in the α-cells, and as previously described the Gcgr$^{-/-}$ mice display α-cell hyperplasia (glucagon staining).To verify the specificity of the applied FGF21 antibody a blocking experiment was performed with rFGF21, the addition of rFGF21 totally blocked the staining of FGF21 on islet sections (data not shown). FGF21 protein expression was also determined in liver and adipose tissue, two other FGF21 expressing tissues. As seen in figure 2A and 2B, FGF21 protein expression was also increased in liver and adipose tissue from Gcgr$^{-/-}$ mice, although only statistically significant for adipose tissue. FGF21 mRNA as well as the mRNAs for the FGF receptors FGFR1, FGFR2, FGFR3, FGFR4 and β-Klotho were also determined in these tissues. FGF21 and FGFR3 mRNAs were significantly increased in liver from Gcgr$^{-/-}$ mice (Table 1). FGFR1 mRNA was significantly

decreased in adipose tissue from Gcgr$^{-/-}$ mice (Table 1). β-Klotho mRNA expression was decreased in adipose tissue (p=0.05) but unchanged in liver.

Gcgr$^{-/-}$ mice display increased circulating FGF21. In agreement with the strong FGF21 immuno-signal observed in the α-cells and increased expression in liver and adipose tissue, plasma FGF21 was significantly increased in the Gcgr$^{-/-}$ mice, reaching levels of 11000 pg/ml (p=0.0073 vs. wild-type) (Fig. 2C). In addition, there was a strong correlation between circulating FGF21 and 4 hour fasting plasma glucose in both wild-type and *Gcgr$^{-/-}$* mice (Supplementary Table 1).

Circulating FGF21 is decreased after high dose STZ treatment. In order to determine whether the increased plasma level of FGF21 could be involved in protecting the Gcgr$^{-/-}$ mice from STZ-induced diabetes, Gcgr$^{-/-}$ and wild-type control mice were treated with multiple high doses of streptozotocin and the in vivo effects on FGF21 were examined. As expected, treatment with multiple high doses of STZ resulted in overt diabetes in wild-type control mice with fasting plasma glucose exceeding 27 mmol/l (500 mg/dL) (Fig. 3A). Consistent with previous findings, Gcgr$^{-/-}$ mice have significantly lower fasting glucose. STZ treatment did result in a minor increase in fasting glucose in Gcgr$^{-/-}$ mice, however they still had significantly lower fasting glucose than untreated wild-type controls (Fig. 3A).

As the effect of STZ on circulating FGF21 is unknown, plasma FGF21 was measured in wild-type and Gcgr$^{-/-}$ mice before and after STZ treatment. Wild-type mice treated with a single dose of STZ at 150 mg/kg showed no differences in plasma FGF-21 compared to untreated mice (Fig. 3B). However, a single dose of STZ at 200 mg/kg resulted in significantly reduced plasma FGF21 levels (Fig. 3B). The multiple high dose STZ regimen (150 mg/kg followed by

100 mg/kg 7 days later) decreased circulating FGF21 in wild-type and Gcgr$^{-/-}$ mice (Fig. 3C,D) but this was only statistically significant for Gcgr$^{-/-}$ mice (Fig. 3D).

Endogenous FGF21 improves glucose tolerance in insulin deficient Gcgr$^{-/-}$ mice. To investigate the role of FGF21 in preventing the development of overt hyperglycemia in the STZ-treated Gcgr$^{-/-}$ mice, an FGF21 neutralizing antibody (Ab) was utilized. The FGF21 neutralizing Ab decreased FGF21-induced signaling *in vitro* (Supplementary Fig. 1) and has previously been shown to neutralize FGF21 *in vivo* (20). As shown in figure 4A, the plasma level of FGF21 is decreased after injection of the neutralizing FGF21 ab and the level of FGF21 after Ab injection is not significantly different than that in control mice. The neutralizing Ab was injected into mice 5 hours prior to the evaluation of glucose tolerance with an oral glucose tolerance test (OGTT). As seen in figure 4B, and in agreement with published data (2), under normal conditions the Gcgr$^{-/-}$ mice display lower fasting plasma glucose and lower glycemic excursions during the OGTT than wild-type controls. As seen in Figure 4B early insulin release in the Gcgr$^{-/-}$ mice is increased compared to the control. Upon STZ treatment the Gcgr$^{-/-}$ mice have slightly increased plasma glucose during the OGTT, but still significantly better glycemic control than untreated wild-type mice (Fig. 4A) despite a near total lack of insulin (Fig. 4B). Neutralization of FGF21 with the FGF21 Ab in STZ-treated Gcgr$^{-/-}$ mice significantly increased the glycemic excursion equaling that of untreated wild-type controls, without significant changes in insulin. As shown in figure 4C and 4D the incretmental area under the curve (AUC) for glucose was significantly increased as a result of FGF21 neutralization in STZ treated Gcgr$^{-/-}$ mice, while insulin was unchanged. In agreement with previous reports (5), the Gcgr$^{-/-}$ mice do not become diabetic upon STZ treatment.

Endogenous FGF21 and GLP-1 act additively to protect Gcgr$^{-/-}$ from insulin deficient diabetes. Despite the fact that immunoneutralization of FGF21 significantly increased glycemia in the STZ treated Gcgr$^{-/-}$ mice, they were not hyperglycemic and had relatively normal glucose elimination. This was despite being near totally insulin deficient. This led us to believe that another factor could be working in addition to FGF21 to aid in glucose elimination and prevent hyperglycemia in the absence of insulin. Gcgr$^{-/-}$ mice have been previously shown to have dramatically elevated levels of GLP-1 (2). Insulin and glucagon independent effects of GLP-1 on glucose homeostasis have been recently demonstrated (22; 23). We thus determined whether GLP-1 contributes to the improved glycemic profile of Gcgr$^{-/-}$ mice, even after β-cell destruction with STZ. GLP-1 receptor signaling was blocked with the GLP-1 receptor antagonist exendin 9-39 (Ex9-39) prior to the OGTT. In STZ treated Gcgr$^{-/-}$ mice given Ex9-39, the glycemic excursion was significantly increased, by 100% compared to STZ treatment alone, without changes in the insulin response (Fig. 5A,B). The resulting incremental AUC for glucose in the STZ Ex9-39 treated Gcgr$^{-/-}$ mice was not significantly different than that of wild-type mice (Fig. 5C), despite the Gcgr$^{-/-}$ mice not having a measurable insulin response (Fig. 5D). STZ treated Gcgr$^{-/-}$ mice were pretreated with the neutralizing antibody to FGF21 and then given Ex9-39 prior to the OGTT. The combination of FGF21 neutralization and GLP-1 receptor antagonism resulted in a significant increase in glucose excursion during the OGTT, by 159% compared to STZ treatment alone, and significantly greater than that of wild-type controls (Fig. 6A,C), without significant changes in insulin levels (Fig. 6B,D).

DISCUSSION

Insulin and glucagon are the two most important hormones for controlling blood glucose levels. Nearly 40 years ago, Unger and colleagues published a series of seminal studies

demonstrating the importance of glucagon action in the pathophysiology of diabetes and the potential of glucagon suppressing agents for the treatment of diabetes (24-26). Recently, this same group revisited the physiological consequences of suppression of glucagon action in near totally insulin deficient states using mice with glucagon receptor deficiency (5; 27). A key finding in these studies was a lack of the lethal metabolic consequences of insulin deficiency in the absence of glucagon action. Another key finding was completely normal postprandial glucose elimination during an OGTT in these insulin deficient mice. It was proposed that another hormone with insulinomimetic properties could be contributing to the regulation of postprandial glycemia in these mice. Leptin and insulin-like growth factor 1 (IGF-1) were investigated but were found not to be altered (5). The involvement of other, as yet undetermined, factors in the diabetes resistance seen in this unique model however, could not be ruled out.

Fibroblast growth factor 21 is one such factor. Recent studies have demonstrated clear, insulin independent, glucose-lowering effects of FGF21 *in vitro* and *in vivo* (15; 28). Circulating FGF21 was dramatically elevated in Gcgr$^{-/-}$ mice and there was abundant FGF21 in β-cells and the hyperplastic α-cells of Gcgr$^{-/-}$ mice. FGF21 protein expression was also increased in adipose tissue, and mRNA expression was increased in the liver of Gcgr$^{-/-}$ mice. It was clear in our study that β-cells express FGF21 protein. In support of our finding, plasma FGF21 was decreased in both wild-type and Gcgr$^{-/-}$ mice after beta cell destruction with high dose STZ treatment. This finding suggests that either β-cells themselves secrete FGF21, that insulin induces FGF21 secretion from insulin responsive tissues or both. In support of this concept, significant decreases in serum FGF21 in human subjects with recent onset type 1 diabetes have been reported (29). Although plasma FGF21 decreased significantly after multiple high dose STZ treatments, it was still more than 10-fold greater in Gcgr$^{-/-}$ mice than that in wild-type mice and thus fully capable of mediating effects on glycemia. This lead us to investigate

whether it might be mediating glucose tolerance in the model even after β-cell destruction with STZ. Another characteristic of Gcgr^{-/-} mice is a compensatory increase in proglucagon gene transcription and a substantial increase in circulating GLP-1 (2; 30; 31). Although the insulin independent effects of FGF21 on glycemia are generally well accepted, insulin independent effects of GLP-1 on glucose lowering have only recently begun to be appreciated. In recent years, several studies in rodents, canines and humans have demonstrated insulin independent effects of GLP-1 on circulating glucose levels (23; 32-34). Thus it stood to reason that one or both of these factors could contribute to the lack of postprandial hyperglycemia seen in insulin deficient Gcgr^{-/-} mice. The Gcgr^{-/-} mice exhibited far lower glucose excursions than wild-type controls. This was still the case after induction of insulin deficiency with STZ. When neutralizing FGF21 with a neutralizing antibody, glucose tolerance was significantly worsened in insulin deficient Gcgr^{-/-} mice, supporting the notion that FGF21 contributes to the diabetes resistance of Gcgr^{-/-} mice. Interestingly, blockade of the GLP-1 receptor alone also worsened glucose tolerance in insulin deficient Gcgr^{-/-} mice. Despite the impairment of glucose tolerance induced in insulin deficient Gcgr^{-/-} mice by both FGF21 neutralization and GLP-1 receptor antagonism individually, insulin deficient Gcgr^{-/-} mice did not have hyperglycemia or diabetes in either case. Only the combination of both FGF21 neutralization and concurrent GLP-1 receptor blockade resulted in significant hyperglycemia in insulin deficient Gcgr^{-/-} mice. This strongly suggests that GLP-1 and FGF21 work in a complementary way to prevent postprandial hyperglycemia in mice lacking insulin and glucagon action. That said, these Gcgr^{-/-} mice had normal fasting glucose, even with near total insulin deficiency and this was unaffected by FGF21 neutralization or GLP-1 receptor blockade. This supports the idea that glucagon is the sole mediator of fasting hyperglycemia in diabetes and chronic blockade of FGF21 and GLP-1 action in insulin deficient Gcgr^{-/-} mice would likely not result in overt diabetes due to the lack of glucagon action.Thus the total

elimination of glucagon action does prevent the lethal catabolic consequences of total insulin deficiency and FGF21and GLP-1, and possibly other factors, contribute to the regulation of postprandial glycemia in insulin deficient states.

In conclusion, genetic ablation of the glucagon receptor results in dramatically increased circulating FGF21, which promotes glucose tolerance in an insulin independent manner. In addition, FGF21 acts together with GLP-1 to prevent hyperglycemia in insulin deficient Gcgr$^{-/-}$ mice. Our results support the idea that pharmacological antagonism of glucagon action could be clinically beneficial for individuals with diabetes.

AUTHOR CONTRIBUTIONS

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FIGURE LEGENDS

FIG. 1. Localization of FGF21 in wild-type (WT) and Gcgr$^{-/-}$ mice (GRKO). Immunohistochemical stainings of control mice (A-D) and Gcgr$^{-/-}$ mice (E-H) pancreatic sections using anti serum against FGF21 (red), insulin (blue) and antibody against glucagon (green). Nuclear staining was done using DAPI (gray). Scale bar is 50 μm.

FIG. 2. Plasma and tissue FGF21 in wild-type and Gcgr$^{-/-}$ mice. Protein expression of FGF21 in A) liver and B) adipose tissue. n = 6-10 mice per group. C) Plasma concentration of FGF21 after a four hour fast in Gcgr$^{+/+}$ (white bars) and Gcgr$^{-/-}$ (black bars) mice n =6-23.

FIG. 3. Effect of high dose streptozotocin treatment on fasting glucose and FGF21. A) Plasma glucose before and after multiple high doses of streptozotocin in Gcgr$^{+/+}$ (white bars) and Gcgr$^{-/-}$ (black bars) mice. B) Plasma FGF21 7 days after single high dose treatment with streptozotocin in C57BL6 mice. n= 10-15 mice per group. C) Plasma FGF21 after multiple high doses of streptozotocin in Gcgr$^{+/+}$ mice. n = 10-23. D) Plasma FGF21 after multiple high doses of streptozotocin in Gcgr$^{-/-}$ mice. n = 6-14. * p < 0.05, ** p < 0.01, *** p < 0.001 versus control group.

FIG. 4. Effect of FGF21 neutralization on oral glucose tolerance in insulin deficient Gcgr$^{-/-}$ mice. A) Plasma FGF21 concentration five hours after treatment with IgG control antibody in Gcgr$^{+/+}$ (white bars) and Gcgr$^{-/-}$ (black bars) or FGF21 antibody in Gcgr$^{-/-}$ mice (hatched bars). Plasma glucose (B) and insulin (C) excursions during the OGTT (75mg/mouse) in Gcgr$^{+/+}$ (black circles), Gcgr$^{-/-}$ (white squares, solid lines), Gcgr$^{-/-}$ FGF21ab treated (white squares, dashed lines), Gcgr$^{-/-}$ STZ (white triangles, solid lines) and Gcgr$^{-/-}$ STZ FGF21ab treated (white triangles, dashed lines) mice. Incremental area under the curve for glucose (D) and insulin (E). * p < 0.05, ** p < 0.01, *** p < 0.001 versus wild-type control group unless otherwise indicated. † p < 0.05. n = 9-27 per group.

FIG. 5. Effect of GLP-1 receptor antagonism on oral glucose tolerance in insulin deficient Gcgr$^{-/-}$ mice. The GLP-1 receptor antagonist Ex9-39 (30 nmol/kg, i.p.) was

injected prior to the OGTT. Plasma glucose (A) and insulin (B) excursions during the OGTT (75mg/mouse) in in Gcgr$^{+/+}$ (black circles), Gcgr$^{-/-}$ (white squares, solid lines), Gcgr$^{-/-}$ STZ (white triangles, solid lines) and Gcgr$^{-/-}$ STZ Ex9-39 treated (white triangles, dashed lines) mice. Incremental area under the curve for glucose (C) and insulin (D). ** $p < 0.01$, *** $p < 0.001$ versus wild-type control group. †† $p < 0.01$. n = 6-27 per group.

FIG. 6. Effects of combined FGF21 neutralization and GLP-1 receptor antagonism on oral glucose tolerance in insulin deficient Gcgr$^{-/-}$ mice. Injections of FGF21 neutralizing antibody and Ex9-39 were given five hours and 10 minutes prior to the OGTT, respectively. Plasma glucose (A) and insulin (B) excursions during the OGTT (75mg/mouse) in in Gcgr$^{+/+}$ (black circles), Gcgr$^{-/-}$ (white squares, solid lines), Gcgr$^{-/-}$ STZ (white triangles, solid lines) and Gcgr$^{-/-}$ STZ FGF21ab/Ex9-39 treated (white triangles, dashed lines) mice. Incremental area under the curve for glucose (C) and insulin (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus wild-type control group. ††† $p < 0.05$ versus the Gcgr$^{-/-}$ STZ group. n = 6-27 per group.

Table 1- mRNA Expression in Liver and Adipose Tissue

	Liver		Adipose Tissue	
mRNA	Gcgr$^{+/+}$	Gcgr$^{-/-}$	Gcgr$^{+/+}$	Gcgr$^{-/-}$
Fgf21	0.036 ± 0.015	0.089 ± 0.011*	0.005 ± 0.003	0.002 ± 0.001
Fgfr1	0.006 ± 0.002	0.006 ± 0.002	0.066 ± 0.001	0.039 ± 0.006*
Fgfr2	0.015 ± 0.004	0.036 ± 0.008	0.007 ± 0.001	0.006 ± 0.001
Fgfr3	0.011 ± 0.001	0.019 ± 0.001**	0.001 ± 0.0001	0.001 ± 0.0001
Fgfr4	0.183 ± 0.013	0.184 ± 0.025	0.0005 ± 0.0001	0.001 ± 0.0001
Bkl	0.064 ± 0.023	0.065 ± 0.007	0.072 ± 0.03	0.020 ± 0.005

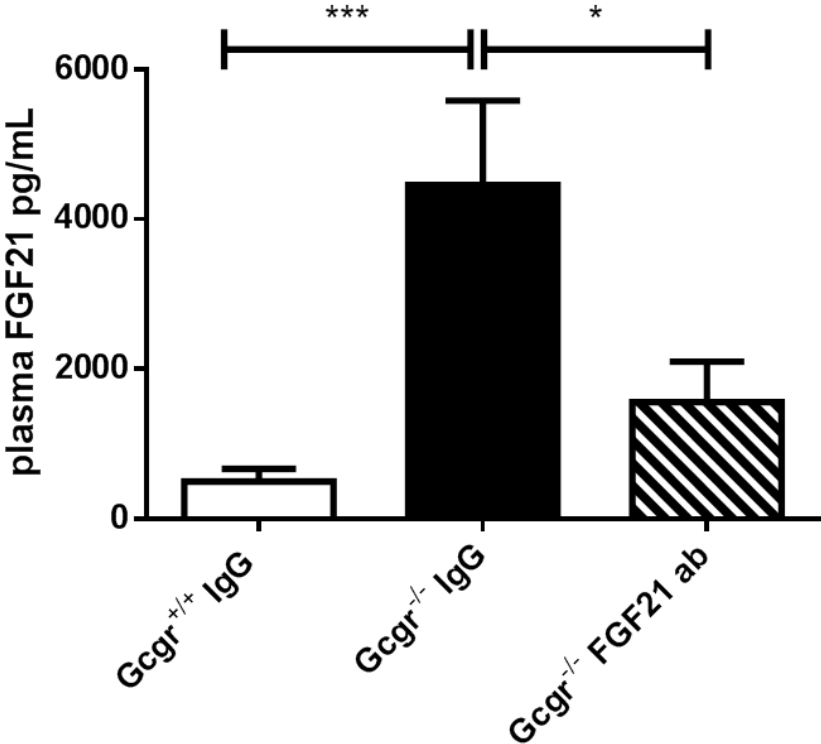Data are mean $\pm$ SEM. n=3 Gcgr$^{+/+}$, n=6 Gcgr$^{-/-}$. * $p < 0.05$, ** $p < 0.01$.
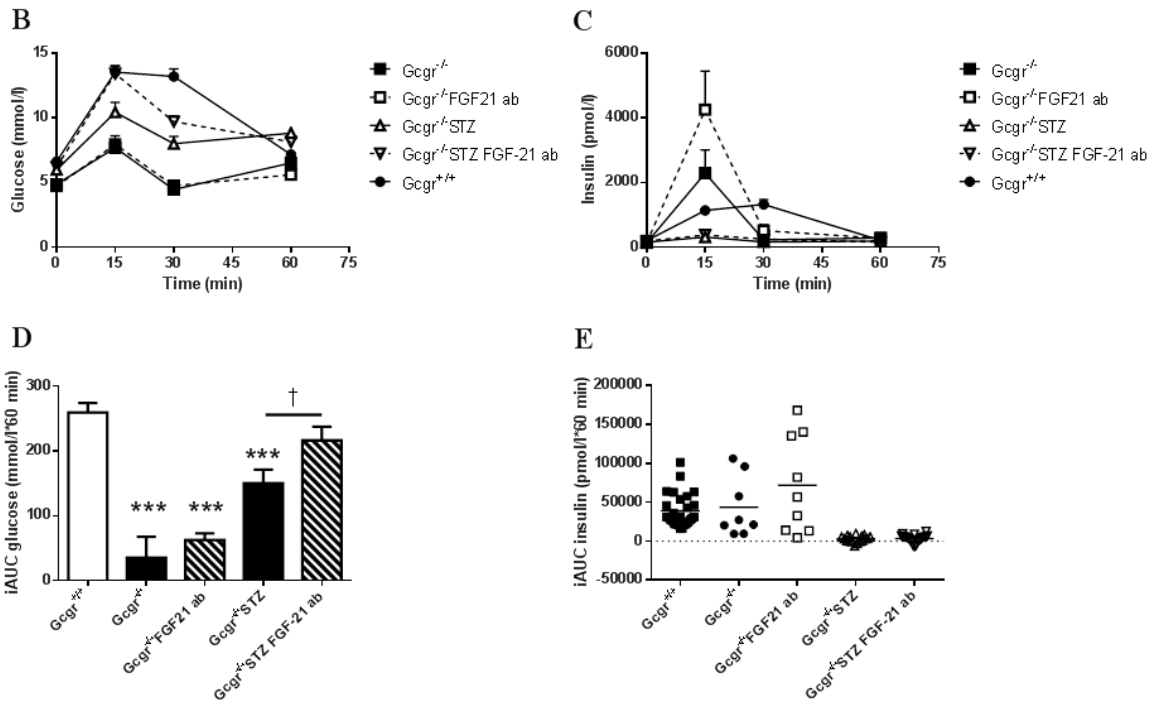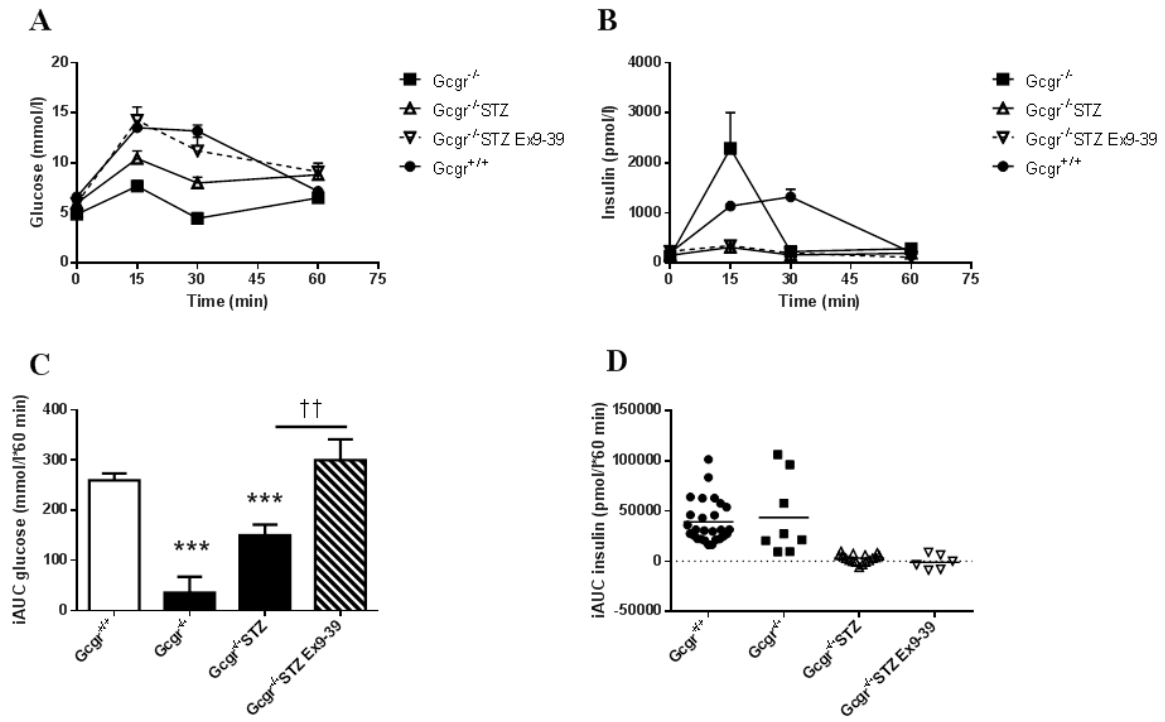
Figure 1
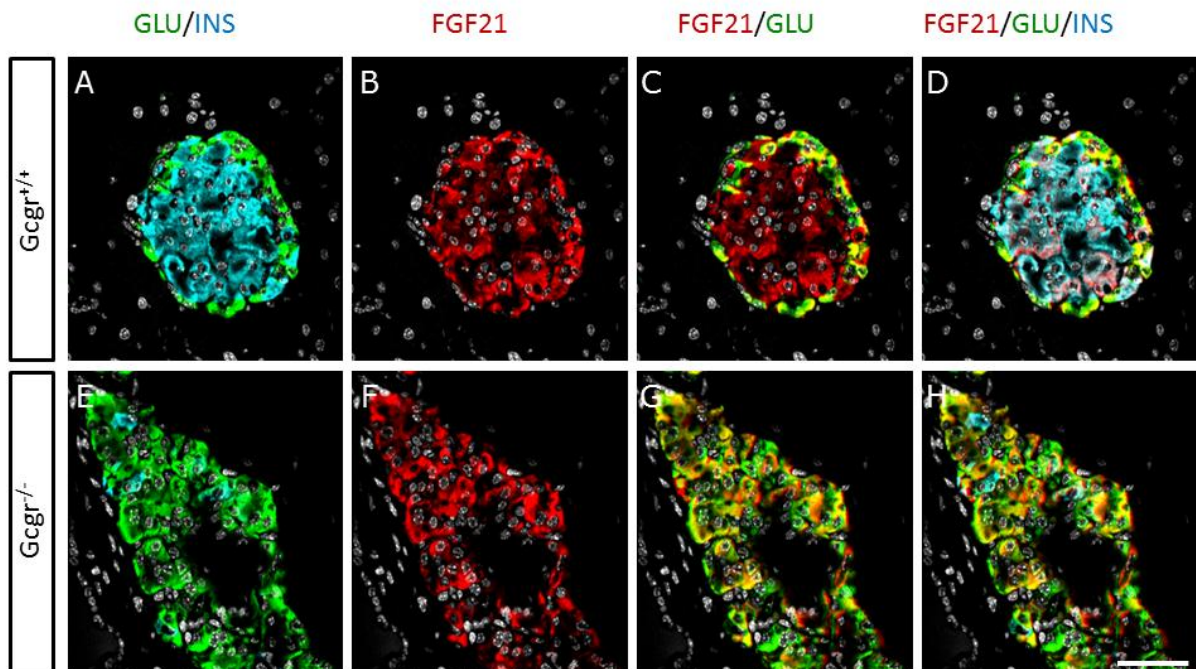
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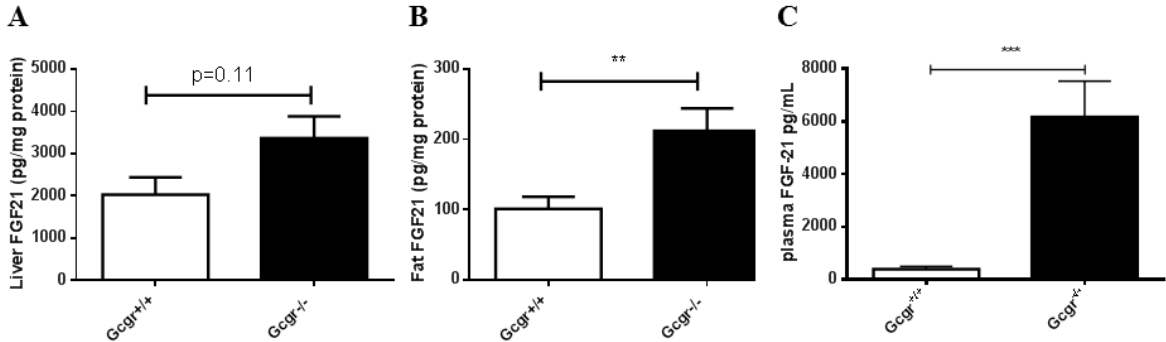
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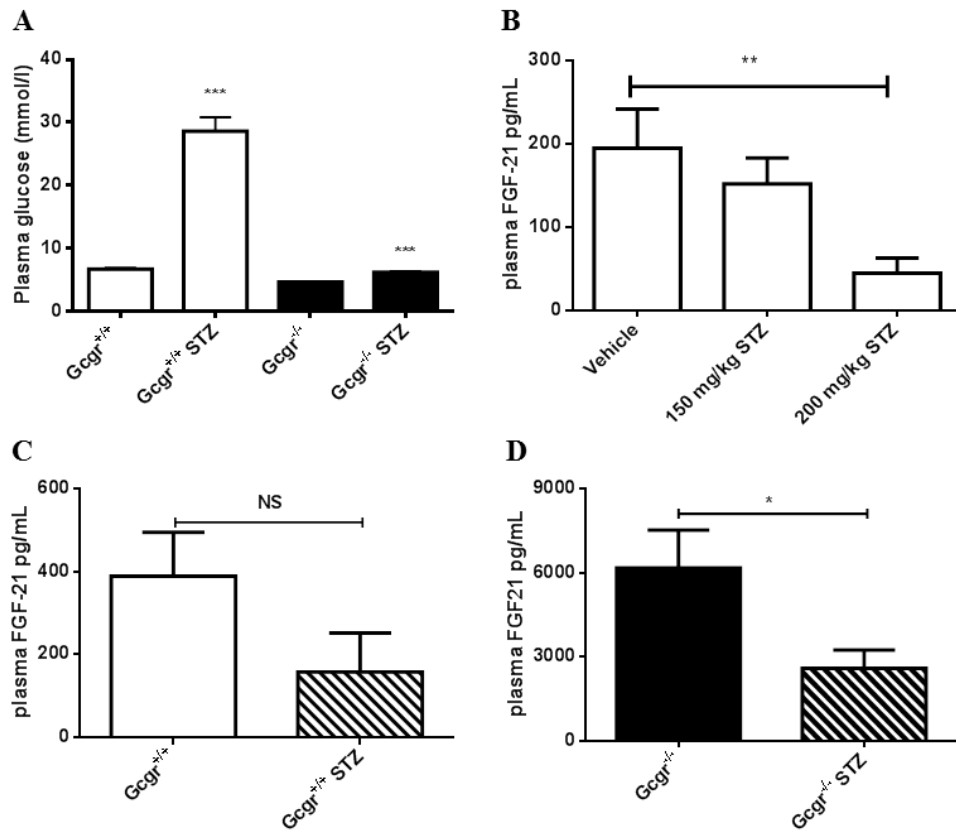
Figure 4A

Figure 4B-D

Figure 5

Figure 6
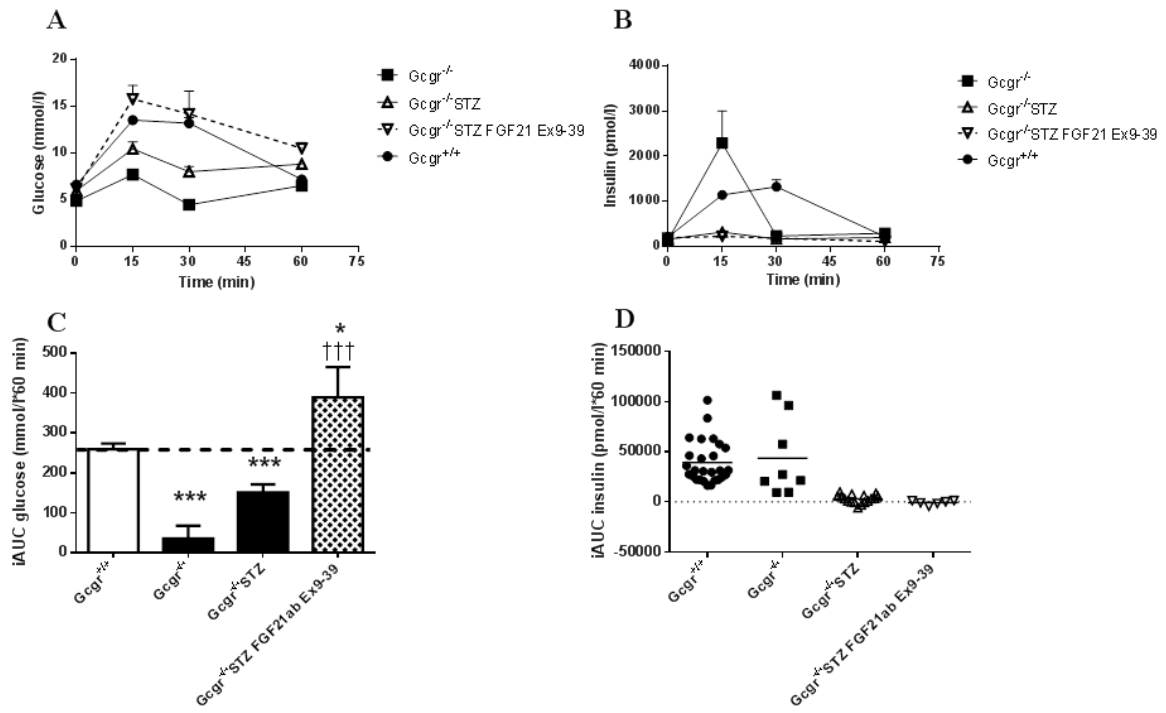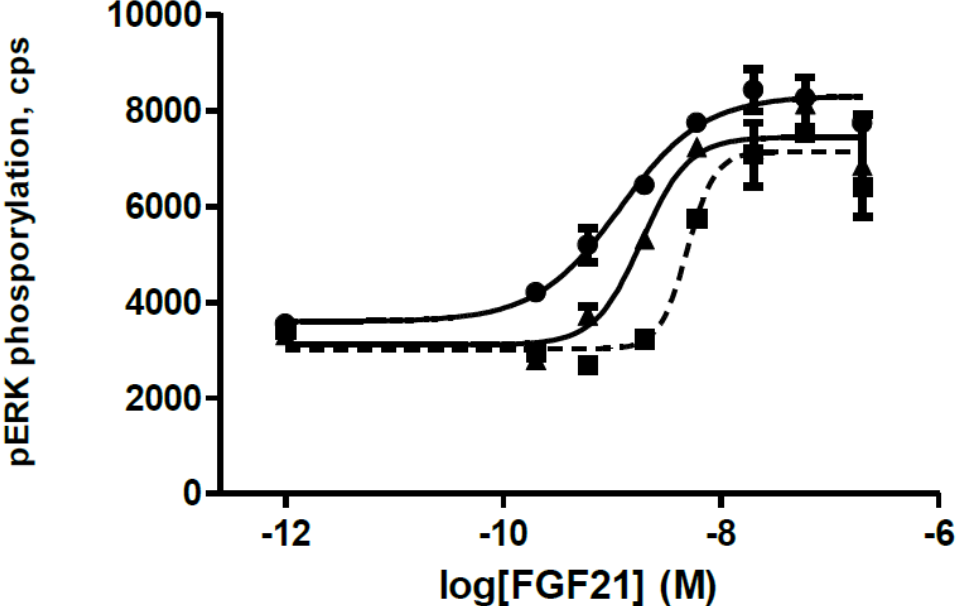
Supplementary Table 1

FGF-21/basal glucose correlation	Spearman r	p-value
Gcgr$^{+/+}$	-0.4506	0.0462
Gcgr$^{-/-}$	-0.8451	0.0008