Disruption of leptin receptor expression in the pancreas directly affects β cell growth and function in mice

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Obesity is characterized by hyperinsulinemia, hyperleptinemia, and an increase in islet volume. While the mechanisms that hasten the onset of diabetes in obese individuals are not known, it is possible that the adipose-derived hormone leptin plays a role. In addition to its central actions, leptin exerts biological effects by acting in peripheral tissues including the endocrine pancreas. To explore the impact of disrupting leptin signaling in the pancreas on β cell growth and/or function, we created pancreas-specific leptin receptor (ObR) KOs using mice expressing Cre recombinase under the control of the pancreatic and duodenal homeobox 1 (*Pdx1*) promoter. The KOs exhibited improved glucose tolerance due to enhanced early-phase insulin secretion, and a greater β cell mass secondary to increased β cell size and enhanced expression and phosphorylation of p70S6K. Similar effects on p70S6K were observed in MIN6 β cells with knockdown of the *ObR* gene, suggesting crosstalk between leptin and insulin signaling pathways. Surprisingly, challenging the KOs with a high-fat diet led to attenuated acute insulin secretory response to glucose, poor compensatory islet growth, and glucose intolerance. Together, these data provide direct genetic evidence, from a unique mouse model lacking ObRs only in the pancreas, for a critical role for leptin signaling in islet biology and suggest that altered leptin action in islets is one factor that contributes to obesity-associated diabetes.

Introduction

The factors that promote β cell failure and increase the incidence of diabetes in obese individuals are not fully understood. The presence of hyperglycemia and hyperphagia in obese individuals, despite the presence of high levels of circulating insulin and leptin, suggests these individuals are resistant to the actions of both hormones (1). The hypothalamic actions of leptin are relatively well characterized; however, the expression of the long form of the leptin receptor (ObRb) in peripheral tissues, including the endocrine pancreas, indicates that leptin can also exert peripheral actions independent of its effects in the hypothalamus (2). For example, in vitro studies have reported inhibitory effects of leptin on insulin gene expression and insulin secretion in β cell lines and isolated murine and human islets (2–4). Furthermore, leptin treatment of *ob/ob* mice reversed hyperinsulinemia (5). Although the *db/db* mouse, which carries a mutation in the ObR gene (6), manifests hyperinsulinemia and hyperplastic islets (7), it is unclear whether the alterations in islet growth and function in *db/db* mice are due to a lack of direct leptin action in β cells or secondary to the effects of insulin resistance in peripheral tissues.

To directly assess the role of leptin signaling in the pancreas, we used the Cre-*loxP* technique to create a mouse model that is

deficient in ObRs only in the pancreas. We report that the expression of *Cre* using the *Pdx1* (pancreatic and duodenal homeobox 1) promoter results in efficient recombination of a *loxP*-containing ObR gene in the pancreas. Mice lacking ObRs in the pancreas (pancreas-ObR-KO) and fed normal chow manifested improved glucose tolerance, enhanced first-phase insulin response to glucose, and islet hyperplasia.

We also report upregulation in the islets of expression and phosphorylation of proteins in the insulin/IGF-I signaling pathway that are known to be important for the maintenance of islet growth and function (8–19). Qualitatively similar signaling data were observed in MIN6 β cells with a knockdown of ObR using siRNA. Challenging the mice with a high-fat diet (HFD), however, led to significantly greater glucose intolerance in the KOs compared with controls. Together, these data provide genetic evidence for cross-talk between leptin and insulin signaling pathways in the regulation of islet biology, with implications for β cell dysfunction in obesity.

Results

Creation of the pancreas-specific ObR-KO mice. We created a mouse with a pancreas-specific KO of *obr* by breeding mice carrying *obr* in which exon 1 was flanked with *loxP* sites (*obr^{lox/lox}*; ObRlox) (20) with mice expressing *cre* driven by the *Pdx1* promoter (21).

Both control (ObRlox) and KO mice were born at the expected Mendelian frequency, survived to adulthood, and were fertile. We performed RT-PCR analysis to evaluate efficiency of recombination of the *ObRlox* allele in the pancreas triggered by *Cre* expression. Expression of the gene coding for ObR was virtually absent in
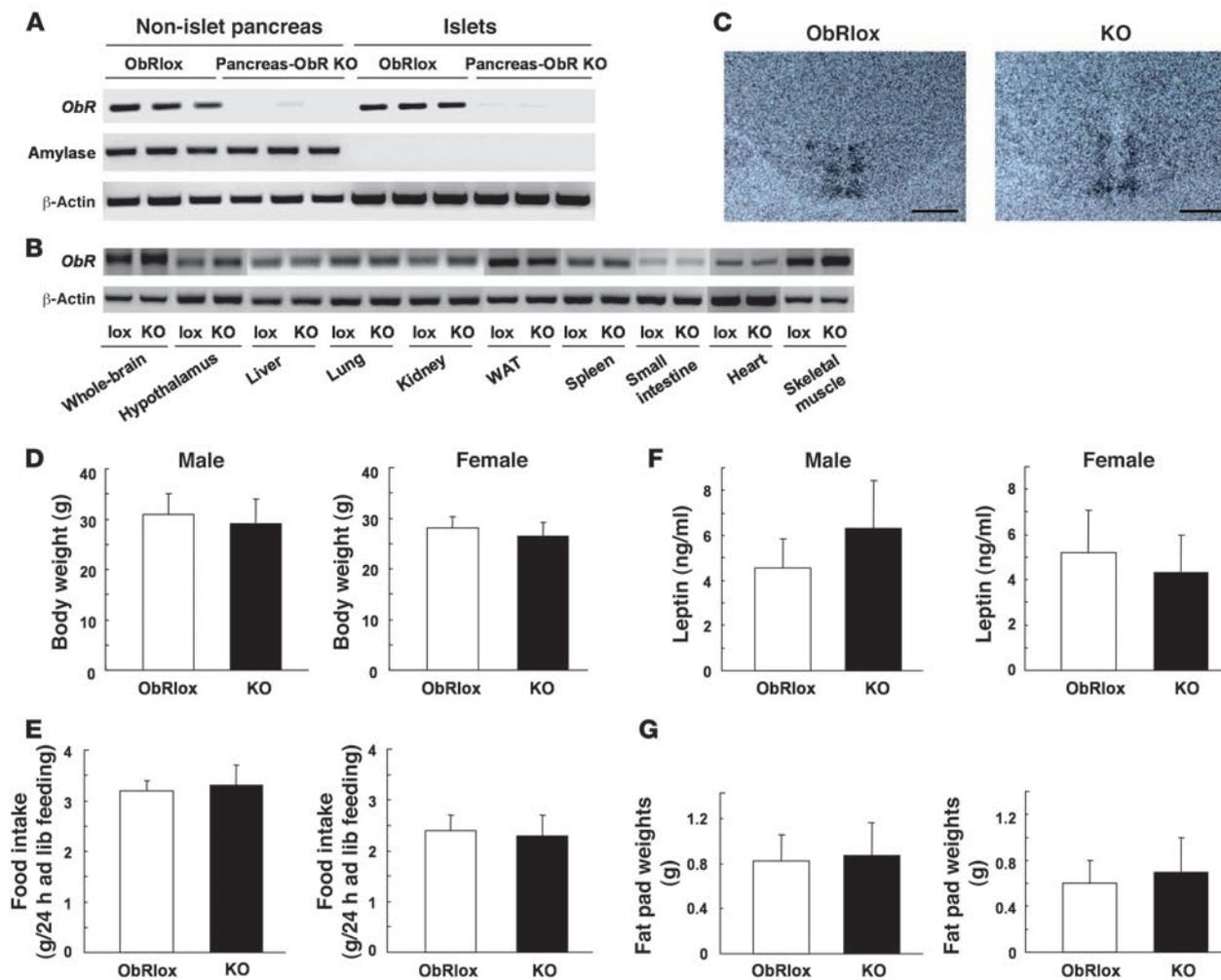
Figure 1
Evidence for deletion of ObR and physiological effects of pancreas-specific disruption of the ObR in pancreas-ObR-KO mice. (**A**) Representative data of expression of ObR gene in islets and non-islet pancreatic tissue in 6-month-old ObRlox and KO mice, as assessed by RT-PCR. Amylase was used as a marker for non-islet pancreas samples and β-actin as an internal control. *n* = 3. (**B**) Representative data of expression of ObR gene assessed by RT-PCR in whole-brain extract, hypothalamus, liver, lung, kidney, white adipose tissue (WAT), spleen, small intestine, heart, and skeletal muscle in 6-month-old ObRlox (lox) and KO mice. *n* = 3. (**C**) In situ hybridization for ObRb showing that it is localized in hypothalamus of both ObRlox and KO mice. Body weight (**D**), 24-hour ad libitum, food intake (**E**), fasting serum leptin concentration (**F**), and perigonadal fat pad weights (**G**) in 4-month-old male and female ObRlox and KO mice. *n* = 4–6; all *P* = NS. Data are shown as mean ± SEM. Scale bars: 500 μm.

the pancreas and islets but unaffected in extrapancreatic tissues in the KOs, indicating pancreas-specific recombination (Figure 1, A and B). The mRNA for ObRb assessed by in situ hybridization was expressed equally in the hypothalamus in both ObRlox and KO mice and confirmed the specificity of recombination (Figure 1C). In all cases, KO mice were compared with ObRlox controls.

Similar body weights, food intake, and fat mass in ObRlox control and KO mice. We next evaluated the physiological actions of leptin in the hypothalamus. Body weight, food intake, serum leptin concentrations, and perigonadal fat pad weights of 4-month-old male and female KO mice were similar to those of age- and sex-matched ObRlox controls (Figure 1, D–G). These findings confirm that ObRs in hypothalamus were functional in KO mice and that KO of ObRs in the pancreas did not modify body weight, food intake, fat mass, or circulating leptin levels in 4-month-old mutant animals.

Enhanced early-phase insulin secretion and improved glucose tolerance in KO mice. Leptin signaling suppresses insulin secretion (2–4). To evaluate the effects of pancreas-specific ObR deletion on β cell secretory function in vivo, we measured acute insulin release in response to glucose stimulation. In male and female ObRlox controls, a significant increase in insulin secretion was observed 2 minutes following i.p. glucose injection, consistent with an acute-phase insulin secretory response (Figure 2A). Interestingly, in KO mice, the acute-phase insulin secretory response to glucose was enhanced by 2-fold in males and 3-fold in females compared with controls (Figure 2A). Consequently, upon i.p. glucose challenge, KO mice showed a significantly lower glucose excursion, consistent with improved glucose tolerance (Figure 2B). Fasting circulating insulin levels in KO mice were approximately 2-fold higher than those in controls; the difference was statistically significant
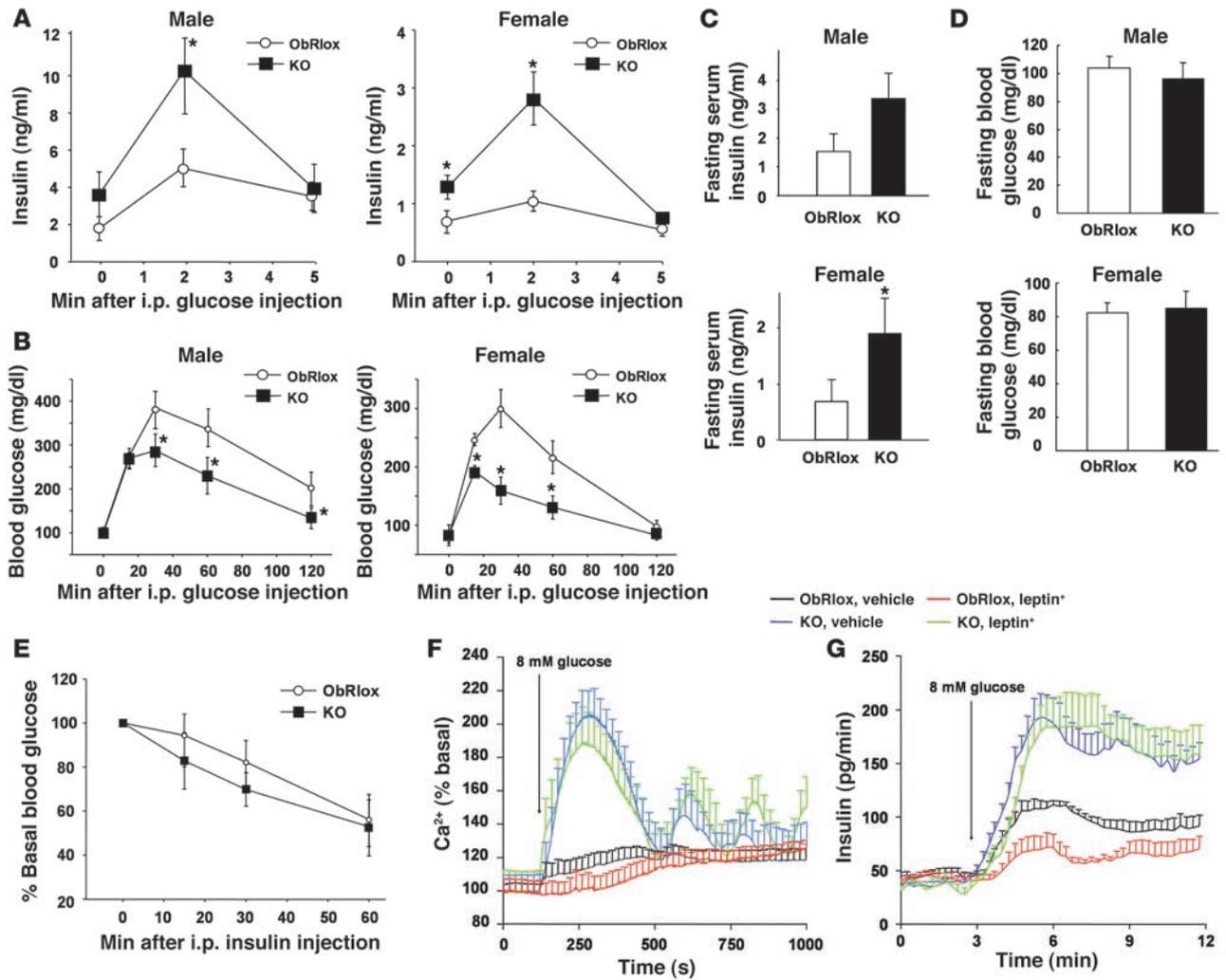
Figure 2
Improved glucose tolerance and enhanced early-phase insulin secretion in pancreas-ObR-KO mice. (**A**) Plasma insulin levels after an i.p. injection of glucose (3 g/kg body weight) to evaluate acute-phase insulin secretion in 6-month-old ObRlox and KO mice. *$P < 0.05$ versus ObRlox controls. $n = 6$. (**B**) Blood glucose after i.p. injection of glucose (2 g/kg body weight) in 6-month-old male and female ObRlox and KO mice. *$P < 0.05$ versus ObRlox controls; $n = 6–7$. Plasma insulin (**C**) and blood glucose (**D**) levels after overnight fasting in 6-month old male and female ObRlox and KO mice. *$P < 0.05$ versus ObRlox controls; $n = 6$. (**E**) Percent change in blood glucose after i.p. injection of insulin (1 U/kg body weight) in 6-month-old male ObRlox and KO mice. $n = 7$; $P = NS$. Data are shown as mean ± SEM. Representative traces of intracellular Ca^{2+} flux (**F**) and insulin secretion (**G**) measured in primary size-matched islets isolated from 6-month-old male ObRlox and KO mice with or without 100 nM (**F**) or 10 nM (**G**) leptin. For area under the curve (AUC), $P < 0.05$, ObRlox versus KO mice in each case; $P < 0.05$, vehicle versus leptin+ in ObRlox mice for both Ca^{2+} and insulin; $P = NS$ for vehicle versus leptin+ in KO mice for both Ca^{2+} and insulin. $n = 6$ islets from 3 individual mice in each group.

in females, and levels tended to be higher in males ($P = 0.07$), indicating increased basal insulin secretion (Figure 2C). Higher circulating levels of insulin were also observed in the random-fed state in KO mice compared with controls (male: 4.7 ± 0.3 versus 2.3 ± 0.4 ng/ml, $P < 0.05$, $n = 5$; female: 3.6 ± 0.4 versus 1.9 ± 0.3 ng/ml, $P < 0.05$, $n = 4–5$). Blood glucose levels in KO mice did not differ from those in ObRlox mice in the fasting state (Figure 1D) and tended to be lower, although the difference was not statistically significant in random-fed states (male: KO, 102 ± 14 versus ObRlox, 110 ± 18 mg/dl, $n = 5$; female: KO, 114 ± 16 versus ObRlox, 131 ± 20 mg/dl, $n = 5$). Whole-body insulin sensitivity evaluated by i.p. insulin tolerance tests (ITTs; 1 U Humulin/kg body weight) showed no differences between the groups (Figure 2E). Fasting plasma glucagon levels did

not show significant differences between groups (male: ObRlox, 66 ± 12 versus KO, 52 ± 14 pg/ml; female: ObRlox, 58 ± 14 versus KO 64 ± 16 pg/ml, $n = 5$; $P = NS$).

To evaluate insulin secretion in vitro, we cultured freshly isolated islets for 48 hours in glucose at physiological concentrations and then subjected single, size-matched islets from ObRlox or KO mice to step-up glucose stimulation. We observed significantly enhanced ($P < 0.05$) Ca^{2+} flux and insulin secretion in response to an increase in glucose concentration from 3 to 8 mM in the KOs (Figure 2, F and G). Further, consistent with previous reports (2, 3), leptin treatment suppressed Ca^{2+} flux and insulin secretion in control islets, while no effects were evident in the KO islets. These results suggest that absence of leptin signaling
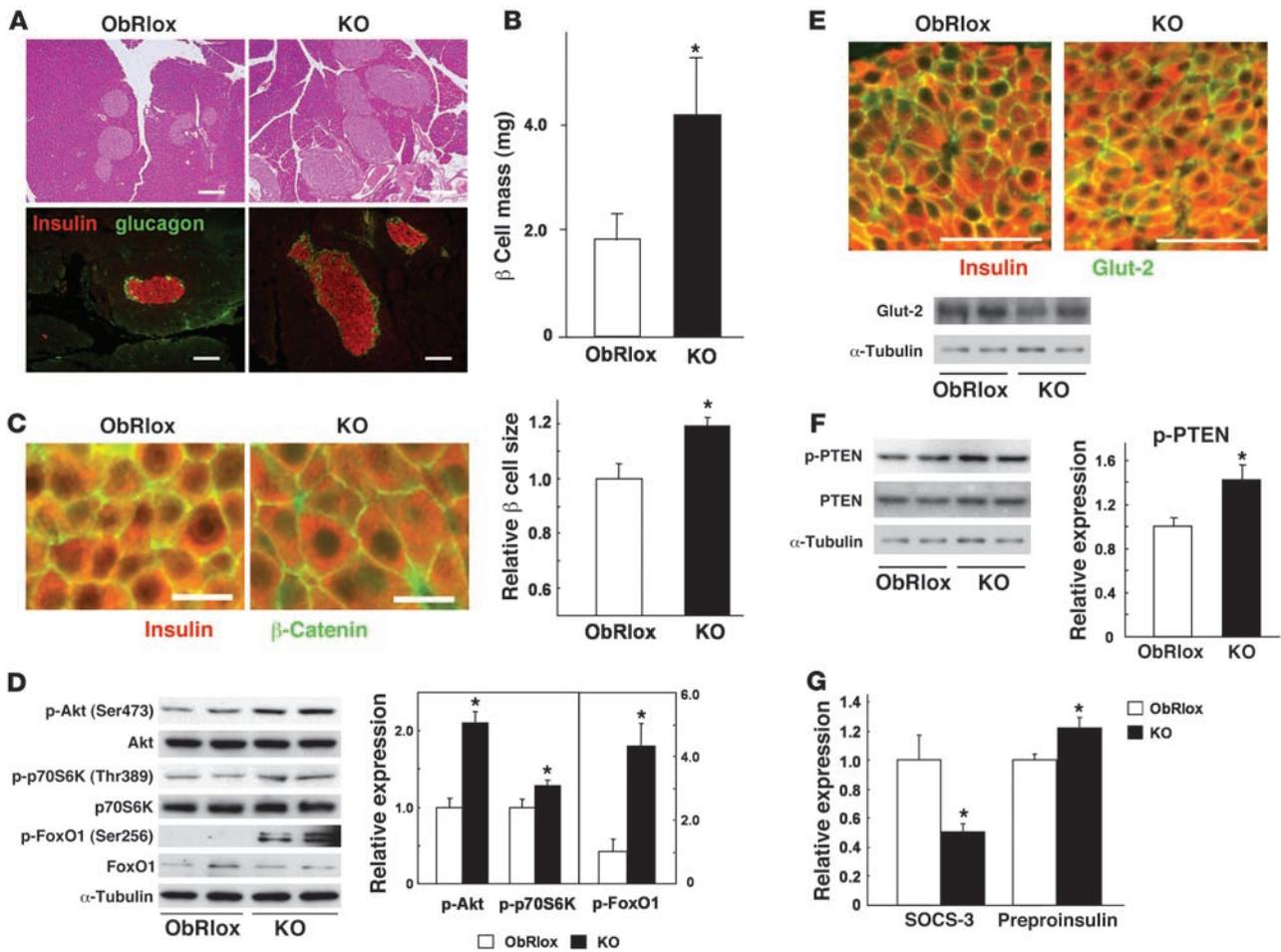
Figure 3

Increase in islet size and β cell mass and enhanced expression of insulin signaling proteins in islets of pancreas-ObR-KO mice. (**A**) H&E staining or immunofluorescence staining for insulin and glucagon in pancreas sections of ObRlox and KO mice. Scale bars: 100 μm. (**B**) β Cell mass estimated by morphometric analysis. *$P < 0.05$ versus ObRlox; $n = 4$. (**C**) Immunofluorescence staining for insulin and β-catenin in pancreas sections to determine β cell size. Relative β cell size (mean from $n ≥ 200$ cells counted) is shown in the graph. *$P < 0.05$ versus ObRlox; mean ± SEM, $n = 3$. Scale bars: 10 μm. (**D**) Western blots of total islet lysates for p-Akt (Ser473), Akt, p-p70S6K (Thr389), p70S6K, p-FoxO1 (Ser256), FoxO1, and α-tubulin as a loading control. The relative expression of p-Akt, p-p70S6K, and p-FoxO1 normalized to each total protein is shown in the graph. *$P < 0.05$ versus ObRlox; $n = 4–6$. (**E**) Immunofluorescence for Glut-2 and insulin in pancreas sections of ObRlox and KO mice. Scale bars: 50 μm. Western blots of total islet lysates for Glut-2 (**E**, lower panel), p-PTEN, and PTEN (**F**), normalized to α-tubulin. The relative expression of p-PTEN normalized to α-tubulin is shown in the graph. *$P < 0.05$ versus ObRlox; $n = 4$. (**G**) Expression of SOCS-3 and preproinsulin in ObRlox and KO islets assessed by quantitative real-time PCR. *$P < 0.05$ versus ObRlox; $n = 4–6$. Data were obtained from pancreas or islet samples from 4- to 6-month-old mice and are shown as mean ± SEM.

allows enhanced insulin secretion from cultured islets, consistent with the observations in vivo.

Islet hyperplasia and increased expression of insulin signaling proteins in KO islets. In addition to growth factors, leptin signaling has also been implicated in β cell growth in vitro (22). To evaluate whether disruption of ObRs in the pancreas affects islet morphology and β cell growth in vivo, we performed immunohistochemical analyses of pancreas sections using a cocktail of antibodies to non-β cell hormones (23, 24). We observed a 2-fold increase in β cell mass in KO mice that was secondary to an increase in β cell size (Figure 3, A–C) but not due to enhanced mitosis (control, 0.27% ± 0.2% versus KO, 0.37% ± 0.2% BrdU+ β cells [total 500 β cells counted], $n = 4$; $P = NS$). We did not observe differences in expression of cleaved caspase-3, suggesting that the increase in

β cell mass observed in KO mice is unlikely due to altered β cell apoptosis (data not shown).

To explore the pathways that mediate an increase in islet hyperplasia, we considered previous reports that proteins in the insulin/IGF-I pathways regulate β cell size and survival (8–19). Western blotting of islet lysates showed significantly enhanced phosphorylation of PKB/Akt at Ser473 (p-Akt), p70 S6 kinase at Thr389 (p-p70S6K), and FoxO1 at Ser256 (p-FoxO1) in KO mice by 2.1-, 1.3-, and 4.3-fold, respectively, compared with controls, with unaltered expression of total Akt, p70S6K, or FoxO1 protein levels (Figure 3D). These results suggest that, in the absence of leptin signaling, the increase in phosphorylation of p70S6K and PKB/Akt, which are important for determining β cell size and survival, respectively (16–18), likely contributes to an increase in β cell size and islet mass in pancreas-ObR-KO mice.
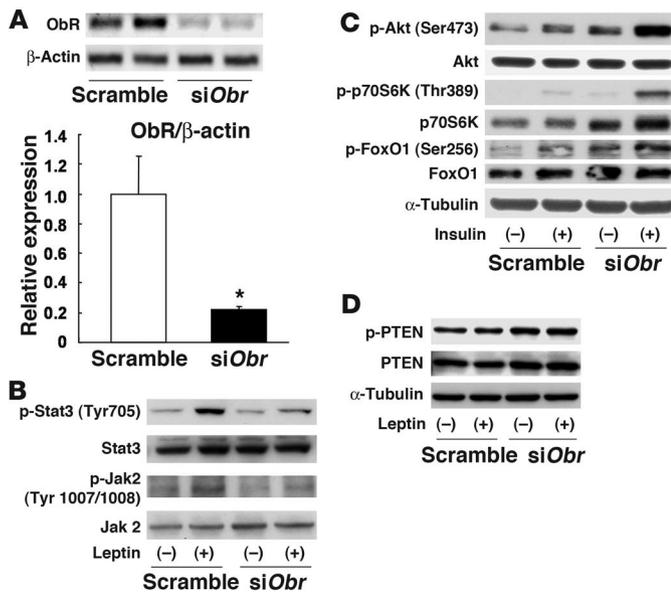
Figure 4
Increased expression and phosphorylation of insulin-signaling proteins in MIN6 β cells with knockdown of ObR gene. (**A**) RT-PCR analysis of ObR and β-actin in MIN6 β cells transfected with scrambled control or siRNA for ObR (si*Obr*) for 48 hours. The relative expression of ObR analyzed by real-time PCR was normalized to β-actin. *$P < 0.01$ versus scrambled control; mean ± SEM; $n = 6$. (**B**) MIN6 β cells were transfected with scrambled control or si*ObR* for 48 hours and treated with or without 10 nM leptin for 15 minutes following overnight serum starvation. Total cell lysates were extracted and subjected to Western blot analysis for p-Stat3, Stat3, p-Jak2, and Jak2. Each experiment was performed at least 3 times. (**C** and **D**) Western blot analyses for p-Akt (Ser473), Akt, p-p70S6K and p70S6K, p-FoxO1 (Ser256), FoxO1 (**C**), p-PTEN, and PTEN (**D**) normalized to α-tubulin protein in total cell lysates of MIN6 β cells transfected with scrambled control or si*ObR* and treated with or without 100 nM insulin (**C**) or 10 nM leptin (**D**) for 15 minutes following overnight serum starvation. Each experiment was performed at least 3 times.

To further explore the possible mechanisms underlying the increased insulin-secretory function with enhanced insulin receptor signaling pathway in KO mouse islets, we evaluated the expression of glucose transporter–2 (Glut-2) in β cells. Immunofluorescence analyses showed similar expression patterns of Glut-2 in both groups (Figure 3E, upper panel). Further, Western blotting of islet lysates showed the tendency of Glut-2 to decrease in KOs, but the difference was not statistically significant (Figure 3E, lower panel), suggesting that alterations in Glut-2 expression are unlikely to contribute substantially to the higher insulin secretion in the KOs.

Since phosphatase and tensin homolog (PTEN), a major negative regulator of the PI3K/Akt signaling pathway, was recently reported to be regulated by leptin in hypothalamic cell lines and pancreatic β cells (25), we evaluated phosphatase activity and protein content in KO islets. By Western blotting, we observed a mild but significant upregulation of PTEN phosphorylation (p-PTEN) in the KOs compared with controls (Figure 3F), while no differences were observed in total PTEN protein expression (KO, 122% ± 14% of control; $n = 4$; $P = NS$). These data are compatible with enhanced PI3K/Akt signaling in the KOs, in part due to the attenuation of PTEN, a negative regulator of insulin signaling.

SOCS-3 also acts as a negative regulator of insulin signaling and has been reported to link cytokine signaling with insulin resistance (26). In β cells, SOCS-3 has been reported to be induced by leptin and inhibit preproinsulin gene expression (27). Consistent with these observations, we observed a significant decrease in the expression of SOCS-3 by quantitative real-time PCR in KO islets that was associated with a mild but significant increase in preproinsulin gene expression (Figure 3G). These results confirm the link between SOCS-3 and insulin gene expression and provide additional evidence for a regulatory role for leptin/insulin signaling in β cell secretory function.

Enhanced insulin signaling in MIN6 β cells with ObR gene knockdown. To further evaluate the potential interactions between insulin and leptin signaling pathways in vitro, we utilized MIN6 β cells with *ObR* gene knockdown using siRNA. Transfection of siRNA oligonucleotides for mouse ObR (si*Obr*) resulted in an approximately 80% reduction in *ObR* gene expression in MIN6 β cells and was

confirmed by quantitative real-time PCR (Figure 4A). In control MIN6 β cells transfected with scrambled RNA, leptin treatment promoted tyrosine phosphorylation of Stat3 (p-Stat3) and Jak2 (p-Jak2), while virtually no effect was observed in si*Obr*-knockdown cells (Figure 4B), indicating that leptin-stimulated Jak2/Stat3 signaling is attenuated in cells with *ObR* knockdown.

Next, to assess alteration in proteins in the insulin-induced signaling pathways in MIN6 β cells with *ObR* knockdown, we treated MIN6 β cells with insulin after transfection of si*Obr* or scrambled RNA. MIN6 β cells with *ObR* knockdown showed an increase in protein expression of p70S6K and FoxO1, in the basal state, and elevated insulin-induced phosphorylation of PKB/Akt (Ser473), p70S6K (Thr389), and FoxO1 (Ser256) (Figure 4C). We also observed mildly elevated PTEN phosphorylation in MIN6 β cells with *ObR* knockdown in both basal and leptin-treated states (Figure 4D). These findings in MIN6 β cells are in agreement with signaling data in islets from pancreas-ObR-KO mice (Figure 3, D and F) and suggest that the phenotypes in KOs are due to a primary defect in β cells and unlikely due to altered leptin action in non-β cells, neural, or other circulating factors. Indeed, no significant differences were detected in circulating glucagon or somatostatin (data not shown) levels between groups.

Impaired glucose tolerance in pancreas-ObR-KO mice induced by HFD feeding. In addition to its role in islet function and survival, leptin modulates lipid metabolism in islets (28, 29). To explore whether the enhanced insulin secretion observed in the KOs on a normal chow diet protects the mutants in the face of caloric excess, we fed control and KO mice with an HFD or normal chow for 12 weeks. After high-fat feeding, as expected, mice in both groups were significantly heavier and exhibited insulin resistance compared with their littermates on normal chow diet, confirming the effects of diet-induced obesity (Figure 5, A and B). Further, the HFD-fed mice in the KO and control groups responded by gaining similar body weights (Figure 5A) and were equally insulin resistant (Figure 5B). Surprisingly, however, the KO mice on HFD exhibited impaired glucose tolerance that was, in part, due to blunted acute insulin response to glucose compared with the controls (Figure 5, C–E). Furthermore, while control mice on HFD developed islet
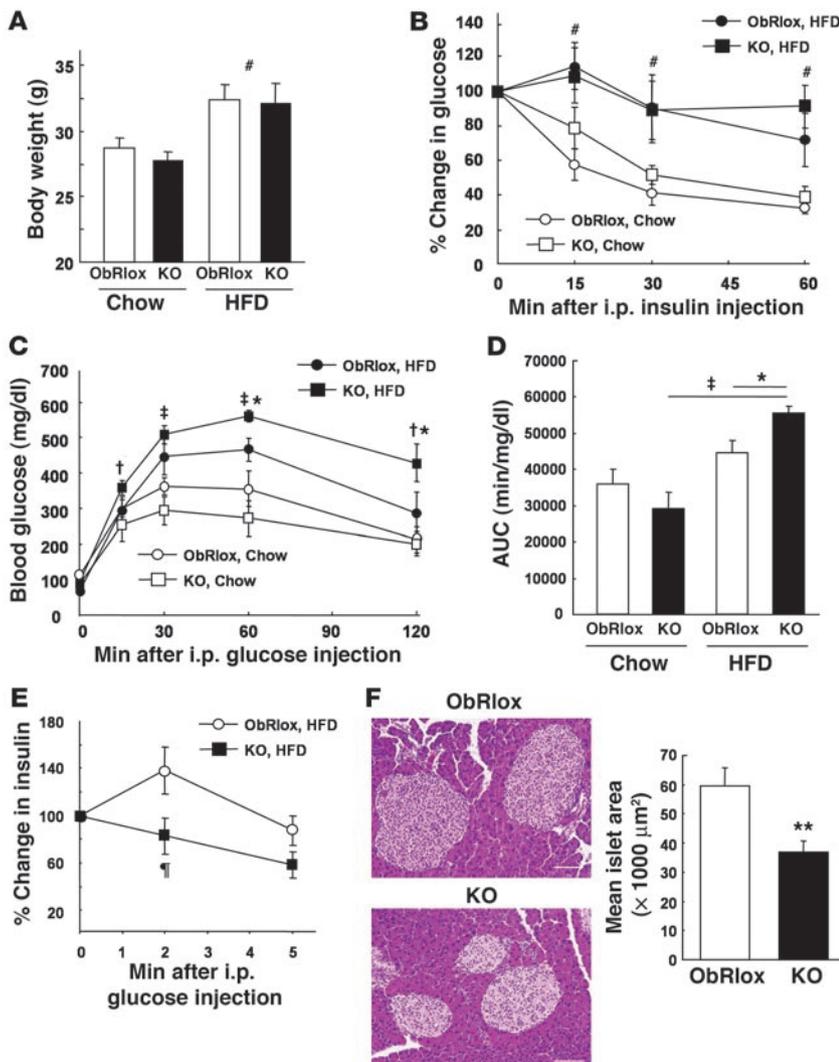
Figure 5
Diet-induced obesity leads to impaired glucose tolerance in pancreas-ObR-KO mice. Body weight (**A**), ITT results (**B**), glucose tolerance test (GTT) results (**C**), and area under the curve for the GTT results (**D**) in 4-month-old male ObRlox and KO mice on regular chow (Chow) or HFD for 12 weeks. #$P < 0.05$, HFD ($n = 10$) versus Chow ($n = 6$); *$P < 0.05$, KO mice on HFD versus ObRlox mice on HFD, $n = 5$; †$P < 0.05$, ‡$P < 0.01$, KO mice on HFD versus KO mice on Chow, $n = 5$ (KO) or 3 (Chow). (**E**) Percent change in plasma insulin levels after i.p. glucose injection (3 g/kg body weight) in ObRlox and KO mice on HFD for 12 weeks. ¶$P = 0.07$; $n = 4$. (**F**) Left: H&E staining in representative pancreas sections of ObRlox and KO mice on HFD for 12 weeks. Scale bars: 100 μm. Right: Mean islet area from at least 10 islets from 5 individual mice for each genotype. **$P < 0.01$ versus ObRlox. Data are shown as mean ± SEM.

hyperplasia to compensate for insulin resistance compared with mice on normal chow, the KO mice showed a significantly reduced islet mass (61.8% of control) after high-fat feeding (Figure 3A and Figure 5F). Leptin has been reported to regulate lipid metabolism in β cells and to protect islets from the effects of lipid overload (28, 30). Lipid accumulation in the islets, in turn, is known to impair islet secretory function and viability (28). Thus, it is possible that switching the diet from normal chow to high-fat in mice lacking leptin signaling in islets leads to lipid accumulation in the islets and consequent poor insulin secretory response and attenuated islet growth, leading to altered glucose homeostasis. Further molecular studies are necessary to dissect the pathways involved in leptin modulation of lipid metabolism and in the cross-talk with growth factor pathways regulating the β cell proliferation response in these mutants in the context of an HFD.

Discussion

To directly assess the role of ObR signaling in the growth and function of pancreatic β cells, we created a genetic model using a mouse expressing *Cre*-recombinase driven by the *Pdx1* promoter for 2 reasons. First, the absence of *Cre* expression in the brain of the Pdx-*Cre* mouse eliminates the possibility of recombination in the hypo-

thalamus, which is rich in ObR-positive neurons (21). Second, this approach allowed us to examine the effect of recombination of the ObR gene around E8.5, when the *Pdx1* promoter is switched on earlier than expression of the rat insulin promoter (21). Our studies provide genetic evidence for a direct role for leptin in the endocrine pancreas, in contrast to a recent report that used a mouse expressing *Cre* recombinase on the rat insulin promoter that is expressed later during development (~E9.5) and is also expressed in some neurons in the hypothalamus (31). Considering the dominant role of leptin in the hypothalamus, it is likely that the islet phenotype in mice generated with the rat insulin promoter–driven Cre is masked by alterations in hypothalamic function affecting insulin sensitivity and fat mass.

Previous studies have reported that leptin signaling interacts with proteins in the insulin-signaling pathway to exert its biological actions in several tissues. For example, leptin activates the IRS/PI3K pathway in hypothalamic neurons to regulate food intake and body weight (1) and to stimulate glucose transport and glycogen synthesis in muscle cells (32). In hepatocytes, leptin activates IRS-associated PI3K and PDE3B to promote inhibition of cAMP elevation by glucagon (33, 34). In β cell lines and isolated islets, leptin inhibits insulin secretion through PI3K-dependent PDE3B
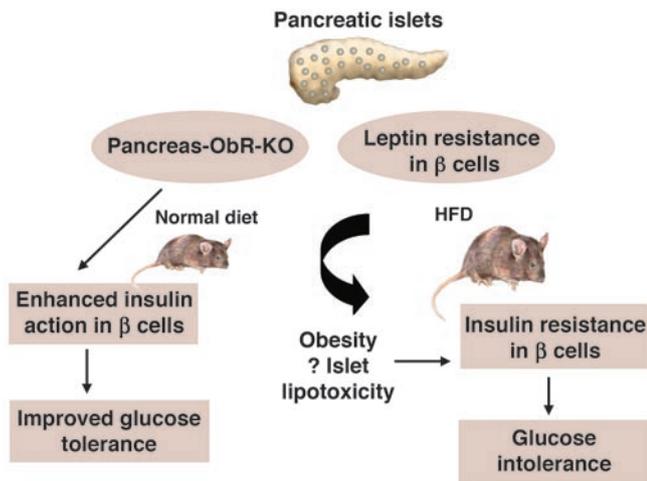
Figure 6
Schematic proposing a potential role for leptin signaling in islets in the development of obesity-associated diabetes.

activation or K_{ATP} channel activation (2). Consistent with an inhibitory role for leptin, pancreas-ObR-KO mice showed enhanced acute-phase glucose-stimulated insulin secretion and elevated fasting plasma insulin concentrations. The lack of a corresponding significant decrease in fasting glucose levels suggests that pathways independent of leptin may play a role during the fasting state. Alternatively, the inhibitory effects of leptin may be more apparent when GLP-1 is secreted during the fed state (2). Furthermore, our studies provide evidence, for the first time to our knowledge, that absence of leptin action promotes β cell growth by affecting the phosphorylation of p70S6K and Akt; the qualitatively similar signaling defects observed in MIN6 β cells with an siRNA knockdown of ObRs suggest that the phenotype in mice in vivo is primarily due to an effect in β cells. Indeed, simultaneous regulation of p70S6K and Akt has been reported to augment growth in other cell types (35). The phenotype in pancreas-ObR-KO mice are independent of direct effects in α cell function since ObRs are not expressed in α cells (2); however, pacarine effects of somatostatin due to altered δ cell function cannot be ruled out. Furthermore, the effects of ObR-KO in the potential modulation of exocrine pancreatic tissue needs further investigation. The findings in the pancreas-ObR-KO mouse provide one explanation for the increase in β cell mass observed at an age that precedes weight gain or hyperinsulinemia in Zucker fatty rats, which also lack leptin action (36). Similarly, the islet hyperplasia observed in mice bearing defects in the leptin (ob/ob) or the ObR (db/db) gene is likely due, in part, to deficient leptin signaling and consequent enhanced insulin action in β cells (7, 37). Thus, it is important to consider the effects of lack of leptin signaling in islets in these widely used naturally occurring rodent models when studying glucose homeostasis.

The reduced SOCS-3 and mildly increased preproinsulin gene expression in the islets of KO mice are compatible with the reports that SOCS-3 is a leptin-induced negative regulator of leptin signaling (1) and an in vitro study showing inhibition of preproinsulin gene expression by leptin induction of SOCS-3 (27). Thus, the reduced expression of SOCS-3 in the KO islets is consistent with enhanced phosphorylation of PI3K/Akt signaling in islets lacking ObR expression (26). The reduced SOCS-3 expression might con-

tribute, in part, to enhanced insulin secretion and islet hyperplasia by upregulating insulin gene expression and insulin signaling in islets lacking leptin signaling. Finally, the elevated PTEN phosphorylation in KO islets and MIN6 β cells with ObR knockdown could indirectly enhance signaling via the PI3K/Akt pathway in the β cells, since phosphorylation of PTEN leads to inactivation of its phosphatase activity. One interpretation of these studies is that absence of leptin signaling allows enhanced action of insulin on β cells in an autocrine/paracrine manner to promote secretory function and/or growth.

To explore whether the effect of enhanced acute-phase secretion and improved glucose tolerance observed in the KO mice on normal chow persists and protects KOs against the effects of diet-induced obesity, we fed the KO mice with an HFD. Surprisingly, we observed glucose intolerance that was significantly worse in the KOs compared to that in the controls. Indeed, these findings bring into context the role of leptin in regulating lipid metabolism and islet function/growth (28, 29). For example, expression of wild-type ObRs in the islets of Zucker diabetic fatty rats attenuates accumulation of lipid content and rescues diabetogenic phenotypes such as impaired insulin secretion and β cell apoptosis (28). Thus, the attenuated acute-phase secretory response and an inadequate compensatory islet growth response in the KOs on the HFD may be due to secondary effects of lipid overload in the islets as a consequence of absent leptin signaling and is one possible mechanism underlying the impaired glucose tolerance in the pancreas-ObR-KO mice (summarized in Figure 6). Alternatively, insulin resistance at the level of the β cell itself in the KOs may directly contribute, in part, to a failure of the β cell to compensate for HFD-induced insulin resistance. In any case, the specific interactions among leptin signaling, lipid metabolism, and growth factor proteins contributing to insulin resistance in β cells in the context of obesity warrants further investigation.

A distinct difference between the pancreas-ObR-KO model reported in this article, created using Pdx-Cre, and that reported by Covey et al, using RIP-Cre (31), is the recombination in the hypothalamus in the latter. Although the mice reported by Covey et al. did not manifest enhanced food intake, the increased body weight, fat mass, serum leptin levels, and plasma and liver triglyceride content, even when the mice were fed normal chow, together can directly contribute to glucose intolerance and poor insulin sensitivity in these mice (20, 38). In contrast, the pancreas-ObR-KOs on normal chow did not manifest alterations in insulin sensitivity but exhibited improved glucose tolerance due to enhanced insulin secretion that is consistent with a lack of tonic inhibitory action of leptin on β cell secretion (2, 3). Furthermore, we also provide evidence for crosstalk between leptin and insulin signaling pathways that contributes to alterations in β cell growth and function. Thus, the phenotype in pancreas-ObR-KO mice reflects the direct effects of absent leptin signaling in the islets, independent of the hypothalamus, and provides a straightforward model to study the role of leptin signaling in the development of obesity-associated diabetes. It will be especially instructive to explore whether disruption of both leptin and insulin signaling in the β cells, akin to leptin and insulin resistance associated with diet-induced obesity, leads to a greater susceptibility to development of diabetes.

In summary, we have created a unique genetic model to directly demonstrate the consequences of lack of functional ObRs in islet β cells. It is tempting to speculate that leptin resistance in pancreatic

β cells is one factor that contributes to the hyperinsulinemia, β cell failure, and consequent glucose intolerance in the obese state.

Methods

Animals and genotyping. ObRlox mice were created by homologous recombination using the ObR gene with *loxP* sites flanking exon 1, as previously described (20), and were maintained on a C57BL/6 background. Transgenic mice expressing *Cre* recombinase under the control of the mouse *Pdx1* promoter (Pdx-*Cre* mice) (21) were maintained on a C57BL/6 background and bred with *Obr* floxed mice. All animals were housed in specific pathogen–free facilities and maintained on a 12-hour light/12-hour dark cycle and fed standard rodent chow at the Foster Animal Laboratory, Brandeis University, Waltham, Massachusetts, USA. We used both male and female mice for all experiments. All protocols for animal use were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and Brandeis University and were in accordance with NIH guidelines.

For the high-fat feeding study, 6-week-old ObRlox control and pancreas-ObR-KO male mice were fed either an HFD or regular chow for 12 weeks as described previously (11). Body weights were followed weekly, and glucose tolerance tests (GTTs) and ITTs were performed during the last week of feeding as described below.

Genotyping was performed on DNA isolated from the tails of 3- to 4-week-old mice by PCR. The primers for the *obrlox* gene were 5′-GTCACCTAGGT-TAATGTATTC-3′ (forward) and 5′-TCTAGCCCTCCAGCACTGGAC-3′ (reverse) and for the *Cre* transgene were 5′-TGCCACGACCAAGTGACAGC-3′ (forward) and 5′-CCAGGTTACGGATATAGTTCATG-3′ (reverse).

Physiological measurements. Food intake of the mice was measured as described previously (39). We determined blood glucose values from whole venous blood using an automated glucose monitor (Glucometer Elite; Bayer) and measured serum insulin and leptin levels by ELISA, using mouse insulin or leptin standards (Crystal Chem Inc.). GTTs and acute insulin secretion tests were performed on animals that had been fasted overnight for 16 hours (8, 9). For GTTs, glucose levels were measured from blood collected from the tail immediately before and 15, 30, 60, and 120 minutes after i.p. glucose injection. For acute insulin secretion tests, blood samples were collected before (time 0) and 2 and 5 minutes after i.p. glucose injection (3 g/kg body weight). ITTs were performed as previously described (9). Blood glucose levels were determined immediately before and 15, 30, and 60 minutes after injection of insulin at random-fed state (1 U/kg body weight) (Humulin R; Lilly).

RT-PCR and quantitative real-time PCR. Islets were isolated from mice as described previously (3). Total RNA from islets, non-islet tissue, and whole-brain extract, hypothalamus, liver, lung, kidney, white adipose tissue, spleen, small intestine, heart, and skeletal muscle was extracted using RNeasy Mini Kit (QIAGEN). Expression of the *obr* gene in the different tissues was detected by RT-PCR (8, 9). The primers for mouse *obr* were 5′-TGATTTGTGTTTGATTTATTGTTT-3′ (forward) and 5′-TATTCGTAGACGTGGGTTGG-3′ (reverse); for mouse β-actin were 5′-AGGGCTATGCTCTCCCTCAC-3′ (forward) and 5′-AAGGAAGGCT-GGAAAAGAGC-3′ (reverse); and for mouse amylase were 5′-ATGTGGT-CAATGGTCAGCCTTTTTCA-3′ (forward) and 5′-TGCCATCACTGCCAA-CATTCACTCTA-3′ (reverse). Real-time quantitative PCR of islet samples or MIN6 β cells was performed as previously described (11). The primers used for real-time PCR for mouse *obr* were 5′-GCTCTTCTGATGTATTT-GGAAATC-3′ (forward) and 5′-ACCTGATATTGAAGCGGAAATGG-3′ (reverse); for mouse SOCS-3 were 5′-GGGGGAGGCAGGAGGTGATGG-3′ (forward) and 5′-GGGCGGGCTGGAGGTGGATT-3′ (reverse); and for mouse insulin I were 5′-CCTGTTGGTGCACTTCCTA-3′ (forward) and 5′-TCTGAAGGTCCCCGGGGGCT-3′ (reverse).

In situ hybridization for ObRb. Mouse brain was dissected and pretreated as described previously (40). For in situ hybridization for ObR mRNA in the mouse brain, we followed the protocol as previously described (41) by using a probe specific for ObRb (42).

Measurement of insulin secretion and Ca²⁺ flux in islets. Mouse islets were isolated as described above, and single size-matched islets were incubated in different concentrations of glucose with or without mouse recombinant leptin (Sigma-Aldrich) as indicated and assayed for insulin secretion and intracellular Ca^{2+} concentration as described previously (10).

β Cell mass, size, and immunohistochemistry. Mice were anesthetized, and pancreas was rapidly dissected, weighed, fixed in Bouin's solution, sectioned, and stained; and β cell mass was calculated by morphometric analysis as described previously (23, 24). Antibodies used for the immunofluorescence staining were as follows; insulin: guinea pig anti–human insulin (Linco) and Texas red–conjugated donkey anti–guinea pig IgG (Jackson ImmunoResearch Laboratories Inc.); glucagon: rabbit anti–glucagon antibody (Zymed) and FITC-conjugated goat anti-rabbit antibody (Zymed); β-catenin: mouse anti–β-catenin antibody (BD Biosciences) and Cy2-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.); Glut-2: rabbit anti–Glut-2 antibody (Alpha Diagnostic International) and Cy2-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.); BrdU: mouse monoclonal BrdU antibody (Dako) and Cy2-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.); cleaved caspase-3: rabbit polyclonal anti–cleaved caspase-3 (R&D Systems) and Texas red–conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc.). β Cell and islet size were analyzed using ImageJ software (NIH; http://rsb.info.nih.gov/ij/). DAPI (Sigma-Aldrich) was used for nuclear staining.

Cell culture and siRNA transfection. MIN6 β cells were incubated at 37°C and 5% CO₂ in DMEM supplemented with 15% FBS and penicillin and streptomycin. Predesigned siRNA for mouse ObR (Ambion) was transfected in MIN6 β cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 100 nM of siRNA was mixed with Lipofectamine 2000 in Opti-MEM I medium and incubated at room temperature for 20 minutes. MIN6 β cells were washed and incubated in antibiotic-free DMEM with 15% FBS before transfection. The siRNA-Lipofectamine mixture was added to the cells and incubated for approximately 8 hours at 37°C and 5% CO₂ before being replaced with normal culture medium. The cells were harvested for protein or RNA extraction 48 hours after transfection.

Western blotting. Protein samples extracted from isolated islets or cell lysates were subjected to SDS-PAGE and immunoblotting (23). Antibodies against Akt, phospho-Akt (Ser473), p70S6K, phospho-p70S6K (Thr389), FoxO1, phospho-FoxO1 (Ser256), phospho-PTEN (Ser380/Thr382/383), PTEN, and phospho-Stat3 (Tyr705) were from Cell Signaling Technology. Anti-α-tubulin antibody was from Abcam. Anti–Glut-2, -Stat3, and -Jak2 antibodies were from Santa Cruz Biotechnology Inc. Anti–phospho-Jak2 (Tyr1007/1008) was from Biosource. HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Santa Cruz Biotechnology Inc. Intensity of the bands was quantified using ImageJ software.

Statistics. All data are presented here as mean ± SEM and were analyzed using an unpaired 2-tailed Student's *t* test or analysis of variance (ANOVA) as appropriate. A *P* value less than 0.05 was considered significant.

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