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PTEN Deletion in Pancreatic α-Cells Protects Against High-Fat Diet–Induced Hyperglucagonemia and Insulin Resistance

Diabetes 2015;64:147–157 | DOI: 10.2337/db13-1715

An aberrant increase in circulating catabolic hormone glucagon contributes to type 2 diabetes pathogenesis. However, mechanisms regulating glucagon secretion and α-cell mass are not well understood. In this study, we aimed to demonstrate that phosphatidylinositol 3-kinase (PI3K) signaling is an important regulator of α-cell function. Mice with deletion of PTEN, a negative regulator of this pathway, in α-cells show reduced circulating glucagon levels and attenuated L-arginine–stimulated glucagon secretion both in vivo and in vitro. This hypoglucagonemic state is maintained after high-fat–diet feeding, leading to reduced expression of hepatic glycogenolytic and gluconeogenic genes. These beneficial effects protected high-fat diet–fed mice against hyperglycemia and insulin resistance. The data demonstrate an inhibitory role of PI3K signaling on α-cell function and provide experimental evidence for enhancing α-cell PI3K signaling for diabetes treatment.

Insulin and glucagon are the two main hormones that regulate glucose metabolism and energy homeostasis. The principal function of insulin is to stimulate the uptake and storage of glucose after food intake while suppressing endogenous hepatic glucose production, whereas glucagon, the main counterregulatory hormone of this anabolic process, stimulates hepatic glycogen breakdown and glucose production. The continual fine balance between the actions of these two hormones is essential for maintaining glucose homeostasis (1).

Insulin-producing β-cells and glucagon-producing α-cells are located in proximity to each other within the pancreatic islets. In mice, β-cells are located centrally within the islet, and α-cells have a satellite distribution in the periphery (2). Murine islet blood supply flows from the center to the periphery, forming the β-to-α-microcirculation within islets and thereby directing insulin to the α-cells (3). In comparison, human islets have much less organized α- and β-cell distribution (4). Nonetheless, in both cases, α-cells are exposed to insulin concentrations that are of magnitudes higher than the level seen in peripheral tissues. Thus, insulin signaling in α-cells may play a critical role in their function. Early studies using either insulin antibody to deplete endogenous insulin or infusion of recombinant exogenous insulin reported an inhibitory function of insulin on glucagon secretion (5,6). However, differentiating the direct effects of insulin per se on α-cell function from the indirect effect of glycemic change in response to insulin is difficult. Kawamori et al. (7), using α-cell–specific insulin receptor knockout mice, showed that genetic ablation of insulin signaling in α-cells leads to hyperglucagonemia and hyperglycemia. Islets from these mice demonstrated enhanced secretion of glucagon under

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Received 8 November 2013 and accepted 28 July 2014.
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-1715/-/DC1.
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both normo- and hypoglycemic conditions. These findings confirmed the inhibitory effects of insulin on glucagon secretion. More importantly, they highlighted the dominant inhibitory role of insulin on α-cell function over the stimulatory role of glucose on α-cells, thus favoring the β-cell switch-off hypothesis that supports insulin signaling as the master regulator of glucagon secretion (8).

Type 1 and type 2 diabetes, although vastly different in their root cause, share features of uncontrolled hyperglycemia and impaired hepatic glucagon response (9). Thus, α-cell secretory defects in combination with the well-known β-cell deficiency together exacerbate hyperglycemia in both conditions. Although the role of insulin in suppression of glucagon secretion is appreciated, the specific downstream signaling transduction pathways that mediate the glucagon inhibition remain elusive. Given that phosphatidylinositol 3-kinase (PI3K) is the major signaling pathway downstream of insulin, we investigated the role of this signaling cascade in α-cells by examining mice with α-cell–specific deletion of PTEN, a potent negative regulator of this pathway.

RESEARCH DESIGN AND METHODS

Pten$^{fl/fl}$ mice (exons 4 and 5 of *Pten* flanked by *loxP* sites by homologous recombination) were mated with GLU*cre* mice (*cre* transgene under the control of the glucagon promoter) (10). GLU*cre*$^{+}$ *Pten*$^{+/fl}$ mice were intercrossed to generate GLU*cre*$^{+}$ *Pten*$^{+/+}$, GLU*cre*$^{+}$ P*ten*$^{+/fl}$, and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice. GLU*cre*$^{+}$ *Pten*$^{+/+}$ mice were used as controls because as we showed previously, GLU*cre*$^{+}$ and GLU*cre*$^{-}$ mice have similar phenotypes (11). Only male mice were used for experiments, and only littermates were used as controls. Genotypes for *cre* and *Pten* gene were determined with PCR (Supplementary Fig. 1*A*) using ear clip DNA as described previously (12,13). All mice were maintained on a mixed 129/J-C57BL/6 background, housed in a pathogen-free facility on a 12-h light-dark cycle, and fed ad libitum with standard irradiated rodent chow (5% fat) (Harlan Teklad, Indianapolis, IN) without restriction to animal activity in accordance with University Health Network Animal Care Facility protocol.

High-Fat–Diet Feeding

A high-fat diet (HFD) (60% kcal from fat; F3282, Bio-Serv, Frenchtown, NJ) for GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ and GLU*cre*$^{+}$ *Pten*$^{+/+}$ mice were started at 2 months of age and continued for 7 months.

Metabolic Studies

All overnight fasts were carried out between 5 P.M. and 10 A.M. All blood glucose measurements, glucose tolerance tests (GTTs), insulin tolerance tests (ITTs), and glucose-stimulated insulin secretion (GSIS) tests were performed in overnight-fasted animals as previously described (14). For L-arginine injection tests, blood glucose levels were measured before and after intraperitoneal injection of L-arginine 3g/kg body weight at 15, 30, 45, 60, and 120 min. Serum insulin and glucagon concentrations were measured before and after intraperitoneal L-arginine injection at 2 and 10 min. Serum insulin was measured by ELISA kit using a rat insulin standard (Crystal Chem, Downers Grove, IL), and serum glucagon was measured by the Hormone Assay & Analytical Services Core at Vanderbilt University (Nashville, TN). GLP-1 was measured using the Active GLP-1 (v2) Kit (Meso Scale Discovery, Gaithersburg, MD) (11).

Small Interfering RNA Transfection and Glucagon Secretion Analysis

Glucagon-secreting In-R1-G9 cells (a gift from Dr. Tianru Jin, Department of Physiology, University of Toronto, Toronto, ON) were cultured as previously described (7). In-R1-G9 cells were replated in 12-well plates/60-mm dishes and transfected with small interfering RNA (siRNA) for PTEN or scrambled control (Ambion; Invitrogen, Carlsbad, CA) with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). After 48-h culture in RPMI medium, cells were harvested for hormone secretion or protein extraction studies. Transfected cells were incubated in glucose or L-arginine at concentrations reported in previous studies, and media were extracted for glucagon measurements after incubation (7,11,15). Cell count was done after 48-h culture.

Immunohistochemistry and Immunofluorescence

Pancreata were fixed in 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) as previously described (12,16). For immunohistochemical and immunofluorescent staining, specimens from each animal were paraffin embedded, and then 7-μm–thick sections from at least three levels, 150 μm apart, were prepared by the University Health Network Pathology Research Program. Antibodies against synaptophysin (Neomarkers, Fremont, CA), insulin (Dako, Glostrup, Denmark), glucagon (Novocastra Laboratories, Newcastle upon Tyne, U.K.), and PTEN (Abcam, Cambridge, U.K.) were used. Total islet area and total pancreas area were determined on insulin immunohistochemically stained sections using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) and expressed as total islet area per total pancreas area. Glucagon- and insulin-positive areas were also determined using Image-Pro Plus. Immunofluorescent staining of dispersed islet cells were performed as previously described (17).

Western Blotting

Liver, muscle, visceral adipose tissue, islets, and hypothalami were isolated and protein lysates obtained as previously described (12). Antibodies against actin (Santa Cruz Biotechnology, Santa Cruz, CA), AMPK, p-AMPK, Akt, p-Akt, FoxO-1, p-FoxO-1, p-CREB, CREB, and PTEN (all from Cell Signaling Technology, Beverly, MA) were used.

Quantitative PCR

Islet RNA was extracted with RNeasy Plus Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized according to the protocol published elsewhere (16). PCR was monitored in real time

using the ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Experiments were performed in triplicate for each sample. Primer sequences for glucose-6-phosphatase (G6Pase), PEPCK, glucokinase, and fatty acid synthase are available upon request.

Statistics

Data are presented as mean ± SEM and were analyzed by one-sample *t* test, independent-sample *t* test, and one-way ANOVA with the post hoc Tukey least significant difference test where appropriate. All data were analyzed using SPSS version 16.0 for Macintosh statistical software.

RESULTS

Generation of Mice With α-Cell–Specific PTEN Deletion

α-Cell–specific PTEN-deleted mice (GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice) were generated by crossing mice carrying the PTEN-floxed gene with mice expressing cre recombinase driven by the glucagon promoter. GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice were born in a Mendelian ratio without any apparent postnatal abnormalities. Previous studies by others and our group using the same GLU*cre*$^{+}$ mice reported an efficient recombination rate (>85% α-cells) (7,11,18). To confirm the efficacy of the gene disruption, we coimmunostained glucagon with cre recombinase and then PTEN in dispersed islet cells. Cre colocalized with all glucagon-positive α-cells but not with glucagon-negative cells, demonstrating efficiency and specificity of glucagon promoter in driving *cre* expression in α-cells (Fig. 1*A*). Furthermore, in islet cells of GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice, PTEN expression was substantially reduced in glucagon-positive α-cells (Fig. 1*B*, Supplementary Fig. 1*B* and *C*). Similarly, immunofluorescent staining of pancreatic sections for glucagon and PTEN showed colocalization in control but not GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (Supplementary Fig. 1*D*). As expected, the whole-islet PTEN expression was not significantly changed in these mice (Supplementary Fig. 1*E*), reflecting the low proportion of α-cells within islets and, importantly, that the majority of non-α-islet cells likely did not have PTEN deletion. Furthermore, PTEN expression remained unchanged in the other tissues examined, including

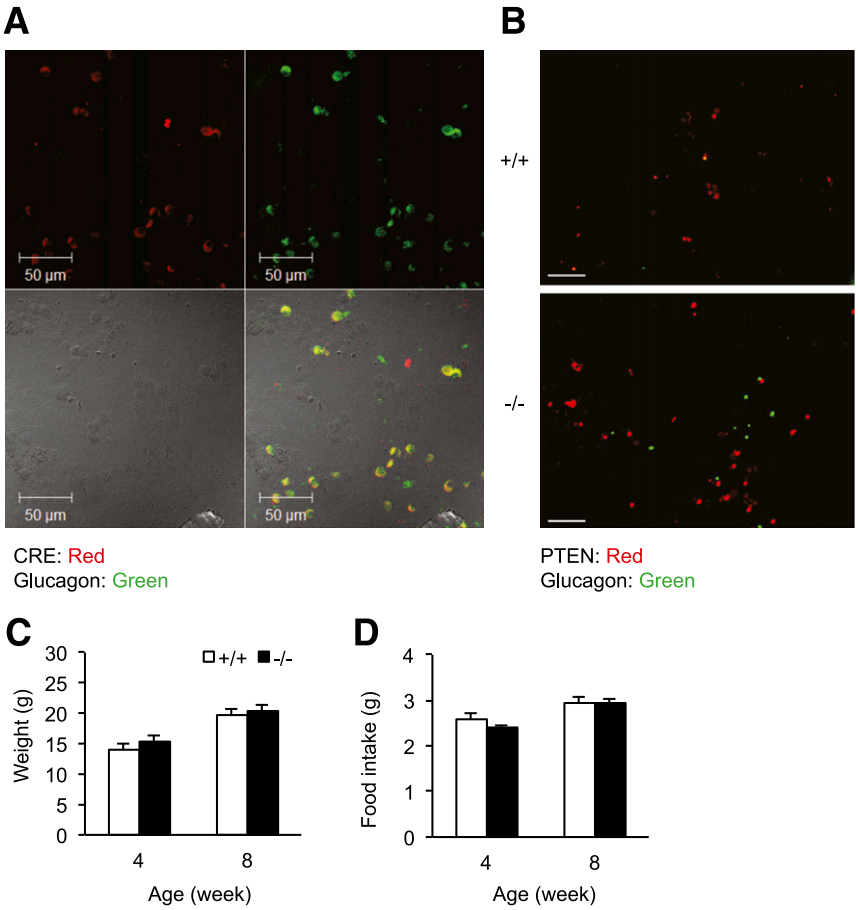

Figure 1—Immunofluorescent staining of dispersed islets and metabolic parameters of GLU*cre*$^{+}$ *Pten*$^{+/+}$ ($^{+/+}$) and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ ($^{-/-}$) mice. *A*: Immunofluorescent costaining of glucagon with cre recombinase in dispersed islet cells from GLU*cre*$^{+}$ mice (magnification ×20). *B*: Immunofluorescent costaining of glucagon with PTEN in dispersed islet cells from GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (magnification ×4, scale bars = 200 μm). *C*: Weight of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at 4 and 8 weeks of age (n = 20). *D*: Daily food intake of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at 4 and 8 weeks of age (n = 20).

hypothalamic, adipose, muscle, and liver tissue, supporting the lack of nonspecific genetic recombination in these metabolic tissues (Supplementary Fig. 1*E*). We also previously showed no significant ectopic cre activity in the brainstem or distal ileum containing GLP-1–secreting L cells (11); moreover, serum active GLP-1 levels from GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at time 0 and 10 min following glucose gavage were not different between GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ and control mice (Supplementary Fig. 1*F*). Consistent with the lack of hypothalamic genetic deletion in this model as shown here and previously by others (7,11), centrally regulated metabolic parameters, such as body weight and food intake, were not significantly different between genotypes (Fig. 1*C* and *D*).

GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ Mice Demonstrate Reduced Serum Glucagon Levels Without Significant Changes in Hepatic Gene Expression Profile of Glucose Metabolism Under Basal Conditions

PTEN deletion in α-cells did not affect blood glucose levels under either fed or fasting conditions (Fig. 2*A* and *B*). Of note, circulating glucagon levels were reduced in chow-fed GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice after fasting (Fig. 2*C*). Fasting insulin levels, on the other hand, remained unchanged in these mice (Fig. 2*D*), and no difference was found in other counterregulatory hormones, such as corticosterone or epinephrine (Supplementary Fig. 2). Given that the liver is the main target for glucagon action, we measured transcript levels of G6Pase and PEPCK, which are involved in glycogenolysis and gluconeogenesis, respectively, as well as glucokinase and fatty acid synthase, which are involved in glucose storage. Among these, only PEPCK was reduced in the liver of GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ after fasting (Fig. 2*E–H*). We next measured proteins involved in insulin signaling and metabolism in the liver, including p-Akt, p-FoxO-1, and p-AMPK, which were similar between genotypes under fasting conditions (Fig. 2*I*). Together, these data show that PTEN deletion in α-cells leads to fasting hypoglucagonemia without significant changes in glycemia, genes involved in hepatic glucose metabolism, or proteins involved in hepatic insulin signaling under basal conditions.

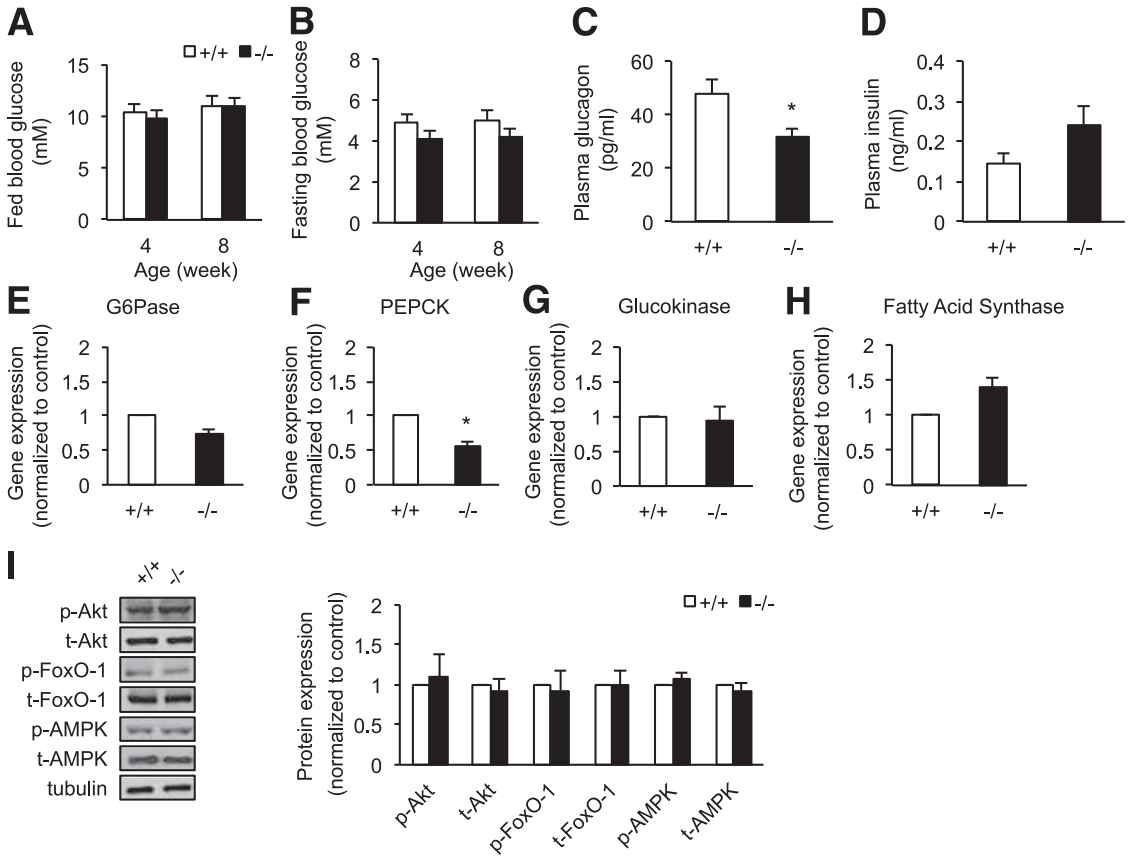

Figure 2—Metabolic parameters and hepatic gene and protein expression of GLU*cre*$^{+}$ *Pten*$^{+/+}$ ($^{+/+}$) and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ ($^{-/-}$) mice. *A* and *B*: Fed blood glucose (*A*) and fasting blood glucose (*B*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at 4 and 8 weeks of age (n = 15). *C* and *D*: Plasma glucagon (*C*) and plasma insulin (*D*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at 8 weeks of age (n = 8). *E–H*: Hepatic gene expression of G6Pase (*E*), PEPCK (*F*), glucokinase (*G*), and fatty acid synthase (*H*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at 8 weeks of age (n = 8). *I*: Representative Western blot (left panel) and quantification (right panel) of p-Akt, total Akt (t-Akt), p-FoxO-1, total FoxO-1 (t-FoxO-1), p-AMPK, and total AMPK (t-AMPK) of liver lysates from GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at 8 weeks of age (n = 6). *$P < 0.05$.

GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ Mice Do Not Show Any Changes in α- or β-Cell Mass or Disruption in Islet Architecture

PI3K signaling is a well-known regulator of cell survival and proliferation (14). Surprisingly, α-cell area was not changed in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice compared with control littermates (Fig. 3*A* and *C*). Furthermore, β-cell area, islet architecture, and overall pancreas weight were not affected by PTEN deletion in α-cells (Fig. 3*A*–*E*).

GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ Mice Demonstrate Reduced L-Arginine–Stimulated Glucagon Secretion

We next challenged these mice with glucose and insulin using GTT and ITT, respectively. GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice showed similar glucose excursion during GTT (Fig. 4*A*) and ITT (Fig. 4*B*) compared with control littermates. Furthermore, in vivo GSIS also showed similar responses between genotypes (Fig. 4*C*). Of note, L-arginine injection, which bypasses the glucose-sensing machinery within islets, led to significantly lower glucose excursion in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (Fig. 4*D*). This was associated with heightened insulin secretion but reduced glucagon secretion in response to L-arginine (Fig. 4*E* and *F*). Together, these results suggest direct effects of PTEN deletion in α-cells, leading to reduced glucagon levels resulting in improved β-cell function.

PTEN Deletion Suppresses L-Arginine–Stimulated Glucagon Secretion and Increases α-Cell Number In Vitro

To assess direct effects of PTEN deletion on α-cells independently of other hormonal and neuronal influences, we performed in vitro siRNA knockdown experiments on

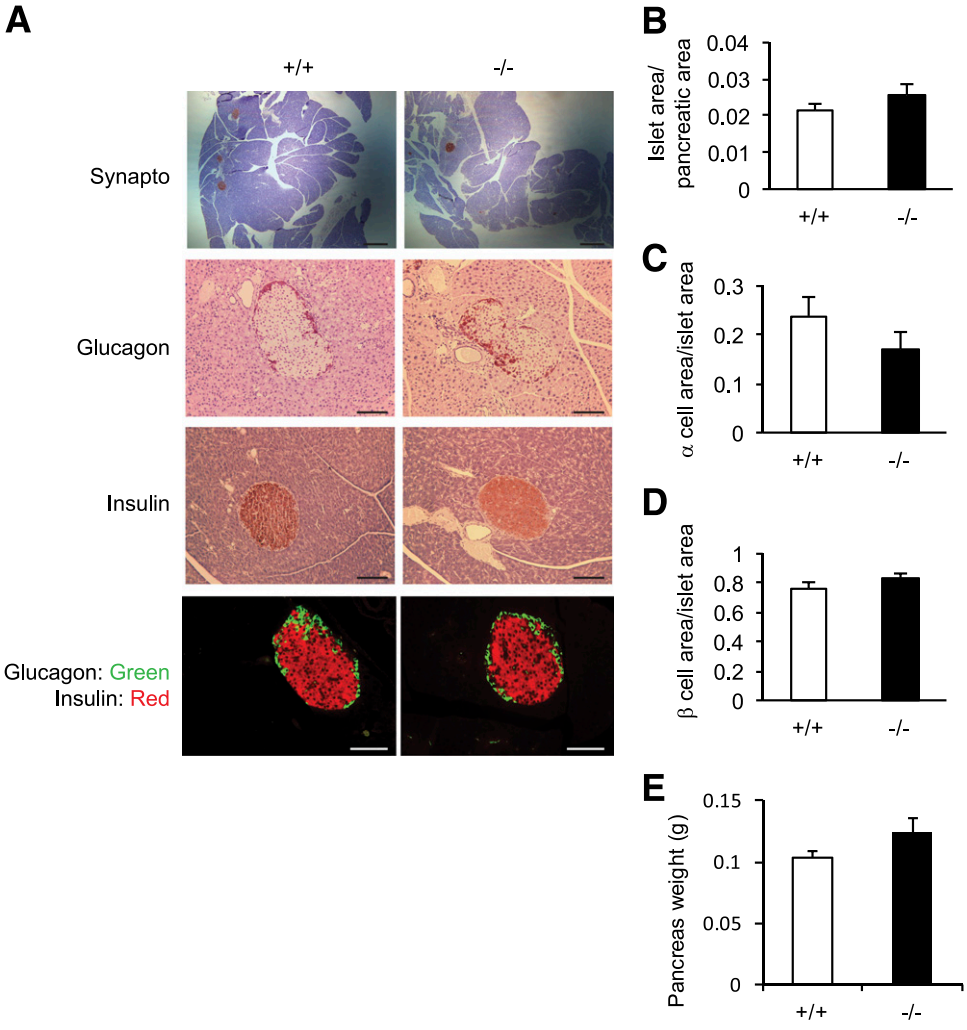

Figure 3—Islet morphology and architecture. *A*: Representative pancreatic sections with immunohistochemical staining of synaptophysin (Synapto) (magnification ×4, scale bars = 400 μm), glucagon (magnification ×20, scale bars = 100 μm), insulin (magnification ×20, scale bars = 100 μm), and immunofluorescent costaining of glucagon and insulin (magnification ×20, scale bars = 80 μm) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ ($^{+/+}$) and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ ($^{-/-}$) mice. *B*: Quantification of islet area (synaptophysin-positive) over total pancreatic area of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (n = 6). *C*: Quantification of α-cell area (glucagon-positive) over islet area of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (n = 6). *D*: Quantification of β-cell area (insulin-positive) over islet area of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (n = 6). *E*: Whole-pancreas weight of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (n = 4–5).

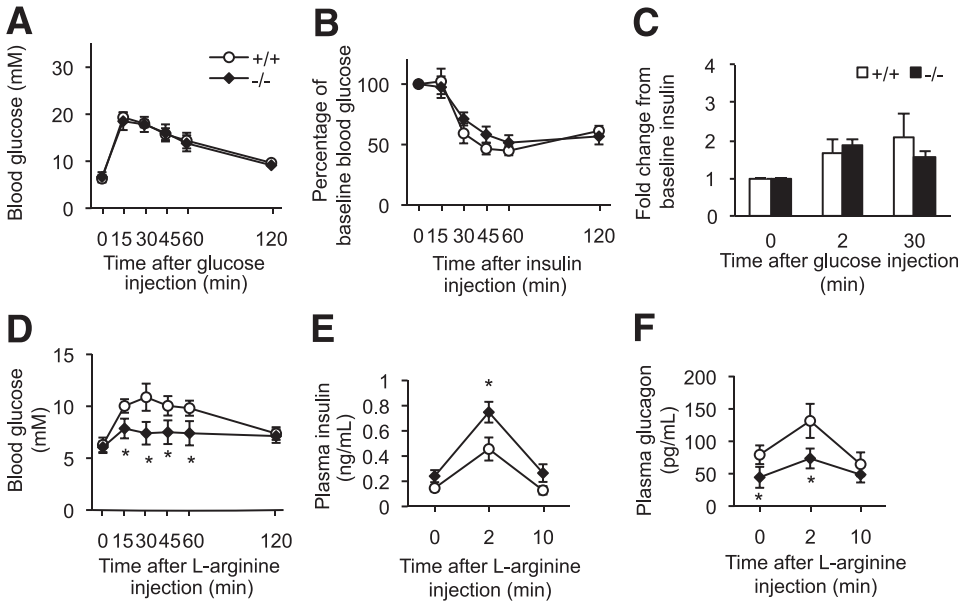

Figure 4—Glucose homeostasis and islet functional parameters in vivo. *A* and *B*: GTT (*A*) and ITT (*B*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ ($^{+/+}$) and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ ($^{-/-}$) mice at 8 weeks of age (n = 15). *C*: GSIS of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice at 8 weeks of age (n = 6). *D–F*: Blood glucose (*D*), plasma insulin (*E*), and plasma glucagon (*F*) concentrations of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice after intraperitoneal L-arginine injection (n = 8). *$P < 0.05$.

an α-cell line, In-R1-G9. Endogenous PTEN was detected in two α-cell lines (In-R1-G9 and αTC) (Supplementary Fig. 3), and siRNA PTEN knockdown in In-R1-G9 cells led to efficient reduction in PTEN protein expression with increased p-Akt and p-FoxO-1 (Fig. 5*A*). Glucagon suppression in response to high-glucose concentration was similar after PTEN knockdown compared with scramble siRNA–treated In-R1-G9 cells (Fig. 5*B*), with similar results seen in isolated islets (Supplementary Fig. 4*A*). Furthermore, the attenuation of glucagon secretion in response to L-arginine that we observed in vivo in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice was also observed after PTEN knockdown in vitro as well as in isolated islets, evident when examined in high-glucose conditions (Fig. 5*B*, Supplementary Fig. 4*B*). On the other hand, glucagon secretion was increased in response to hypoglycemia during ITT, likely in response to lower glucose levels achieved in response to insulin in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice (Supplementary Fig. 5). These results show that PTEN deficiency in α-cells leads to attenuation in glucagon secretion in response to glucose or arginine, which is associated with overall lower glucagon levels, whereas physiological response of glucagon with hypoglycemia remains intact.

GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ Mice Are Protected From Hyperglycemia and Insulin Resistance With Reduced Islet Expansion After High-Fat Feeding

Given the emerging role of α-cell dysregulation in the pathogenesis of type 2 diabetes (9), we next challenged GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice with prolonged HFD starting at 2 months of age for up to 7 months (9 months of age). GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice demonstrated similar weight gain as controls on HFD (Fig. 6*A*). However, these mice were protected against both hyperglycemia (Fig. 6*B*) and glucose intolerance (Fig. 6*C*) after prolonged HFD. They also remained more insulin sensitive compared with HFD-fed controls as shown by improved glucose-lowering response to insulin during ITT (Fig. 6*D*). In agreement with their insulin-sensitive state, β-cell expansion was attenuated in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice as shown by decreased islet-to-whole pancreas area (Fig. 6*E* and *F*). However, the proportion of α- and β-cells within islets remained similar between genotypes on an HFD (Fig. 6*E*, *G*, and *H*).

HFD-Induced Hyperglucagonemia Is Attenuated in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ Mice

Circulating glucagon in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice remained lower than that in control littermates after prolonged feeding with an HFD (Fig. 7*A*). Furthermore, in line with their increased insulin sensitivity, fasting insulin levels remained lower in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice than in control littermates after prolonged HFD (Fig. 7*B*). Furthermore, β-cell function was preserved in these GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice, as demonstrated by high insulin secretion during GSIS, in contrast to attenuation in insulin response to glucose observed in control mice after prolonged HFD (Fig. 7*C*). In line with reduced serum glucagon levels and preserved insulin sensitivity, activation of hepatic glucagon signaling was decreased (Supplementary Fig. 6), and genes involved in hepatic glucose production, such as G6Pase and PEPCK, were reduced in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ liver (Fig. 7*D* and *E*), whereas genes involved in glycogen synthesis and glucose storage, including glucokinase and fatty acid synthase, were increased

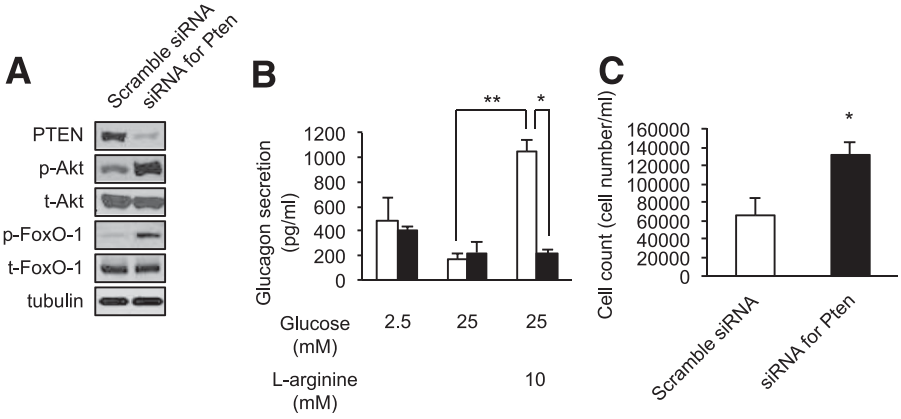

Figure 5—In vitro effect of PTEN siRNA knockdown in α-cells on glucagon secretion and proliferation. *A*: Western blotting of PTEN, p-Akt, total Akt (t-Akt), p-FoxO-1, and total FoxO-1 (t-FoxO-1) in In-R1-G9 cells treated with scramble siRNA and siRNA for PTEN (n = 3). *B*: Glucagon secretion of In-R1-G9 cells treated with scramble siRNA and siRNA for PTEN after incubation with glucose and L-arginine (n = 3). White bars, scramble siRNA; black bars, siRNA for PTEN. *C*: Total cell count of In-R1-G9 cells treated with scramble siRNA and siRNA for PTEN (n = 3). $^*P < 0.05$, $^{**}P < 0.01$.

compared with control littermates (Fig. 7*F* and *G*). Furthermore, GLU*cre*$^+$ *Pten*$^{fl/fl}$ mice were protected from HFD-induced hepatosteatosis, which was present in HFD-fed control mice (Fig. 7*H*). Associated with protection against fatty liver and hepatic insulin resistance, GLU*cre*$^+$ *Pten*$^{fl/fl}$ mice showed preserved hepatic insulin signaling as evidenced by increased p-Akt, p-FoxO-1, and p-AMPK compared with controls even after 7 months of an HFD (Fig. 7*I*). Collectively, these data suggest that PTEN deletion in α-cells leads to reduction in glucagon secretion, which is associated with protection against HFD-induced insulin resistance and hyperglycemia.

DISCUSSION

In this study, we investigated the direct role of PI3K signaling in α-cell function and survival by genetic deletion of PTEN, a potent negative regulator of this signaling pathway. Mice with PTEN-deficient α-cells showed reduced circulating glucagon levels and attenuated glucagon secretion after L-arginine stimulation. Similarly, knockdown of PTEN in the In-R1-G9 α-cell line decreased L-arginine–stimulated glucagon secretion. Moreover, deletion of PTEN in α-cells protected mice from HFD-induced hyperglycemia, glucose intolerance, and insulin resistance. Overall, these data support the importance of PI3K signaling in α-cells and demonstrate that activation of this pathway can attenuate hyperglucagonemia to improve glucose metabolism.

Glucagon and its glucose-raising properties were described by Kimball and Murlin in the 1920s. However, historically, its main counterregulatory hormone insulin has been a greater focus of diabetes research (19,20). The discovery that hyperglycemia could result from increased glucagon production in pancreatic α-cells and other tissues renewed interest in this hormone (21–23), particularly when glucagon suppression restored normoglycemia (24). Accordingly, mice with genetic ablation of glucagon signaling have improved peripheral insulin sensitivity (25,26) and are protected against both type 1 and type 2 models of diabetes (27,28). In both settings, hyperglycemia is prevented primarily by reduced glucagon-mediated hepatic glucose production. Therefore, glucagon is critical for modulating glucose metabolism in both physiological and pathological settings. Notably, human islets have significantly more α-cells than mouse islets (4); thus, the clinical significance of α-cells in glucose homeostasis may be underestimated. As such, blocking glucagon secretion or action could potentially provide effective novel therapeutic tools in treating diabetes.

Glucagon secretion by α-cells is under intense hormonal and neuronal control. Endocrine secretions from neighboring islet cells, including insulin from β-cells and somatostatin from δ-cells, directly inhibit glucagon secretion (8). Of note, the common intracellular signaling pathway activated by both these hormones is the PI3K signaling pathway, suggesting PI3K activation in α-cells as a potential therapy for treating hyperglucagonemia and diabetes.

We previously studied the role of the potent negative regulator of insulin signaling PTEN in pancreatic β-cells and RIPcre-expressing neurons (14,29) and showed that PTEN deletion in β-cells improved glucose intolerance and β-cell function in HFD-fed or *db/db* mice compared with controls. In the present work, we further elucidate the role of PI3K signaling in α-cells, demonstrating an important parallel and complementary role for this pathway in glucagon secretion and mediation of insulin sensitivity.

Accordingly, we found that GLU*cre*-mediated PTEN deletion in α-cells reduces serum glucagon levels and glucagon response to L-arginine stimulation, importantly, without disrupting response to hypoglycemia. This reduction in glucagon secretion may result from distinct mechanisms. First, activation of the PI3K/Akt pathway opens

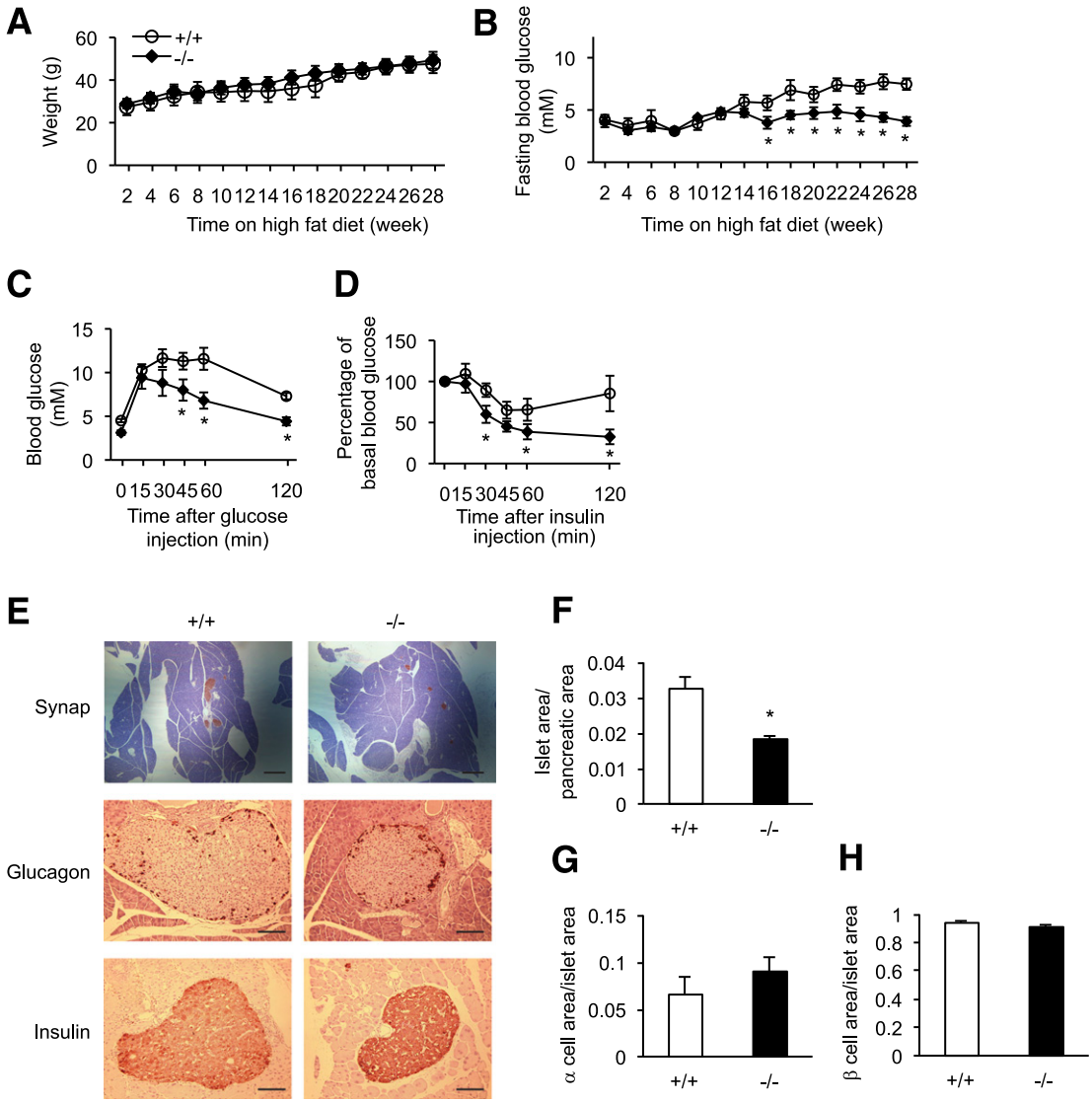

Figure 6—Glucose homeostasis and islet morphology of GLU*cre*$^{+}$ *Pten*$^{+/+}$ ($^{+/+}$) and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ ($^{-/-}$) mice after HFD. *A–D*: Weight (*A*), fasting blood glucose (*B*), GTT (*C*), and ITT (*D*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice on HFD ($n > 15$). *E*: Representative pancreatic sections of immunohistochemical staining of synaptophysin (Synap) (magnification ×4, scale bars = 400 μm), glucagon (magnification ×20, scale bars = 100 μm), and insulin (magnification ×20, scale bars = 100 μm) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice. *F*: Quantification of islet area (synaptophysin-positive) over total pancreatic area ($n = 6$). *G* and *H*: Quantification of α-cell area (glucagon-positive) over islet area (*G*) and quantification of β-cell area (insulin-positive) over islet area (*H*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice ($n = 6$). *$P < 0.05$.

ATP-sensitive K^{+} channels, culminating in hyperpolarization and inhibition of glucagon secretion (30). Second, autocrine glucagon action on α-cells has been shown to have a positive effect on glucagon secretion (31); thus, reduced circulating glucagon may further augment the inhibitory effect of PI3K activation on glucagon release. Although GLU*cre* may theoretically have some activity in a small number of L cells in the intestine, which secrete GLP-1 (32), we did not detect any elevation in GLP-1 and have previously shown minimal GLU*cre* activity in intestinal cells (11), suggesting that alterations in GLP-1 are a less likely mechanism for the improvements in glucose homeostasis seen in the GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice.

Similar to β-cell dysfunction, dysregulation of glucagon secretion by α-cells is increasingly recognized as one of the pathologies associated with diabetes. In human subjects with type 2 diabetes, impaired glucagon response has been reported such that hyperglycemia and hyperinsulinemia failed to suppress hepatic glucose production, leading to further elevation in blood glucose (9). This increase in circulating glucagon associated with insulin resistance is attenuated in GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice fed a prolonged HFD. With diminished glucagon action in the liver, hepatic gene expression shifted from those involved in glucose production to those of glucose storage. This was associated with better glucose tolerance in these mice

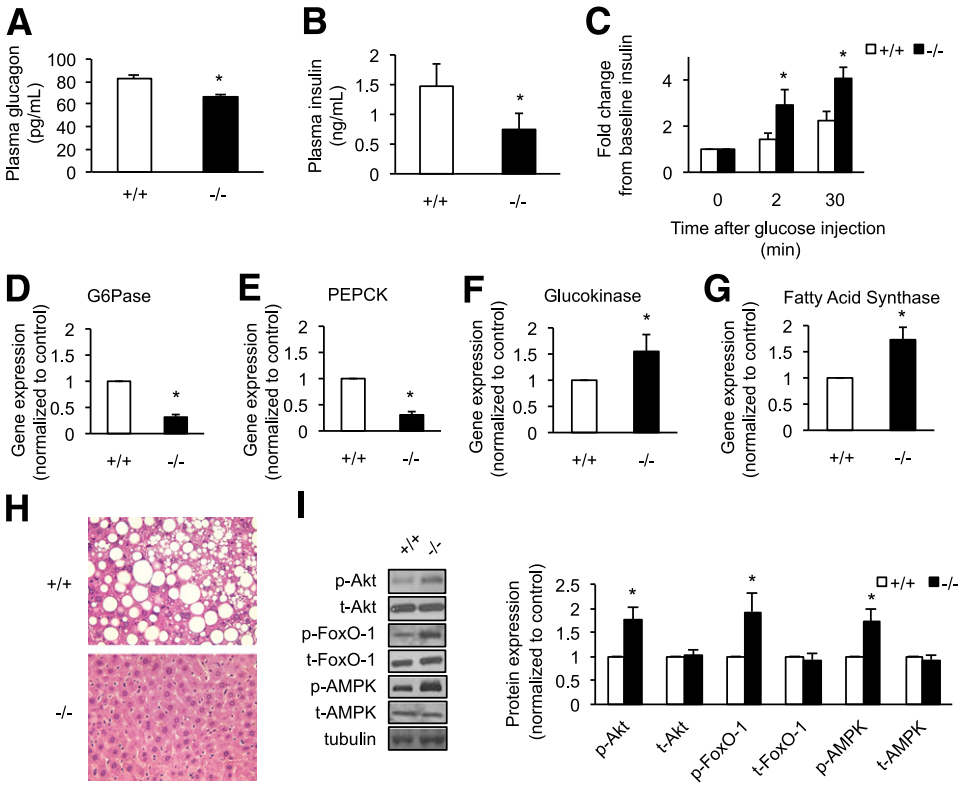

Figure 7—Serum hormone levels and hepatic gene and protein expression of GLU*cre*$^{+}$ *Pten*$^{+/+}$ ($^{+/+}$) and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ ($^{-/-}$) mice after HFD. *A–C*: Plasma glucagon (*A*), plasma insulin (*B*), and GSIS (*C*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice after HFD (n = 8). *D–G*: Hepatic gene expression of G6Pase (*D*), PEPCK (*E*), glucokinase (*F*), and fatty acid synthase (*G*) of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice after HFD (n = 6). *H*: Representative liver hematoxylin-eosin–stained sections of GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice after HFD. *I*: Representative Western blot (left panel) and quantification (right panel) of p-Akt, total Akt (t-Akt), p-FoxO-1, total FoxO-1 (t-FoxO-1), p-AMPK, and total AMPK (t-AMPK) of liver from GLU*cre*$^{+}$ *Pten*$^{+/+}$ and GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice on HFD (n = 6). *$P < 0.05$.

compared with controls. Furthermore, the low level of glucagon can potentially require less insulin to counterregulate the actions of this hormone, which could at least partly account for lower serum insulin levels and improved insulin sensitivity in HFD-fed GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice. In contrast to phenotypes resulting from direct liver-specific manipulation of PTEN or PI3K signaling (33–35), GLU*cre*$^{+}$ *Pten*$^{fl/fl}$ mice have decreased fatty liver and improved hepatic insulin sensitivity consistent with improved whole-body insulin sensitivity (29,36,37). Thus, α-cell–specific deletion of PTEN results in minimal perturbations in hormone levels under basal conditions and protection against insulin resistance when challenged with metabolic stress through prolonged HFD.

PI3K signaling has been implicated in cell function and proliferation (38). Here, we demonstrate that increased PI3K activation in PTEN-deficient α-cells leads to decreased glucagon secretion in vivo and in vitro. Of note, this signaling pathway did not appear to have a major impact on estimated α-cell mass in animals on both chow and HFD, although an increase in cell number was observed in the PTEN-deficient α-cell line (Fig. 5*C*). In our mice, no evidence of malignant lesions on gross pathology or pancreatic histology were seen up to 9 months of age, which is in keeping with our previous findings that RIPcre-mediated PTEN deletion in β-cells also did not result in tumor formation (14,39). Although we did not specifically study proliferation, these findings might be consistent with α-cells representing a small percentage of the total islet population such that increased proliferation may be difficult to detect in vivo. Of note, previous studies suggested a direct negative effect of glucagon signaling on α-cell mass regulation such that α-cell hyperplasia was observed in glucagon receptor null mice but not in mice with liver-specific glucagon receptor knockdown (25,40). These two observations suggest the importance of the autocrine effects of glucagon on α-cells rather than effects of other glucagon-responsive tissues responsible for inhibiting α-cell proliferation. α-Cell mass remained unchanged despite hypoglucagonemia, which further emphasizes the primary function of PTEN in regulating glucagon secretion or α-cell function rather than α-cell mass.

The present data complement and build on the study by Kawamori et al. (7), which showed that knockout of the insulin receptor in α-cells results in mild glucose intolerance, hyperglycemia, and hyperglucagonemia. Conversely,

we activated downstream insulin signaling specifically through the PI3K pathway by deleting PTEN, a negative regulator of this pathway, and showed reduced serum glucagon levels and protection from HFD-induced glucose intolerance and hyperglycemia.

In summary, we provide genetic evidence supporting the critical role of PI3K signaling in modulating α-cell function. We show that PTEN deletion in pancreatic α-cells inhibits glucagon secretion and provides protection against HFD-induced hyperglucagonemia, insulin resistance, and hyperglycemia. The data therefore support the essential role of α-cell dysregulation in the pathogenesis of type 2 diabetes, and as such, suppression of glucagon secretion through PTEN deletion may represent a viable therapeutic strategy for this chronic disease.

Acknowledgments. The authors thank Tianru Jin (Department of Physiology, University of Toronto) for providing the In-R1-G9 cells, as well as Manuel Gil-Lozano and Patricia L. Brubaker (Department of Physiology, University of Toronto) for advice and technical assistance with the Meso Scale Discovery Active GLP-1 Assay Kit and MESO SECTOR.

Funding. L.W. was supported by the Canadian Institutes of Health Research (CIHR) Frederick Banting and Charles Best Canada Graduate Scholarship and the Comprehensive Research Experience for Medical Students Scholarship from the Faculty of Medicine, University of Toronto. C.T.L. was supported by the Eliot Phillipson Clinician Scientist Training Program and postdoctoral fellowships from the Banting & Best Diabetes Centre and the Canadian Diabetes Association (CDA). E.P.C. was supported by the CDA Doctoral Student Research Award. S.Y.S. was supported by the Canadian Liver Foundation Graduate Studentship, CDA Doctoral Student Research Award, and CIHR Frederick Banting and Charles Best Canada Graduate Scholarship. This work was supported by grants to M.W. from the CIHR (MOP-81148) and CDA. M.W. was supported by the Canada Research Chair in Signal Transduction in Diabetes Pathogenesis.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. L.W. contributed to the generation and analyses of research data and preparation of the manuscript. C.T.L. and E.P.C. contributed to the generation of research data and the manuscript. S.A.S., E.M.A., and S.Y.S. contributed to the generation of research data. M.B.W. designed experiments and supervised students. H.Y.G. provided the GLU*cre* mice and contributed to the study concept. M.W. designed the experiments, supervised students, contributed to the study concept, interpreted data, and reviewed and edited the manuscript. M.W. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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