Growth hormone receptor regulates β cell hyperplasia and glucose-stimulated insulin secretion in obese mice

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Insulin, growth hormone (GH), and insulin-like growth factor–1 (IGF-1) play key roles in the regulation of β cell growth and function. Although β cells express the GH receptor, the direct effects of GH on β cells remain largely unknown. Here we have employed a rat insulin II promoter–driven (RIP-driven) Cre recombinase to disrupt the GH receptor in β cells (βGHRKO). βGHRKO mice fed a standard chow diet exhibited impaired glucose-stimulated insulin secretion but had no changes in β cell mass. When challenged with a high-fat diet, βGHRKO mice showed evidence of a β cell secretory defect, with further deterioration of glucose homeostasis indicated by their altered glucose tolerance and blunted glucose-stimulated insulin secretion. Interestingly, βGHRKO mice were impaired in β cell hyperplasia in response to a high-fat diet, with decreased β cell proliferation and overall reduced β cell mass. Therefore, GH receptor plays critical roles in glucose-stimulated insulin secretion and β cell compensation in response to a high-fat diet.

Introduction

β Cell mass changes according to insulin demand, and loss of β cell hyperplasia in the face of insulin resistance is fundamental to the pathogenesis of type 2 diabetes (1). Although β cell hypertrophy and neogenesis contribute to enlargement of β cell mass, growing evidence indicates that β cell hyperplasia is mainly controlled by β cell proliferation as demonstrated in the cyclin D2–knockout mouse (2). Previous studies have implicated the insulin receptor (IR), but not the IGF-1 receptor (IGF-1R), in the maintenance of β cell mass and adaptation to high-fat diet (HFD) feeding (3). β Cell–specific IR-knockout (βIRKO), IGF-1 receptor–knockout (βIGFRKO), and double-knockout mutants exhibited normal growth and development of β cells (4), as did mice with knockout of IR substrate–1 (IRS-1) (5). However, βIRKO, but not βIGFRKO, mice exhibited an age-dependent decrease in β cell mass and eventually developed diabetes (6), suggesting that the IR plays a role in the maintenance of adult β cell mass. When challenged with HFD, βIRKO mice showed poor islet compensatory growth as compared with βIGFRKO and control mice (3), suggesting a major role for the IR in compensatory increases in β cell mass.

The role of the growth hormone receptor (GHR) in β cells is not well understood. Previous reports have shown that growth hormone (GH) stimulates insulin gene expression, biosynthesis, and release in β cells of rodents and humans. The first physiological evidence for a role of GHR in β cells came from GHR- (7) and prolactin receptor–null (PRLR-null) (8) mice, which exhibited reduced β cell mass, impaired glucose tolerance, and increased insulin sensitivity. However, these mice displayed compromised growth and significant changes in body adiposity; thus, a direct causal effect could not be established. To resolve these systemic effects, Lee et al. specifically ablated the downstream mediator of GH, STAT5a/b, in β cells using the Cre transgene (9). *Pdx-Cre-Stat5*$^{fl/fl}$ mice developed functional islets, which suggested that STAT5 is not essential for β cell proliferation or function. Nonetheless, with age *Pdx-Cre-Stat5*$^{fl/fl}$ mice developed mild glucose intolerance, probably due to increased body adiposity. In contrast, *RIP-Cre-Stat5*$^{fl/fl}$ mice exhibited mild obesity, hyperglycemia, and glucose intolerance, and it was suggested that these were secondary to partial *Stat5* gene ablation in the hypothalamus. Similar findings were demonstrated by a dominant negative form of STAT5 under the RIP promoter (10), while expression of constitutively active STAT5 counteracted these effects (10). Altogether, STAT5 may have negligible effects on β cell mass or function under normal conditions, but during obesity it may play a role in preserving β cell mass and function. Nonetheless, STAT5 can also be activated by other stimuli, such as IL-2, IL-3, the type 1 interferon receptor, and leptin (11); thus, these studies merely allude to the role of GHR in β cells. Furthermore, the effects of GH on lipid accumulation in β cells and its adverse effects of lipids during obesity may not be mediated solely via STAT5. Thus, we took a direct approach, inactivating GHR in β cells to unequivocally dissect the role of GHR in determining β cell mass and function during normal and pathophysiological conditions.

Results and Discussion

Generation of β cell–specific GHR KO (βGHRKO) mice. Specific *Ghr* inactivation in β cells was achieved using the Cre/loxP system (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI45027DS1). Specificity of *Ghr* gene recombination was validated by PCR using genomic DNA extracted from islets dissected by laser capture microdissection (Figure 1A) and other tissues by standard protocols. In control mice, homozygous for the floxed *Ghr* allele, and RIP-Cre transgenic mice, the recombinant allele was undetectable in all tissues (Figure 1B), while in β cell–specific GHR–knockout (βGHRKO) mice, the *Ghr* recombinant allele (null allele) was detected only in islets and

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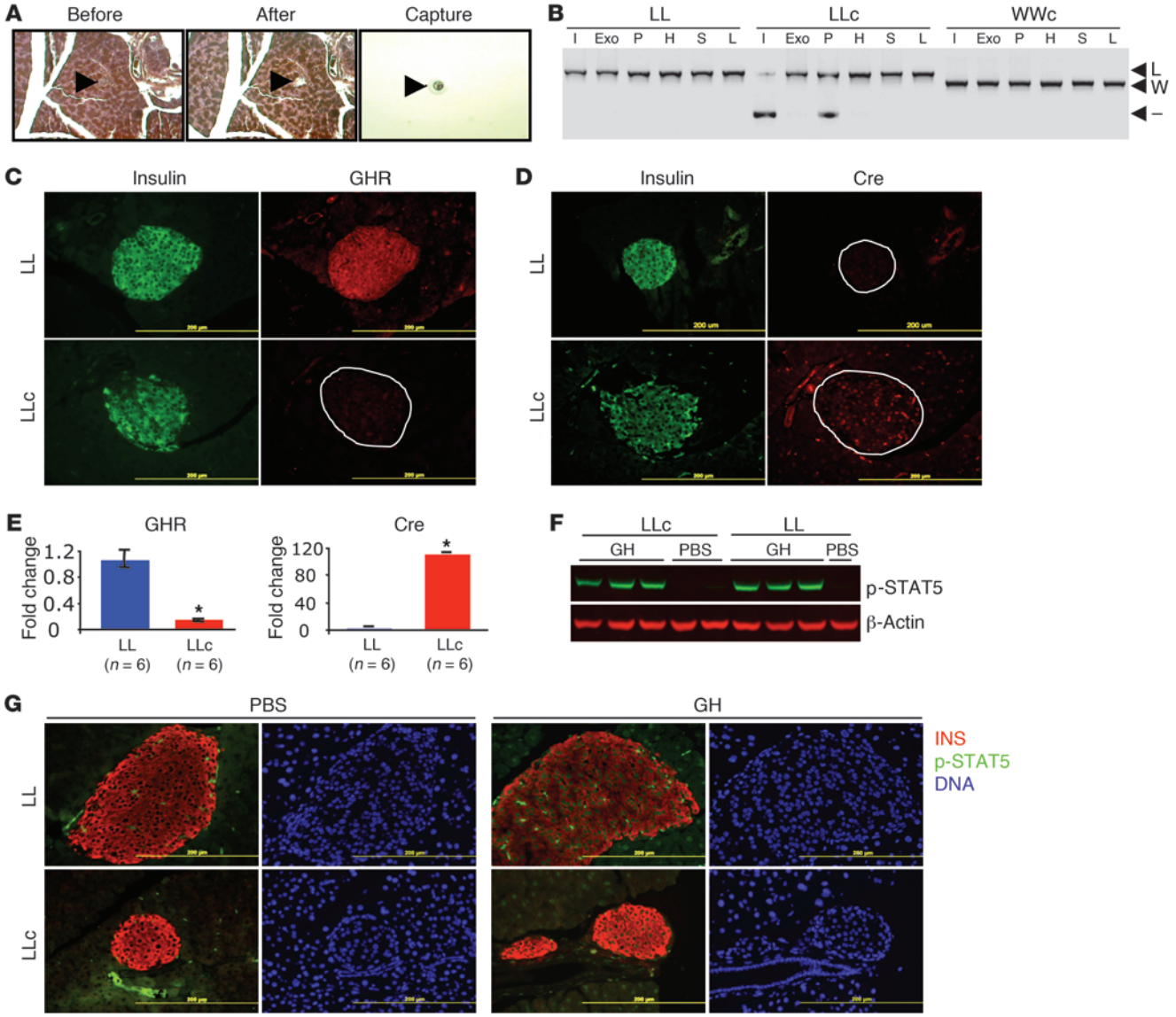

Figure 1

Assessment of *Ghr* excision and expression in βGHRKO mice. (**A**) Isolation of the islets from pancreas by laser capture microdissection. Original magnification, ×10. (**B**) PCR analysis of GHR excision in different tissues of βGHRKO mice using primers S6 and R2 (see Supplemental Figure 1A). LL, mice homozygous for the floxed *Ghr* allele; LLc, βGHRKO mice; WWc, RIP-Cre transgenic mice; I, islet; Exo, exocrine; P, pancreas; H, hypothalamus; S, spleen; L, liver. "L" arrowhead indicates the floxed allele, "W" indicates the wild-type allele, and "–" indicates the recombinant allele. (**C**) Immunofluorescence analysis of GHR protein (red) and insulin (green) in pancreatic sections. (**D**) Immunofluorecence analysis of Cre (red) and insulin (green) in pancreatic sections. (**E**) Gene expression of *Ghr* and the *Cre* transgene in islets analyzed by real-time PCR. **P* < 0.05. (**F**) Phosphorylation of STAT5 in liver upon intravenous injection of GH to obese control and βGHRKO mice, as assessed by Western immunoblotting. (**G**) Immunofluorecence analysis of phosphorylated STAT5 protein in pancreatic sections from obese mice (insulin [INS], red; pSTAT5, green; and nucleus, blue). Scale bars: 200 μm (**C**, **D**, and **G**).

whole pancreas, but not in exocrine pancreas (Exo), hypothalamus, spleen, or liver, indicating β cell–specific Cre-mediated excision of the *Ghr* gene (Figure 1B).

Immunostaining of whole pancreas with anti-GHR antibody was positive in control mice, while no staining of GHR was detected in islets of βGHRKO mice (Figure 1C). Furthermore, immunostaining with anti-Cre recombinase antibody (Figure 1D) was positive in islets of βGHRKO mice but not in controls. Expression of the *Ghr* gene according to real-time PCR (Figure 1E) decreased by 95% in βGHRKO as compared with controls. Additionally, we could not find evidence for *Ghr* gene recombination in the hypothalamus, as no differences were found in the immunostaining pattern of GHR, CRE, NPY, POMC, or AGRP (Supplemental Figure 2, A and B), nor in their expression levels assessed by real-time PCR (Supplemental Figure 2, C–E).

Last, we tested STAT5 phosphorylation and nuclear localization in liver and pancreas, respectively. GH injected (12.5 μg/100 g body weight) intravenously stimulated STAT5 phosphorylation in liver

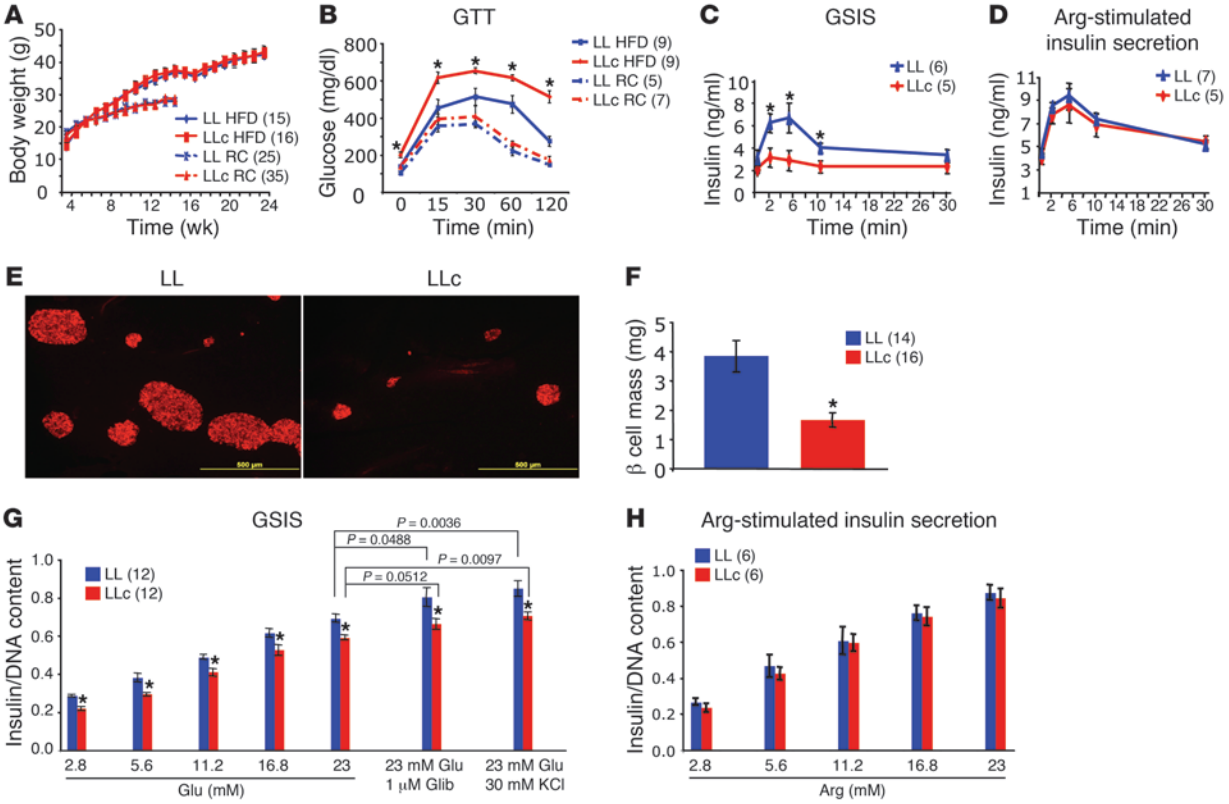

Figure 2

HFD-induced obesity causes impaired glucose tolerance and insulin secretion in βGHRKO mice. (**A**) Body weight of male control and βGHRKO mice fed regular chow (RC) or HFD. (**B**) GTT in male mice after 24 weeks on HFD. βGHRKO mice did not exhibit first-phase GSIS (**C**), but displayed normal first-phase arginine-stimulated (Arg-stimulated) insulin secretion (**D**). Numbers in parentheses indicate sample size. (**E**) HFD-induced islet hyperplasia is shown by immunostaining using anti-insulin antibody. Scale bars: 200 μm. (**F**) β Cell mass was quantified in insulin-stained pancreas sections. (**G**) GSIS in isolated islets from obese mice in response to different glucose (Glu) concentrations and in the presence of KCl or glibenclamide (Glib). (**H**) Arginine-simulated insulin secretion in isolated islets from obese mice. *$P < 0.05$.

of both control and βGHRKO mice (Figure 1F). However, while in control islets STAT5 translocated into the nucleus following GH injection, in βGHRKO mice we hardly detected nuclear STAT5 staining (Figure 1G). Together, our results show that GHR action in βGHRKO mice is ablated in β cells but is intact in other tissues.

Ghr inactivation in β cells does not affect islet size or insulin content in mice fed chow. Male (Supplemental Figure 3A) and female (Supplemental Figure 3B) mice were followed from 2 to 15 weeks of age. Body weight and body composition (assessed by MRI) of βGHRKO mice were indistinguishable from those of controls throughout the study. Serum IGF-1 and insulin levels tested at 8 and 16 weeks were similar between the groups (Supplemental Figure 3, C and D), indicating that *Ghr* inactivation in β cells does not affect growth and development.

Overexpression of GH in mice resulted in increased β cell mass (12), while *Ghr*-null mice (7) as well as GH antagonist (13) transgenic mice exhibited significant reductions in islet size and number. In vivo experiments with rats bearing GH-secreting tumors (14) also showed β cell hyperplasia, as seen in patients with acromegaly (15). We therefore sought to determine whether β cell ablation of GHR affected islet size. Sections throughout the pancreatic head, body, and tail revealed no differences in H&E staining (Supplemental Figure 3, E and F) or in insulin staining (Supplemental Figure 3G) between controls and βGHRKO mice at 16 weeks. This was in accordance with similar levels of insulin in serum of control and βGHRKO mice (Supplemental Figure 3D) and islet insulin content measured in islet extracts by RIA (data not show), suggesting that loss of *Ghr* in β cells does not affect islet development.

The metabolic consequences of GHR ablation in β cells were determined by intraperitoneal glucose tolerance and insulin tolerance tests (GTT and ITT) in males at 16 weeks, which revealed no differences between βGHRKO and control mice (Supplemental Figure 3, H and I). These data are in agreement with data obtained from mice expressing a dominant negative form of STAT5b (10), or mice with β cell–specific ablation of STAT5b (9) fed chow. However, we found that the first phase of insulin secretion, observed shortly following glucose bolus, was blunted in βGHRKO mice (Supplemental Figure 3J), suggesting that when mice are challenged with high glucose, maximal insulin secretion is low, while insulin secretion at a more physiologic (meal) glucose level is normal. In contrast, arginine-stimulated insulin release from the islets was normal in βGHRKO mice (Supplemental Figure 3K), indicating that GHR in β cell is involved in glucose-stimulated first-phase insulin secretion.

βGHRKO mice exhibit impaired β cell hyperplasia when fed HFD. β Cell hyperplasia is compromised in βIRKO mice when fed HFD (4). To investigate whether βGHRKO mice manifest β cell hyperplasia, we fed mice with HFD from weaning. Both control and βGHRKO mice fed HFD exhibited a similarly marked increase in body weight and body adiposity (Figure 2A). GTT after 24 weeks of

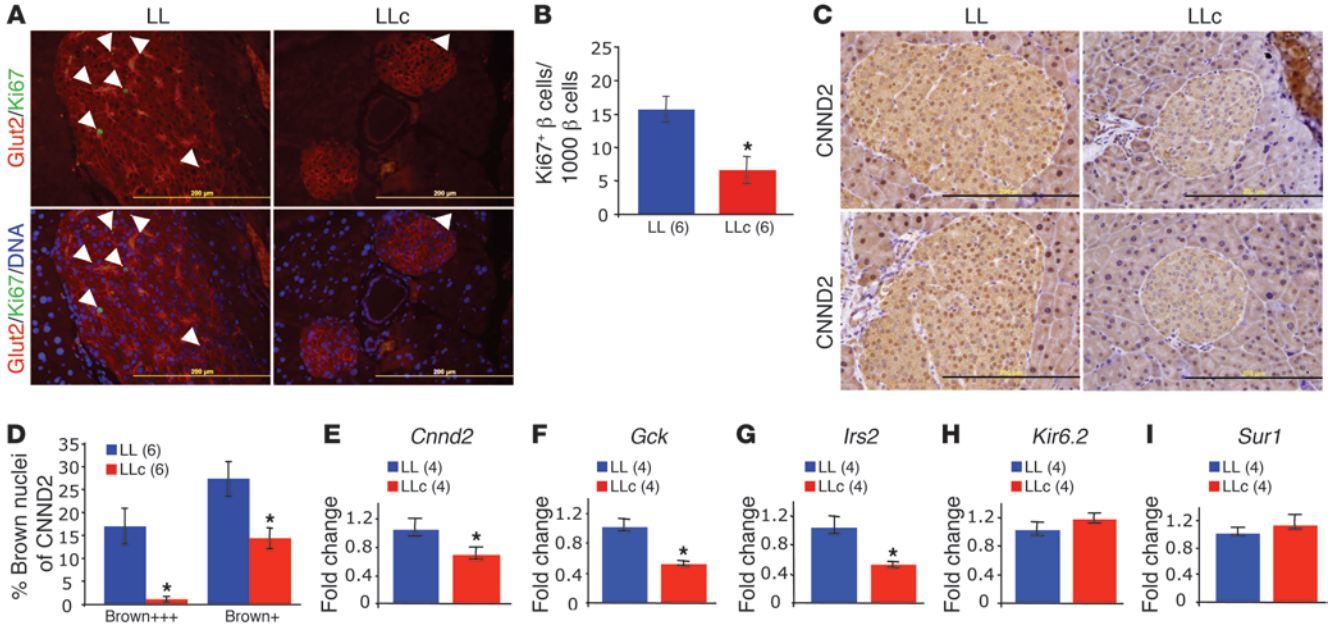

Figure 3

Reduced β cell proliferation in obese βGHRKO mice. (**A**) Immunofluorecence analysis of Ki67 in pancreatic sections. Ki67 is stained green, GLUT2 red, and the nucleus blue. (**B**) Quantification of Ki67-positive cells per 1,000 cells based on 20–25 islets in each group. (**C**) Immunohistochemical staining for cyclin D2 in pancreas sections from obese control and βGHRKO mice. Top and bottom rows each show representative images of the two groups. (**D**) Quantification of nuclear cyclin D2 staining, with determination of strong nuclear staining (Brown+++) and weak nuclear staining (Brown+). (**E–I**) Gene expression of cyclin D2 (*Cnnd2*), *Gck*, *Irs2*, *Kir6.2*, and *Sur1* in isolated islets from obese control and β-GHRKO mice. Scale bars: 200 μm (**A** and **C**). Numbers in parentheses indicate sample size. *$P < 0.05$.

HFD showed that both obese control and βGHRKO mice became glucose intolerant (Figure 2B), but obese βGHRKO mice showed significantly higher glucose levels. ITT indicated that obese βGHRKO mice responded to exogenous insulin in the same manner as controls (data not show). Obese βGHRKO mice (40 weeks on HFD) exhibited severely blunted first-phase glucose-stimulated insulin secretion (GSIS) (Figure 2C) but showed normal arginine-stimulated insulin secretion (Figure 2D). While β cell mass in obese control mice markedly increased in response to high-fat feeding, obese βGHRKO mice showed no increase in β cell mass, resulting in an about a 2.5-fold decrease in mean β cell mass (Figure 2, E and F), suggesting an impaired ability to adapt to the higher insulin demand in response to a HFD. Interestingly, islet insulin content did not differ between obese control and obese βGHRKO mice (Supplemental Figure 4), suggesting an insulin secretion defect in βGHRKO mice. Ex vivo GSIS in isolated islets revealed increased insulin secretion in response to elevations in glucose concentrations in both groups (Figure 2G). However, when corrected to islet DNA, islets isolated from obese βGHRKO mice secreted significantly less insulin than those isolated from obese controls (Figure 2G), while arginine-stimulated insulin secretion was not affected (Figure 2H). To verify the integrity and activity of the K_{ATP} channels in islets, we studied GSIS in the presence of KCl or glibenclamide. We found that islets from obese controls and βGHRKO significantly increased insulin secretion in response to both stimuli, suggesting that both channels are intact.

Obese βGHRKO mice exhibit decreased β cell proliferation. Islets from obese βGHRKO mice exhibited significant reductions in cellular proliferation, as evident by significant decrease in Ki67- (Figure 3, A and B) or PCNA-positive (data not shown) cells. This correlated with a decreased percentage of cyclin D2–positive cells in islets of obese βGHRKO mice (Figure 3, C and D). Cyclin D2 gene expression, assessed by real-time PCR, was also decreased in isolated islets of obese βGHRKO mice (Figure 3E). Additionally, insulin, glucagon, *Glut2*, caspase-3, *P16*, or *P27* gene expression revealed no differences between islets from obese controls or βGHRKO mice (data not shown). We speculate that the defect in β cell hyperplasia in obese βGHRKO mice is due to decreased proliferation, which may partially be cyclin D2 mediated. Interestingly, however, data from mice expressing a dominant negative STAT5b (dnSTAT5b) specifically in β cells (10) show that pancreatic insulin content and the relative β cell mass significantly increased in response to high-fat feeding. Nonetheless, similar to our findings, β cell replication was lower and associated with decreased islet expression of cyclin D2 in obese dnSTAT5b mice (10). This may suggest that β cell compensatory response to HFD is not mediated by STAT5b. In support of this, studies with rat β cells have shown that activation of GHR results in increased PKC activity, leading to elevations in Ca^{2+} and concomitant increases in diacylglycerol (DAG) that together play a role in transmitting the mitogenic effects of GH into a proliferative response (16). Other studies with rat β cells have shown that upon stimulation with GH, there was a rapid stimulation of JAK2 and Src protein phosphorylation and a rise in intracellular Ca^{2+}, which is required for cell replication (17).

To begin to understand the molecular mechanism involved in impaired insulin secretion, we performed gene expression studies in isolated islets. The expression levels of glucokinase (*Gck*), which plays a key role in glucose metabolism, were reduced

in islets of obese βGHRKO mice (Figure 3F). Previous studies showed that IRS-2 is a crucial regulator of β cell survival and function (18). Thus, β cell–specific *Irs2* gene inactivation resulted in reduced β cell mass and GSIS (19). Accordingly, we found that the expression level of the *Irs2* was significantly reduced in islets of obese βGHRKO mice (Figure 3G). Immunostaining with anti-GCK and -IRS2 antibodies showed significant reductions in fluorescent signal in βGHRKO islets (Supplemental Figure 5). Our results are consistent with the phenotype of $Gck^{+/-}$ mice, which showed insufficient β cell hyperplasia when fed HFD (20). Overexpression of IRS-2 in β cells partially rescued the diabetic phenotype of HFD-fed $Gck^{+/-}$ mice, indicating that IRS-2 is the downstream mediator of glucose signaling and β cell replication. Last, expression levels of the SUR/KIR K_{ATP} channels, which are crucial for the regulation of glucose-induced insulin secretion and are the target for the sulfonylureas, were similar in obese control and βGHRKO mice (Figure 3, H and I).

In conclusion, deletion of GHR specifically in pancreatic islet β cells was associated with a lack of compensatory hyperplasia in response to HFD-induced obesity. Furthermore, GHR is apparently important for GSIS from the β cells. While the exact mechanism(s) involved are undefined, results from the present study suggest that GHR signaling for these important processes is not solely dependent on STAT5.

Methods

Animals. All mice were on the C57BL/6 genetic background. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine (New York, New York, USA). Generation of *Ghr*-floxed mice and the *Ghr* β cell–specific knockout (βGHRKO) mice is detailed in Supplemental Methods. HFD, 60% (wt/wt) fat content, was obtained from Research Diets Inc.

Intraperitoneal glucose and insulin tolerance tests. GTTs and ITTs were performed as detailed previously (21).

Serum insulin and IGF-1. Serum insulin and IGF-1 levels were determined using RIA (Millipore).

Glucose- and arginine-stimulated insulin secretion. Glucose (3 g/kg) or L-arginine (15 mM/l in 0.2 M PBS) was injected intraperitoneally to overnight fasted mice. Blood glucose was measured at the indicated time points. Ex vivo GSIS and arginine-stimulated insulin secretion were done using a modified protocol detailed in Supplemental Methods.

Histology and immunohistochemistry. Histology and immunohistochemistry were performed in paraffin-embedded tissue sections. Antibody description is provided in Supplemental Methods. β Cell mass was calculated from 3–5 pancreatic sections per mouse stained with anti-insulin antibody. Relative β cell area was calculated using NIH ImageJ software, and β cell mass was calculated as β cell area multiplied by pancreatic weight of 6 mice per group.

Gene expression. RNA was isolated using TRIzol (Invitrogen). RNA integrity was verified using Bioanalyzer (Agilent Technologies). cDNA was generated using oligo(dT) primers (Invitrogen). Real-time PCR was performed with the QuantiTect SYBR Green PCR kit (QIAGEN) in ABI PRISM 7900HT detection systems (Applied Biosystems). Sequences of primers used for real-time PCR are presented in Supplemental Table 1.

Statistics. All data are expressed as mean ± SEM and were analyzed using unpaired 2-tailed Student's *t* test. A *P* value less than 0.05 was considered significant.

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