Normal Glucagon Signaling and β-Cell Function After Near-Total α-Cell Ablation in Adult Mice

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OBJECTIVE—Evaluate whether healthy or diabetic adult mice can tolerate an extreme loss of pancreatic α-cells and how this sudden massive depletion affects β-cell function and blood glucose homeostasis.

RESEARCH DESIGN AND METHODS—We generated a new transgenic model allowing near-total α-cell removal specifically in adult mice. Massive α-cell ablation was triggered in normally grown and healthy adult animals upon diphtheria toxin (DT) administration. The metabolic status of these mice was assessed in 1) physiologic conditions, 2) a situation requiring glucagon action, and 3) after β-cell loss.

RESULTS—Adult transgenic mice enduring extreme (98%) α-cell removal remained healthy and did not display major defects in insulin counter-regulatory response. We observed that 2% of the normal α-cell mass produced enough glucagon to ensure near-normal glucagonemia. β-Cell function and blood glucose homeostasis remained unaltered after α-cell loss, indicating that direct local intraislet signaling between α- and β-cells is dispensable. Escaping α-cells increased their glucagon content during subsequent months, but there was no significant α-cell regeneration. Near-total α-cell ablation did not prevent hyperglycemia in mice having also undergone massive β-cell loss, indicating that a minimal amount of α-cells can still guarantee normal glucagon signaling in diabetic conditions.

CONCLUSIONS—An extremely low amount of α-cells is sufficient to prevent a major counter-regulatory deregulation, both under physiologic and diabetic conditions. We previously reported that α-cells reprogram to insulin production after extreme β-cell loss and now conjecture that the low α-cell requirement could be exploited in future diabetic therapies aimed at regenerating β-cells by reprogramming adult α-cells.

In rodents, glucagon-producing α-cells are the second most abundant endocrine cell type in pancreatic islets of Langerhans, after the insulin-producing β-cells. In human islets, α-cells are nearly as abundant as β-cells (1,2). They secrete glucagon in response to reduced blood glucose to promote gluconeogenesis and glycogenolysis in the liver (3). Proper control of blood glucose level thus relies on insulin action and the counter-regulation mediated by glucagon signaling. Besides this, insulin and glucagon reciprocally regulate α- and β-cell function through local, intraislet paracrine signaling (4).

Excess of plasma glucagon (hyperglucagonemia) is frequently reported in diabetic patients, a deregulation that exacerbates hyperglycemia and triggers ketoacidosis, a major complication of diabetes (5). A recent study showed that streptozotocin (STZ)-induced diabetes is prevented in glucagon receptor–knockout (GcgR−/−) mice, suggesting that in diabetes, hyperglycemia is largely due to glucagon action (6). Therefore, reducing the α-cell mass to limit glucagon production may represent an interesting approach to prevent glucagon excess in diabetes. Nevertheless, it is unknown how a severe decrease in pancreatic α-cell mass would be tolerated in physiologic or in diabetic conditions. Indeed, whereas a strong deficit in β-cell mass triggers diabetes, less is known regarding the requirement of maintaining intact the adult α-cell population.

Complete disruption of glucagon signaling in GcgR−/− mice is associated with defects in endocrine cell differentiation and increased embryonic lethality (7–9). In addition, mice that are unable to process proglucagon in its mature and active form due to prohormone convertase 2 (PC2) inactivation also display altered islet cell differentiation (10,11). GcgR−/− and PC2−/− adult mutant mice both exhibit an expanded α-cell mass, which is associated with lower blood glucose levels.

Mice constitutively lacking aristaless-related homeobox (ARX) in the pancreas from early development display an α-cell deficit associated with abnormally increased non–α-cell numbers and hypoglycemia (12). In this situation, the altered metabolic status of adult individuals may reflect adaptation phenomena subsequent to developmental defects. Collectively, these studies that focused on the effect of glucagon deficiency did not directly address whether functional α-cells are essential per se for proper β-cell function and blood glucose homeostasis in physiologic conditions in adulthood.

In the current study, we have analyzed a model of inducible, selective α-cell loss that we previously used in studies involving β-cell regeneration (13). Here, we examine the short- and long-term influence on blood glucose homeostasis of an acute, rapid, near-total α-cell ablation induced in healthy normal adult mice. We show that a very limited number of adult α-cells, some 2% of the normal α-cell mass, is sufficient to preserve a normal counter-response to insulin in basal conditions, without affecting β-cell function or longevity. Furthermore, we report that extreme α-cell removal, contrary to what happens in GcgR−/− mice (6), does not prevent hyperglycemia after β-cell loss, indicating that the few remaining α-cells still mediate normal glucagon signaling in diabetes.

RESULTS Ablation of α-cells. We previously generated the Glucagon-DTR mouse line in which the administration of diphtheria toxin (DT) induces selective and massive ablation of adult α-cells (10). This is achieved through the transgenic
expression of human DT receptor (DTR) on the α-cell surface, driven by a 1.6-kb proximal fragment of the rat glucagon promoter (Fig. 1A).

DT-untreated adult Glucagon-DTR mice were healthy and showed normal blood glucose homeostasis, like nontransgenic controls. For this reason, they were used as DT, indicating that the effects of α-cell ablation had no effect on global insulin production (Fig. 3A). Glucagon-DTR mice were also able to recover normoglycemia after glucose challenge (glucose tolerance test), either 1 week or 6 months after DT (Fig. 3B and Supplementary Fig. 4) and did not exhibit any defects in basal or glucose-stimulated insulin secretion, as shown by pancreas perfusion experiments (Fig. 3C). These results, together with the long-term follow-up of glucagon in α-cell–depleted Glucagon-DTR mice (Supplementary Table 2), suggested that the few newly formed α-cells do not affect blood glucose homeostasis or β-cell function.

**Limited α-cell regeneration after extreme α-cell ablation.** One week after DT administration, the total pancreatic glucagon content had dropped to 0.86% of control level, but 1 and 6 months later, it was increased by a factor of 6.7- and 14-fold, respectively (Fig. 4A and Table 1). Basal glucagonemia was normal in Glucagon-DTR mice (n = 4) 6 months after α-cell destruction (63 ± 0.9 vs. 64.2 ± 0.4 pmol/L in controls, n = 3; Fig. 2D). These observations are consistent with 1) increased glucagon production and secretion with time, by the few remaining α-cells, and 2) the regeneration of new α-cells, or both. To explore these possibilities, we assessed the number of α-cells present in the pancreas 1 week, 1 month, and 6 months after DT (Supplementary Table 1). The total number of α-cells, as calculated between 1 and 6 months after DT, was doubled at 6 months, from 0.17 at 1 month to 0.35 cells/mm² 6 months later (P = 0.0286; Fig. 4B and Table 1). The number of islets was similar between untreated and DT-treated animals at all intervals, suggesting that new α-cells were formed after α-cell ablation (Fig. 4C and Table 1). The number of islet sections containing at least 1 α-cell did not increase during the regeneration period under study; the percentage of sections containing α-cells dropped from 20% 1 week after DT, to 6% 6 months after DT, with few remaining α-cells, and did not exhibit any defects in basal or glucose-stimulated insulin secretion, as shown by pancreas perfusion experiments (Fig. 3C). These results, together with the long-term follow-up of glucagon in α-cell–depleted Glucagon-DTR mice (Supplementary Table 2), suggested that the few newly formed α-cells do not affect blood glucose homeostasis or β-cell function.

**β-Cell function is unaltered after extreme α-cell ablation.** α-Cells interact with β-cells, most likely by local intraislet interactions, and α- and β-cells express insulin and GcgR, respectively (3,4,16–18). We thus assessed whether α-cells are important for adequate β-cell function. Pancreatic insulin expression was unchanged during the entire period of analysis (Fig. 3A), suggesting that the few newly formed α-cells do not affect blood glucose homeostasis or β-cell function.

**Altogether, these results suggest that the few newly formed α-cells do not increase during the regeneration period under study; the percentage of sections containing α-cells dropped from 20% 1 week after DT, to 6% 6 months after DT, with few remaining α-cells, and did not exhibit any defects in basal or glucose-stimulated insulin secretion, as shown by pancreas perfusion experiments (Fig. 3C). These results, together with the long-term follow-up of glucagon in α-cell–depleted Glucagon-DTR mice (Supplementary Table 2), suggested that the few newly formed α-cells do not affect blood glucose homeostasis or β-cell function.**
islets, and 3) the β-cell lineage-tracing revealed that β-cells do not trans-differentiate spontaneously into α-cells.

Near-total α-cell loss does not prevent diabetes. Inhibition of glucagon signaling (GcgR−/− mice) was recently shown to prevent STZ-induced diabetes (6). We thus assessed whether near-total α-cell ablation in Glucagon-DTR mice affects or prevents hyperglycemia. For this purpose, Glucagon-DTR mice were treated 1 week after DT with a high dose of STZ (200 μg/g) to induce ~90% β-cell removal (Fig. 6A–G). Interestingly, mice that had only residual α-cells (arrowheads in Fig. 6D and G) became overtly hyperglycemic and lost weight after β-cell destruction, like diabetic mice with normal α-cell mass (Fig. 6H and I). Similarly, the simultaneous coablation of β- and α-cells (in Glucagon-DTR, RIP-DTR double transgenic mice) (13) also induced

FIG. 1. α-Cell ablation in Glucagon-DTR mice. A: DT injection triggers α-cell ablation in Glucagon-DTR transgenic mice but not in wild-type mice. Two days after DT injection, dying cells are detected by TUNEL, mainly at islet periphery in Glucagon-DTR animals (middle). At 1 week after DT injection, islets in transgenic mice are devoid of glucagon-expressing cells (right). The dashed lines delineate islets. Scale bars = 20 μm. B: At 1 week after DT treatment, 97.9% of glucagon-expressing cells were destroyed (*P = 0.05, one-tailed Mann-Whitney test; values in Table 1). C: At 1 week after DT, pancreatic glucagon content was reduced to 0.86% of control (***P = 0.0001, one-tailed Mann-Whitney test; values in Table 1). (A high-quality digital representation of this figure is available in the online issue.)
Extrainsular a-cells/mm² pancreas

Table 1

<table>
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<tr>
<th>Variable</th>
<th>Control mice</th>
<th>DT-treated mice</th>
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<td>Time post-DT</td>
<td>Mean ± SEM</td>
<td>n</td>
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<td>Pancreatic glucagon content</td>
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<td>(pg/mg pancreas)</td>
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<td>a-Cell number/mm² pancreas</td>
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aP ≤ 0.005. †P ≤ 0.01. ‡P ≤ 0.05.

Hyperglycemia and cachexia (Supplementary Fig. 7). These results show that near-total a-cell ablation does not prevent diabetes, contrary to what happens in GcgR−/− mice (6). This was consistent with the unaltered glucagon secretion and counter-regulatory response after removal of 98% of a-cells under physiologic conditions.

Discussion

We have shown that near-total a-cell loss in adult mice has little effect on glucagonemia and no apparent effect on b-cell function or glucose homeostasis. This reveals that 2% of the normal a-cell mass is astonishingly sufficient to produce enough glucagon to ensure glycemic control under basal conditions. Massive a-cell loss is associated with a rapid enhancement of glucagon sensitivity, which allows increased glucose mobilization, even if the decrease in glucagonemia is very mild.

A corollary of this observation is that the normal amount of pancreatic a-cells apparently exceeds the physiologic requirement for proper blood glucose homeostasis. Indeed, the total glucagon found in the pancreas when only 2% of the a-cells remain was sufficient to maintain normogluconagemia. A simple calculation reveals that if all pancreatic glucagon found 1 week after a-cell loss (~7,400 pg; Supplementary Table 2) were entirely released at once into the circulation, glucagonemia would be more than 120-fold higher than the normal value of about 60 pg (Supplementary Table 2). The amount of glucagon measured in plasma after 8% a-cell ablation (~40 pg; Supplementary Table 2) only represents 0.5% of the residual pancreatic glucagon content produced by the remaining 2% of a-cells (7,400 pg). This suggests that the intracellular glucagon store in the remaining a-cells is, in principle, sufficient to maintain basal glucagonemia. However, because plasma glucagon has a half-life of only 2 min (20–22), sustained glucagon biosynthesis after a-cell ablation is mandatory to maintain glucagonemia and pancreatic content over time. Indeed, in absence of glucagon biosynthesis, all pancreatic glucagon stocks found after DT should theoretically be finished within ~6 h vs. ~17 days in control mice with normal a-cell mass (estimated considering the observed pancreatic glucagon contents and glucagon half-life). In agreement with this, we found that although the number of a-cells had not increased 1 month after the ablation, the glucagon content per cell had doubled. In this situation, the lack of response of the a-cell–ablated pancreata to arginine suggests that the few remaining a-cells have reached a maximum secretion capacity or that glucagon biosynthesis is rate limiting, or both. The inability of Glucagon-DTR mice to ensure normogluconagemia 1 week after DT, despite having sufficient pancreatic glucagon, indicates that glucagon secretion is limited; however, because two-thirds of normal plasma glucagon level is ensured by just 2% of normal a-cell mass, this indicates that a-cells display a huge secretory capacity. This property may contribute to the excessive glucagon secretion observed in diabetic patients. Alternatively, our observations also suggest that in normal conditions, with a normal a-cell mass, glucagon secretion (relative to a-cell number) is very low.

It is therefore intriguing to observe that a-cells are so numerous. They may somewhat be required for proper endocrine pancreas development. Alternatively, or in addition, their numbers may reflect the effect of selection pressure during evolution: most species must often thrive through long periods of sustained starvation, and glucagon facilitates glucose mobilization during food deprivation. In the latter perspective, the massive loss of a-cells, which we report here, was associated with a rapid enhancement of glucagon sensitivity, which allows an increased glucose mobilization likely through glycogenolysis, rather than gluconeogenesis, as revealed with glucose tolerance tests and a tendency to express higher levels of liver GcgR and GP.

Interestingly, we have seen that extreme a-cell removal in stressful situations, such as prolonged starvation and insulin-induced hypoglycemia, has no obvious consequences...
FIG. 2. Extreme α-cell loss moderately decreases circulating glucagon and has no effect on the counter-regulatory response. Evolution of body weight (A) and glycemia (B) in fasted controls (n = 3; DT-untreated, black ◆) and DT-treated (n = 3; red ▲) Glucagon-DTR mice. C: Glycemia after insulin-induced hypoglycemia 1 week after α-cell ablation. Blood glucose increased in both DT-treated mice (red ▲) and controls (black ◆) after an insulin challenge (n = 3/group). D: Plasma glucagon 1 week and 6 months after DT (red □) injections and in control (black □) mice. Glucagonemia was significantly reduced by 35% 1 week after DT (***P = 0.001) but returned to normal levels 6 months later (values in Supplementary Table 2). Confocal images of pancreatic sections stained for insulin (green) and glucagon (red) in controls (E) and Glucagon-DTR mice (F). Very few pancreatic α-cells can be observed 1 week after DT. E’ and F’: higher magnification of the dotted areas depicted in E and F, respectively. Scale bars = 100 µm in E and F and 20 µm in E’ and F’. G: Arginine-induced glucagon secretion from perfused pancreas 1 week after DT or in controls. H: Quantification of arginine-induced glucagon secretion upon arginine stimulation (1,290.2 ± 281.9 for controls, and 423.3 ± 85.3 pg/mL for DT-treated Glucagon-DTR mice; *P = 0.014). (A high-quality digital representation of this figure is available in the online issue.)
on fasting blood glucose and normoglycemia recovery. Furthermore, Unger and colleagues (6) reported that inhibition of glucagon signaling prevented STZ-induced diabetes in mice. By contrast, we show here that near-total α-cell ablation does not prevent hyperglycemia, indicating that a minimal fraction of the α-cell mass is sufficient to mediate normal glucagon signaling. Together, these combined observations strongly suggest that a total α-cell loss, or glucagon-signaling blockade, would be required to prevent hyperglycemia and diabetes after massive β-cell destruction.

Cell–cell signaling between α- and β-cells is thought to be essential for proper blood glucose homeostasis (4), but the direct, physical influence of α-cells on glucose-stimulated insulin release has never been studied in vivo. We know β-cell activity is independent of glucagon signaling because it remains unaltered upon treatment with GcgR antagonists or in GcgR−/− mice (8,23), that is, in situations where α-cells are abundant or expanded. In DT-treated Glucagon-DTR mice, however, almost all islets are totally devoid of α-cells, which prevents any paracrine intraislet direct interaction between these two cell types. Yet, glucose homeostasis in this situation is not affected under basal conditions, thus suggesting that local α-cell–β-cell interactions are dispensable for adequate β-cell function. This conclusion could not be reached in previous studies because the animal model reported here is the first in which adult mice have nearly normal glucagonemia, yet lack most α-cells.

α-Cell hyperplasia has been reported in patients bearing mutations in the GcgR gene (24,25) and in mice exhibiting glucagon deficiency, such as in glucagon−/−, GcgR−/−, and PC2−/− mice, as well as in wild-type mice treated with GcgR-neutralizing antibody (7,8,26–29). In all these situations, the formation of new α-cells likely represents a compensatory response to deficient glucagon signaling.

The origin of newly formed α-cells in glucagon-deficient conditions is not known. The continuous emergence of α-cells from pancreatic ductal Neurog-3-expressing cells was reported in mice expressing the β-cell–specific transcription factor paired box gene 4 (PAX4) in embryonic α-cells (30). In these animals, α-cells reprogram toward the β-cell phenotype upon PAX4 misexpression, leading to a substantial reduction in postnatal α-cell numbers. The authors proposed that glucagon deficiency was the driving force for the continuous appearance of α-cells, yet glucagonemia was not measured in their study, and the α-cell deficit was similar to the one observed here in DT-treated Glucagon-DTR mice. By contrast, there is no evidence for ductal α-cell neogenesis in Glucagon-DTR mice: the rare α-cells found after DT were never preferentially located within or near ducts. This difference may be attributed to the perturbed metabolic status of mice over-expressing PAX4, which exhibit strong β-cell hyperplasia and very high insulinemia, likely resulting in an abnormally elevated insulin-to-glucagon balance.

We recently reported that adult α-cells reprogram to insulin production after extreme β-cell ablation (13). Here, we explored whether β-cells can reprogram to produce glucagon after massive α-cell loss and failed to observe any such β- to α-cell conversion. However, the interconversion between α- and β-cells may occur in both directions when conditions are appropriate. Indeed, it has been shown that I) the ectopic expression of the α-cell–specific transcription factor ARX or 2) the conditional inactivation of the DNA methyltransferase gene Dnmt1 in embryonic β-cells converts them to the α-cell phenotype (31,32).

After β-cell destruction in RIP-DTR mice, ~5% of adult α-cells spontaneously undergo cell reprogramming; this limited number of cells probably determines the extent of β-cell recovery in RIP-DTR transgenic mice (13). This observation alone makes us speculate that promoting the conversion of most α-cells to β-cells, if it could be achieved, might represent an attractive therapeutic strategy for diabetes, once we learn how to modulate autoimmunity. In this regard, it was unclear what the minimal α-cell mass is to ensure enough glucagon production should α-cell reprogramming become a therapy to treat diabetes. The present work reveals that 2 to 4% of functional α-cells is sufficient to guarantee the glycemic control, at least under basal conditions. Reduction of the α-cell pool by reprogramming to β-cells could have the additional beneficial effect of preventing the glucagon excess typical of type 1 diabetes, which enhances the risk for hyperglycemia and ketoacidosis in patients afflicted with the disease (5).

**RESEARCH DESIGN AND METHODS**

**Mice.** The Glucagon-DTR transgenic mice were generated by pronuclear injection, as described (13). Briefly, heparin-binding epidermal growth factor–like growth factor (i.e., the natural receptor to DT, DTR) cDNA was subcloned...
FIG. 4. Changes in pancreatic glucagon content and α-cell number after near-total α-cell loss. A: Pancreatic glucagon content was increased almost sevenfold between 1 week and 1 month (from 23.52 to 157.9 pg/mg; **P = 0.0048, one-tailed Mann-Whitney test) and doubled between 1 and 6 months (157.9 to 331.4 pg/mg; *P = 0.0317 one-tailed Mann-Whitney test) in DT-treated animals.

B: Pancreatic α-cell number was not increased between 1 week and 1 month after DT but was doubled at 6 months. C: The total number of islets (defined as clusters of at least 3 β-cells) remained unchanged after α-cell ablation. D: The number of islet sections containing at least 1 α-cell was dramatically reduced after DT treatment, throughout the whole period of analysis. E: The number of α-cells in islet sections containing α-cells after DT treatment was almost doubled at 6 months. F: The number of α-cells located outside of islets was always lower in DT-treated mice than in controls and did not increase significantly with time after DT. A–F: Black ◆: control; red ▲: DT-treated mice.
FIG. 5. Newly formed α-cells after ablation are not reprogrammed β-cells. A: Transgenes required for the inducible β-cell tracing and α-cell ablation. B: Experimental design for irreversible labeling of adult β-cells before α-cell ablation. C–F: Almost all β-cells were YFP-labeled after tamoxifen (TAM) administration in control animals (DT-untreated). G–J: At 6 months after DT, none of the very rare glucagon-expressing cells were YFP+ (the arrowhead points to one α-cell, also shown in the top right inset at higher magnification). Scale bars = 20 μm. (A high-quality digital representation of this figure is available in the online issue.)
FIG. 6. α-Cell ablation does not prevent streptozotocin (STZ)-induced diabetes. A: Experimental design. One high dose of STZ (200 μg/g of mouse weight) was administered to Glucagon-DTR mice to ablate β-cells 1 week after massive DT-mediated α-cell removal. Animals were killed 2 weeks after STZ. B–G: Confocal images of pancreatic sections show DT-mediated α-cell ablation and STZ-mediated β-cell removal. White arrowheads show remaining α-cells after DT. Scale bars = 20 μm. H: Follow up of glycemia. All STZ-treated mice become hyperglycemic irrespective of DT administration. By contrast, animals that did not receive STZ remain normoglycemic (red ◆: Glucagon-DTR mice treated with both DT and STZ, n = 6; black ▼: Glucagon-DTR mice treated only with STZ, n = 6; black ■: untreated Glucagon-DTR mice, n = 3; red ▲: Glucagon-DTR mice treated only with DT). I: Body weight 15 days after STZ. All mice treated with STZ lose weight and develop typical diabetes symptoms. (A high-quality digital representation of this figure is available in the online issue.)
downstream of a 1.6-kb-long rat glucagon promoter fragment (14). The RITR, RIP-CreERT, and R26-YFP mice were previously described (33,34). Animals were housed and cared for according to the guidelines of the Direction Générale de la Santé of the Canton of Genève.

Glucagon, pyruvate, insulin tolerance tests. Glucocose (1.1 g/kg of body wt), pyruvate (2 g/kg; P2256, Sigma, St Louis, MO) and glucagon (1 mg/kg; H-6790, Bachem, Babendorf, Switzerland) were injected intraperitoneally after overnight fasting, when indicated. Insulin (0.5 units/kg; Actrapid, Novo Nordisk A/S, Bagsværd, Denmark) was injected after a 6-h fast.

Pancreas perfusion. Mice were anesthetized with sodium pentothal (10 mg/kg i.p.) and prepared for pancreas perfusion, as described (35,36). Pancreata were perfused with modified Krebs-Ringer HEPES buffer containing 4% dextran (Pharmacosmos, Holbaek, Denmark) and 0.25% BSA at 37°C; the perfusion rate was 1.5 mL/min. For equilibration, the pancreas was perfused for 30 min before collecting the efflux. Glucose and arginine were added to the perfusion buffer as indicated in the Figures. Insulin was measured using an enzyme immunoassay kit (SPI-BIO, Massy, France). Glucagon was determined by radioimmunoassay using an antiligogon antibody (Daiko Diagnostics, Zug, Switzerland), as described previously (36).

Hormone measurements, immunofluorescence, and morphometric analyses. Pancreatic glucagon and insulin (immunoassays) and morphometric analyses were performed as described (13,37,38). Paraffin and cryostat sections were 5 μm or 10 μm thick, respectively. The antibodies used were: guinea pig anti-pancreatic insulin (1:400; Dako), mouse anti-pancreatic glucagon (11,000, Sigma), mouse anti-somatostatin (1:200, Beta Cell Biology Consortium, Nashville, TN), goat anti-green fluorescent protein (1:200, Molecular Probes Inc, Eugene, OR), and anti-phospholipid bis (1:400, Millipore Corp., Billerica, MA). Secondary antibodies were coupled to Alexa fluor dyes 488, 568, or 647 (Molecular Probes), to Cy3, or to Cy5 (Jackson Immunoresearch Laboratories Inc, West Grove, PA). Transferase-mediated dUTP nick-end labeling assay was performed according to manufacturer's instructions (Apotag, Chemicon, Temecula, CA).

Sections were examined with a Leica TCS SPE confocal microscope (Leica Microsystems, Bannockburn, IL).

DT, STZ, and tamoxifen treatments. DT (D0564, Sigma) was given to 2-month-old mice in three intraperitoneal injections during 5 days, the second and third doses on days 3 and 5, respectively. Each injection (200 μg of DT in 0.9% NaCl) was given to 504 ng of DT in 0.9% NaCl. STZ (SO130; Sigma) was administrated in one injection (200 μg i.p.) after a 4-h fast.

RIP-CreERT mice were given a freshly prepared tamoxifen suspension with a gastric catheter, as previously described (13).

Quantitative PCR. Liver samples were frozen in liquid nitrogen and kept at −80°C until use. Fragments of 30 μg were subsequently used for total RNA isolation using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) with small modifications: due to high glycogen content in hepatocytes, we used 50% ethanol instead of 70% ethanol, as recommended. Four samples per group were analyzed in triplicates, and in a minimum of three independent runs. The real-time PCR was performed with modified Krebs-Ringer HEPES buffer containing 4% dextran (Pharmacosmos, Holbaek, Denmark) and 0.25% BSA at 37°C; the perfusion rate was 1.5 mL/min. For equilibration, the pancreas was perfused for 30 min before collecting the efflux. Glucose and arginine were added to the perfusion buffer as indicated in the Figures. Insulin was measured using an enzyme immunoassay kit (SPI-BIO, Massy, France). Glucagon was determined by radioimmunoassay using an antiligogon antibody (Daiko Diagnostics, Zug, Switzerland), as described previously (36).

REFERENCES