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Pancreatic β Cell Dedifferentiation in Diabetes and Redifferentiation following Insulin Therapy

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SUMMARY

Diabetes is characterized by "glucotoxic" loss of pancreatic β cell function and insulin content, but underlying mechanisms remain unclear. A mouse model of insulin-secretory deficiency induced by β cell inexcitability (K_{ATP} gain of function) demonstrates development of diabetes and reiterates the features of human neonatal diabetes. In the diabetic state, β cells lose their mature identity and dedifferentiate to neurogenin3-positive and insulin-negative cells. Lineage-tracing experiments show that dedifferentiated cells can subsequently redifferentiate to mature neurogenin3-negative, insulin-positive β cells after lowering of blood glucose by insulin therapy. We demonstrate here that β cell dedifferentiation, rather than apoptosis, is the main mechanism of loss of insulin-positive cells, and redifferentiation accounts for restoration of insulin content and antidiabetic drug responsivity in these animals. These results may help explain gradual decrease in β cell mass in long-standing diabetes and recovery of β cell function and drug responsivity in type 2 diabetic patients following insulin therapy, and they suggest an approach to rescuing "exhausted" β cells in diabetes.

INTRODUCTION

Type 2 diabetes is characterized by β cell dysfunction, the mechanism of which is controversial (Ahlqvist et al., 2011; Butler et al., 2003; Hur et al., 2010; Nolan and Prentki, 2008; Prentki and Nolan, 2006; Puri and Hebrok, 2012; Robertson et al., 2004; Talchai et al., 2012b; Wajchenberg, 2007). When faced with persistent hyperglycemia, the normal pancreatic β cell first responds with compensatory increase in insulin secretion and β cell mass (Ahrén, 2005; Bernal-Mizrachi et al., 2000; Heit et al., 2006; Jhala et al., 2003). However, chronic hyperglycemia gradually also leads to a paradoxical "glucotoxic" loss of β cell mass and insulin content that has typically been attributed to enhanced β cell apoptosis (Butler et al., 2003; Lupi and Del Prato, 2008; Poitout and Robertson, 2008; Porat et al., 2011; Prentki and Nolan, 2006). Progressive deterioration in β cell function and marked reduction of β cell mass are classic findings in type 2 diabetic human islets, regardless of the therapy (Cnop et al., 2005; Del Prato et al., 2007; Sakuraba et al., 2002; UK Prospective Diabetes Study Group, 1998a, 1998b), and reduced glucose-stimulated insulin secretion (GSIS) as well as increased rates of β cell apoptosis and decreased β cell survival have been detected in islets from human diabetic pancreases (Butler et al., 2003; Tanaka et al., 2002; Weinberg et al., 2007). In general, however, the impairment of β cell function in diabetic islets may be much greater than could be explained by the observed increase in the rate of apoptosis (Butler et al., 2003), and β cell death may not be the main contributor to the marked loss of β cell mass.

An alternative mechanism for diabetic loss of insulin content has recently received attention (Talchai et al., 2012b). The transcription factor FoxO1 is a major determinant of cell fate in enteroendocrine cells. In islets that lack FoxO1 in β cells, Talchai et al. (2012b) demonstrated β cell dedifferentiation to endocrine progenitor-like cells during stress-induced hyperglycemia. In addition to processes impinging on β cell survival and, hence, on islet mass, β cell dedifferentiation can also be observed in vitro (Weinberg et al., 2007). Dedifferentiation in common forms of β cell failure has also been inferred from partial pancreatectomy studies (Jonas et al., 1999). Together, these studies raise the possibility that dedifferentiation and conversion into other endocrine cell types may be an underrecognized mechanism of β cell failure in multiple forms of diabetes and, moreover, that this process might conceivably be reversible.

Insulin secretory failure due to inexcitability is a major cause of monogenic neonatal diabetes (Flanagan et al., 2009; Gloyn et al., 2004) and a prominent contributor to human type 2 diabetes (Nielsen et al., 2003; Riedel et al., 2005; Villareal et al., 2009). Our studies reveal that a major mechanism of β cell loss in diabetes resulting from secretory failure due to inexcitability (Remedi et al., 2009) is also dedifferentiation. Even more striking, additional experiments show that intensive insulin therapy, by reversing the hyperglycemia, leads to redifferentiation to mature β cells. These results provide a potential explanation for gradual decrease in β cell mass in long-standing and poorly controlled human diabetes, as well as for recovery of β cell function and sulfonylurea responsivity, as can be observed in type 2 diabetic patients after intensive insulin therapy (Torella et al., 1991; Wajchenberg, 2007).

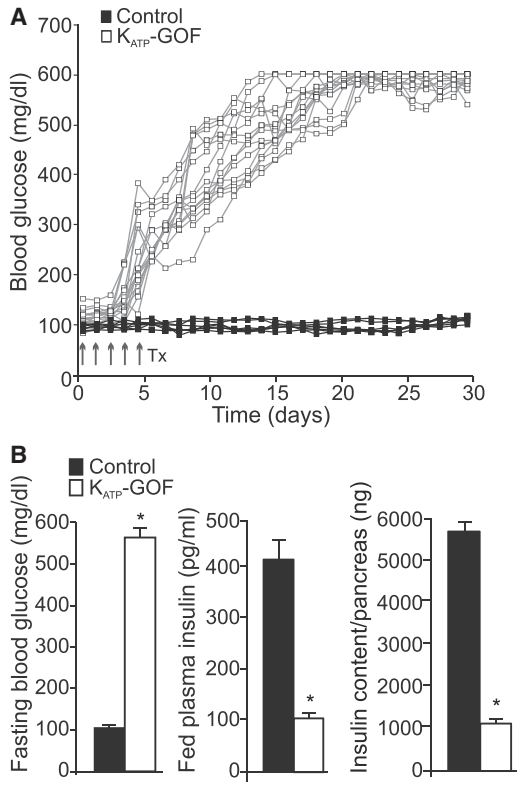

Figure 1. K_{ATP}-GOF Mice Develop Profound Diabetes

(A) Fed blood glucose (individual traces) from control (black squares) and K_{ATP}-GOF (white squares) mice after tamoxifen induction of transgene expression. (B) Fasting blood glucose, plasma insulin, and total insulin content per pancreas in control (black) and K_{ATP}-GOF (white) mice 30 days after tamoxifen induction (n = 10–12 mice per group, mean ± SEM; *p < 0.05, with respect to control).

RESULTS

K_{ATP}-GOF Mice Develop Diabetes with Dramatic Loss of Insulin Content

Following tamoxifen injection, 2-month-old Pdx1^{PB}Cre^{ER}TM Kir6.2[K185Q,ΔN30] (K_{ATP}-GOF) mice express the ATP-insensitive Kir6.2[K185Q,ΔN30] transgene, as well as an EGFP reporter. The animals develop severe diabetes within 2 weeks after tamoxifen induction (Figure 1A), as a result of the loss of glucose-dependent insulin secretion (Remedi et al., 2009, 2011). Fed and fasting blood glucose rise to >500 mg/dl in all K_{ATP}-GOF mice within ~20 days after tamoxifen induction of transgene expression and remain high thereafter (Figures 1A and 1B). Insulin secretion is extremely low, and insulin content is markedly decreased in K_{ATP}-GOF animals with respect to control mice (Figure 1B). These findings thus reiterate key features of human neonatal diabetes resulting from severe K_{ATP}-GOF mutations (Flanagan et al., 2009; Gloyn et al., 2004; Matthews et al., 1998; Nolan et al., 2011; Pearson et al., 2006; Shimomura et al., 2007), as well as the consequences of K_{ATP}-GOF that result from the type 2 diabetes-associated polymorphism (E23K) in the Kir6.2 subunit of the K_{ATP} channel (Nielsen et al., 2003; Villareal et al., 2009).

Insulin Content and Insulin-Positive β Cells Are Restored in K_{ATP}-GOF Diabetic Mice after Chronic Insulin Therapy

Following the induction of diabetes in K_{ATP}-GOF mice, the reduction of plasma insulin level was accompanied by gradual loss of islet insulin content and insulin-positive β cells (Figures 2B and 2C) (Remedi et al., 2009). We previously showed that this secondary loss could be avoided by maintenance of normoglycemia during and following disease induction, either by syngeneic islet transplantation or by sulfonylurea treatment, if initiated at disease onset (Remedi et al., 2009, 2011). However, once the disease has developed, sulfonylurea treatment is relatively ineffective, readily explained as a consequence of the marked loss of islet insulin content that rapidly develops (Remedi et al., 2009). Similar processes may also underlie gradual loss of drug responsivity in long-term or poorly controlled human diabetes, and this raises the possibility that loss of insulin content might actually be restorable if glucose levels are normalized and that drug responsivity may then also be restored.

To examine this possibility directly, we divided severely diabetic K_{ATP}-GOF mice (blood glucose >500 mg/dl for ~3 weeks) into two groups: (1) untreated and (2) chronically treated with insulin by implantation of slow-release insulin pellets in an attempt to restore normoglycemia. Untreated K_{ATP}-GOF mice maintained persistently elevated blood glucose (Figure 2A). Insulin-treated K_{ATP}-GOF mice all demonstrated a marked reduction in blood glucose (Figure 2A), although the degree of normalization was variable; some mice essentially achieved normoglycemia with only one pellet implanted, whereas others required a second pellet to achieve a sustained lowering of blood glucose levels (Figure 2A).

In untreated diabetic K_{ATP}-GOF pancreases, immunohistochemistry reveals disrupted architecture with a marked loss of insulin content and insulin-positive β cells in islets from K_{ATP}-GOF mice that were sacrificed at 30 days following tamoxifen induction (Figures 2B and 2C). However, insulin content and insulin-positive β cells were fully restored in islets from the second set of diabetic K_{ATP}-GOF mice that were treated with insulin by implantation of slow-release pellets at 30 days following disease induction and then sacrificed 40 days later (i.e., after 40 days of insulin treatment; Figure 2C). Notably, islets from insulin-treated mice, in which normalization of glycemic control was incomplete, showed a less pronounced recovery of insulin content than islets from mice in which glucose was almost fully normalized (Figure 2C). Moreover, total insulin content per pancreas, which was markedly reduced from 574 ± 21 ng in control mice to 102 ± 6 ng in untreated K_{ATP}-GOF mice (Figure 1B), was almost completely restored (to 495 ± 14 ng) in insulin-treated K_{ATP}-GOF mice.

β-Cell Death May Not Explain the Marked Loss of Insulin Content and Insulin-Positive β Cells in K_{ATP}-GOF Islets

Increased apoptosis may contribute to β cell loss in type 2 diabetes (Butler et al., 2003; Poitout and Robertson, 2008; Prentki and Nolan, 2006), and the marked reduction of insulin content in diabetic K_{ATP}-GOF islets (Figures 2B and 2C) raises the possibility that increased apoptosis might be responsible. Apoptosis was therefore assessed in pancreatic sections from control and K_{ATP}-GOF (untreated and insulin-treated) mice by terminal

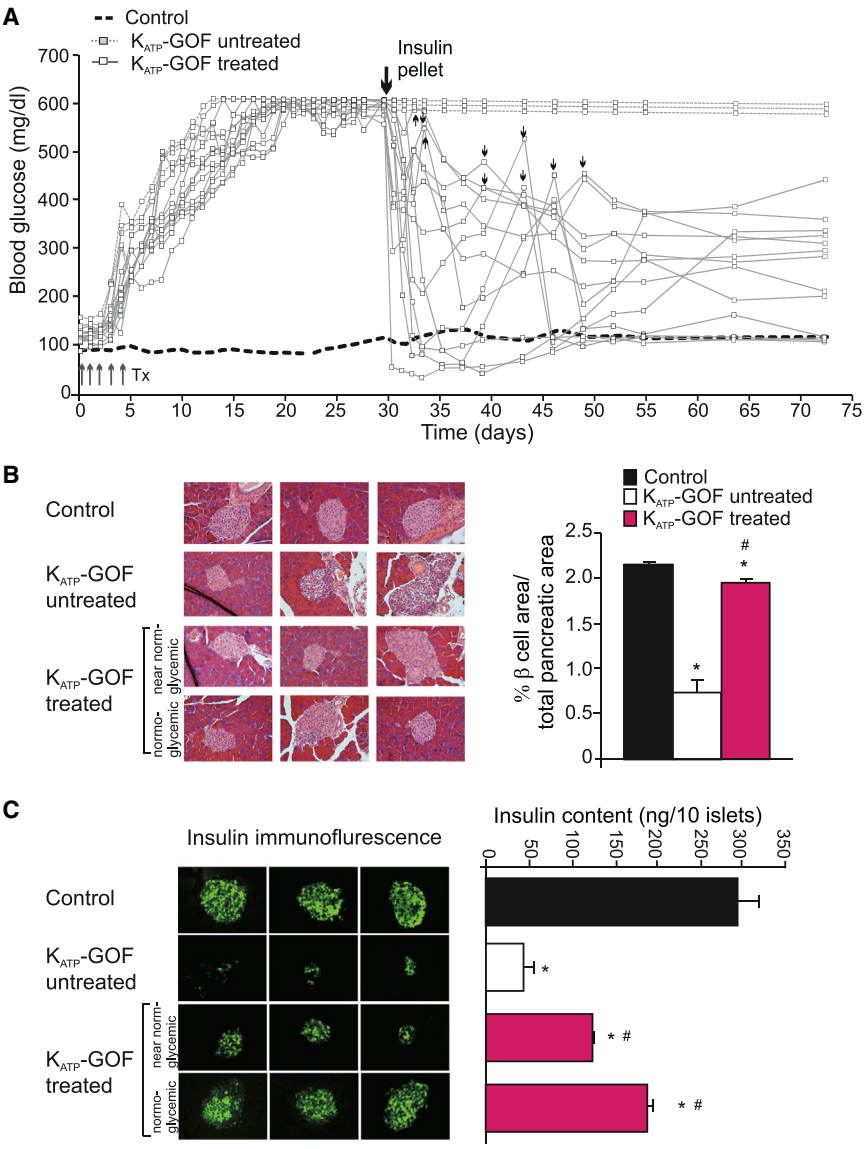

Figure 2. Insulin Therapy Restores Endogenous Insulin Content in Diabetic K_{ATP}-GOF Mice

(A) Fed blood glucose in control (average, black dashed line) and in K_{ATP}-GOF untreated (white, dashed line) and insulin-treated (white, solid line) mice after tamoxifen induction of transgene expression. Big arrow indicates first insulin pellet implantation, and small arrows a second insulin pellet implanted in individual mice as necessary (blood glucose > 400 mg/dl).

(B) Panels on the left show high-magnification sections of pancreases stained with hematoxylin and eosin from control and K_{ATP}-GOF untreated and insulin-treated mice. Bar graph on the right indicates the pancreatic β cell area from (C) in control (black), K_{ATP}-GOF untreated (white), and insulin-treated (pink) mice.

(C) Insulin immunofluorescence (left) and insulin content (right) per islet in control (black) and K_{ATP}-GOF mice, untreated 30 days after tamoxifen injection (white) or insulin treated (70 days after tamoxifen, 40 days after insulin pellet implantation; pink) (n = 3–6 mice per group, mean ± SEM). Insulin-treated K_{ATP}-GOF mice were divided in two groups at the time of sacrifice following the criteria of blood glucose levels: near normoglycemic and normoglycemic. Significant differences: *p < 0.05, with respect to control mice and #p < 0.05, with respect to untreated K_{ATP}-GOF mice.

deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) and cleaved caspase-3 assays. TUNEL staining showed only a slight (nonsignificant) increase in the number of apoptotic cells within diabetic islets from untreated K_{ATP}-GOF mice, compared with islets from control and insulin-treated mice (Figure 3A). Since TUNEL detects DNA strand breaks in terminal cell stages, it is possible that the frequency of TUNEL-positive cells does not accurately reflect the level of apoptosis in these samples; therefore, we additionally assessed the levels of cleaved caspase-3. Cleaved caspase-3 positivity showed a small but significant increase in untreated K_{ATP}-GOF islets but was not different between control and insulin-treated islets (Figure 3B).

Hyperglycemia Drives Dedifferentiation of K_{ATP}-GOF Pancreatic β Cells to an Insulin-Negative, Neurogenin3-Positive Phenotype

Given the only slight increase in apoptosis in even severely diabetic K_{ATP}-GOF islets, the recent findings of Talchai et al. (2012b) raise the possibility that dedifferentiation might be an alternative mechanism underlying the dramatic loss of insulin-positive β cells in these mice. Pancreatic sections from untreated and insulin-treated K_{ATP}-GOF mice were immunostained for neurogenin3 (Ngn3), a marker of islet progenitor cells (Gu et al., 2002; Xu et al., 2008). There was essentially no Ngn3 positivity in control islets, but we observed a high number of dedifferentiated, Ngn3 positive, cells in diabetic K_{ATP}-GOF mouse islets using both Santa Cruz and BCBC Ngn3 antibodies (Figure 4; Figure S3A available online). Importantly, almost all Ngn3-positive cells were insulin-negative (Figure 4). These results indicate that, in the diabetic condition, a significant number of mature insulin-containing β cells are replaced by insulin-negative and Ngn3-positive cells, which might actually have arisen by dedifferentiation from mature β cells. In order to lineage trace the loss of insulin-positive cells as well as the origin of Ngn3-positive cells, pancreatic sections were double immunostained for insulin or Ngn3 and EGFP, which is coexpressed with the K_{ATP}-GOF mutant transgene following tamoxifen induction (Remedi et al., 2009) and therefore indicates cells that are, or were, mature insulin-producing β cells. Double immunostaining for insulin and EGFP shows that almost all cells in the islet core are EGFP positive in untreated diabetic K_{ATP}-GOF islets, but only ~40% express insulin (Figure 5A). Moreover, in these severely diabetic K_{ATP}-GOF islets, essentially all Ngn3-positive cells also express EGFP (Figure 5B), indicating that the

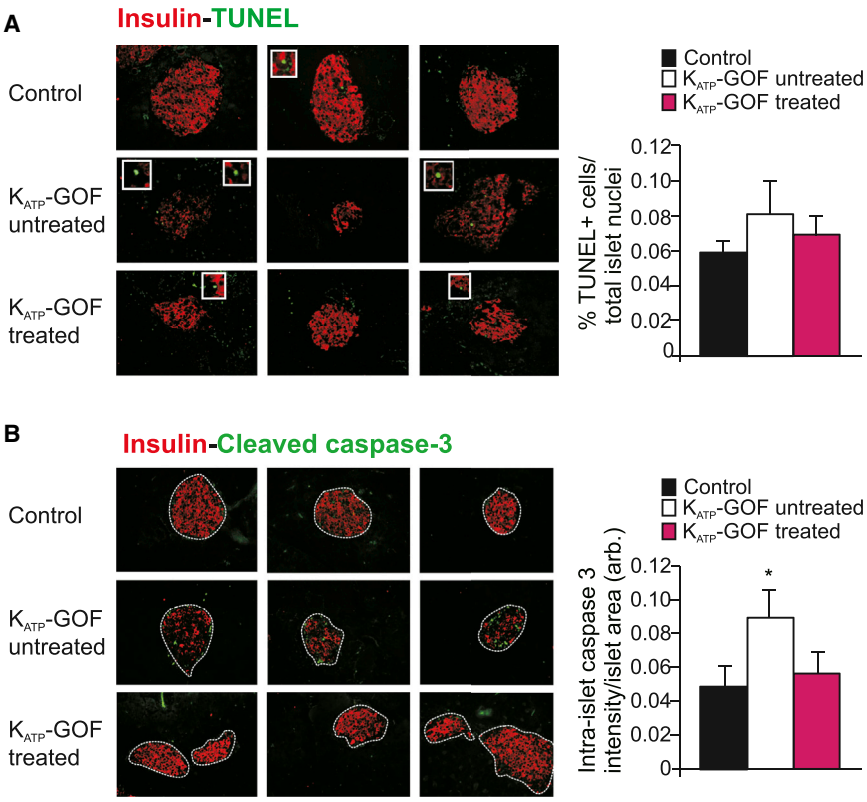

Figure 3. Small Increase in Apoptosis in K_{ATP}-GOF Diabetic Mice

(A and B) Representative pancreatic sections (left panels) and quantification of apoptosis (bar graphs at right) from control (black) and K_{ATP}-GOF untreated (white) and insulin-treated (pink) mice immunostained for insulin (red) and apoptosis (green) using TUNEL (A) and cleaved caspase-3 (B). Data represent mean ± SEM, n = 4 mice per group, five pancreatic sections per mouse. *p < 0.05, with respect to control. arb., arbitrary units.

Ngn3-positive cells were also originally mature β cells and providing compelling evidence for dedifferentiation as the primary mechanism for the loss of β cell phenotype. A question arises as to what happens to the insulin, since these islets are electrically inexcitable and lack glucose-dependent insulin secretion. Clearly, the cells are depleted of insulin, but this is presumably either through intracellular degradation or depletion through basal secretion once the cells turn off insulin production (i.e., as they dedifferentiate). To examine potential plasticity and pluripotency of β cell dedifferentiation, we also looked for expression of other stem cell markers in diabetic K_{ATP}-GOF islets. Quantitative real-time PCR shows an increase in mRNA expression levels of islet progenitor markers Ngn3, Nanog, and L-Myc (Figure S2D) and a corresponding decrease in the levels of β cell-specific markers Pdx1, Nkx6.1, and MafA (Figure S2C) in diabetic islets, consistent with β cell dedifferentiation to endocrine progenitor cells in the diabetic state. Immunohistologically, we detected Oct4, Nanog, and Nestin expression in control embryonic day (E) 15.5 fetal tissue, although we were unable to detect these markers in adult K_{ATP}-GOF diabetic islets (Figure S2B); thus, we cannot confirm that the elevated message levels are reflected in elevated protein levels.

Redifferentiation to Insulin-Producing β Cells following Chronic Lowering of Blood Glucose

As shown earlier, Ngn3-positive cells were not observed in islets from control islets, nor were they observed in insulin-treated K_{ATP}-GOF islets from mice after reduction of blood glucose levels (Figure 5B). Instead, following lowering of blood glucose in treated mice, EGFP and insulin are again expressed throughout the core (Figure 5). This suggests that substantial reduction of systemic hyperglycemia—in this case, by insulin therapy—actually permits redifferentiation of the Ngn3-positive cells to mature, insulin-containing β cells. Tamoxifen can remain in the body for an extended period and may continue to label significant numbers of cells for a few weeks after treatment (Reinert et al., 2012), but the finding that almost all cells in the islet core stain positive for the EGFP reporter, 70 days after initial tamoxifen induction (Figure 5) strongly argues that these were the same cells that were originally induced to express EGFP as mature β cells. Notably, mRNA expression levels of the β cell markers Pdx1, Nkx6.1, and MafA are restored to normal levels in insulin-treated islets (Figure S2C), with a concomitant decrease in the progenitor markers Ngn3, Oct4, Nanog, and L-Myc (Figure S2D) consistent with dedifferentiated cells redifferentiating to mature β cells.

To investigate the alternative possibility that an abnormally high β cell proliferation might underlie restoration of the insulin-expressing cells, we performed immunostaining for the cell cycle marker Ki67. The fraction of Ki67-positive β cells per islet was not different between control, diabetic, or treated conditions (Figure S1), providing evidence of similarly low rates of proliferation in each and suggesting that the reappearance of mature β cells is not likely to be the result of proliferation of residual, preexisting β cells.

Endogenous Insulin Secretion in Response to Sulfonylureas, but Not to Glucose, Is Restored in Redifferentiated Islets from Insulin-Rreated K_{ATP}-GOF Mice

The aforementioned results imply that the restoration of islet insulin content, following normalization of glycemia, results from redifferentiation of preexisting K_{ATP}-GOF β cells that had dedifferentiated to insulin-negative, Ngn3-positive cells. If this is correct, then these cells, in addition to still expressing EGFP, should also still be expressing the K_{ATP}-GOF transgene and, hence, should remain electrically inexcitable and nonresponsive to glucose but responsive to sulfonylureas. Insulin secretion in low (1 mM) glucose or in response to the sulfonylurea glibenclamide was not different between control and insulin-treated

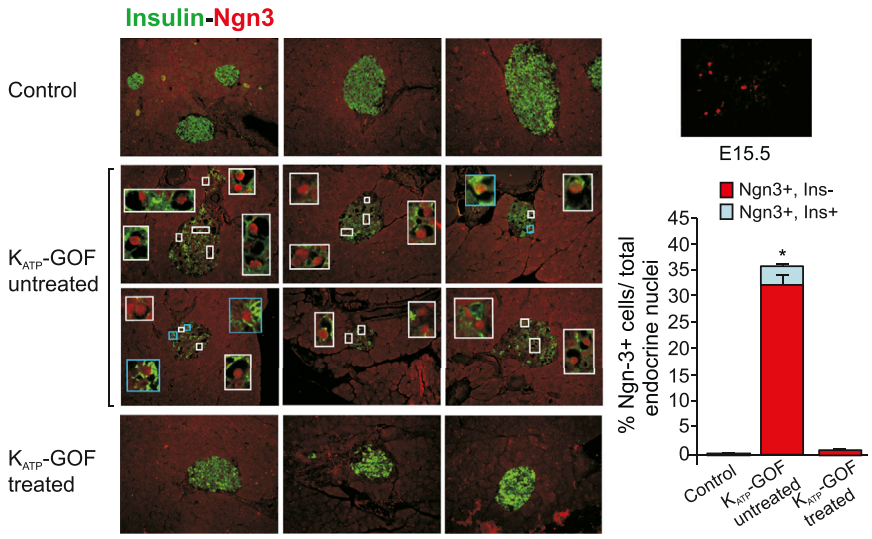

Figure 4. β Cell Dedifferentiation in Severely Diabetic K_{ATP}-GOF Mice and β Cell Redifferentiation in Islets from K_{ATP}-GOF Insulin-Treated Mice

Panels at left show representative pancreatic sections from control and K_{ATP}-GOF untreated and insulin-treated mice double immunostained for insulin (green) and Ngn3 (Santa Cruz antibody; red). White-bordered insets show Ngn3-positive and insulin-negative cells within islets. Blue-bordered insets show occasional Ngn3-positive and insulin-positive cells. Panel at top right shows Ngn3 positivity in E15.5 fetal tissue. Bar graph at bottom right indicates percentage of Ngn3-positive cells, either insulin negative (Ins−; red) or insulin positive (Ins+; light blue from each condition; mean ± SEM). Data represent n = 5–8 mice per group, five pancreatic sections per mouse. Significant differences: *p < 0.05, with respect to control and K_{ATP}-GOF insulin-treated mice.

K_{ATP}-GOF islets (Figure 6A), but, in excellent agreement with the aforementioned prediction, robust glucose-dependent secretion was present in control islets but not in insulin-treated and control islets (Figure 6A). Together with the immunohistochemical findings, these results provide strong evidence that dedifferentiated and then redifferentiated cells were originally K_{ATP}-GOF mutant β cells.

Sulfonylurea-Dependent Insulin Secretion Is Reinstated in K_{ATP}-GOF Mice after Insulin Therapy

Restoration of sulfonylurea-sensitive insulin secretion in insulin-treated K_{ATP}-GOF islets would also suggest that in vivo sulfonylurea-sensitive secretion should also be restored. Artifactually high insulin levels are measured in the blood of insulin-treated mice (3,251 ± 487 ng/ml), which obviates assessment of the levels of endogenous insulin itself. We therefore measured C-peptide levels in mice following injection with the sulfonylurea glibenclamide. Control mice all showed high (400–600 pmol/l) plasma C-peptide levels (Figure 6B). Thirty days after tamoxifen injection, untreated diabetic K_{ATP}-GOF mice showed very low levels of C-peptide (30 days after treatment; Figure 6B), consistent with the marked increase in the number of dedifferentiated Ngn3-positive cells (Figures 4 and S3A) and decrease in insulin content within their islets (Figures 2B and 2C). However, C-peptide levels were considerably higher in the same K_{ATP}-GOF mice after a subsequent 10 days with insulin treatment (total, 40 days after tamoxifen injection; Figure 6B) and even higher after 40 days of insulin treatment (total, 70 days after tamoxifen injection; Figure 6B). In a subset of K_{ATP}-GOF mice, C-peptide levels were measured both before and 30 min after glibenclamide injection (Figure 6C). In untreated diabetic K_{ATP}-GOF mice, there was no significant response to the drug, but there was increasingly marked glibenclamide response with time after insulin treatment. These results nicely correlate with the increase in insulin content and number of insulin-positive β cells that was observed in insulin-treated islets. Notably, and as predicted, both K_{ATP}-GOF untreated or insulin-treated (10 days or 40 days of insulin treatment) mice demonstrated low C-peptide levels in fed conditions, and only mice that had been treated with insulin showed restoration of C-peptide release in response to sulfonylurea stimulation (Figure 6C; C-peptide release after glibenclamide over basal). Together, these results demonstrate that restoration of β cell insulin positivity and islet insulin content in diabetic mice by insulin therapy indeed leads to reestablishment of antidiabetic drug responsivity.

Dedifferentiated β Cells Do Not Transdifferentiate to Glucagon-Producing α Cells

As reported previously (Remedi et al., 2009), severely diabetic K_{ATP}-GOF mice demonstrate a significant increase in glucagon immunoreactive area, as well as elevated plasma glucagon levels, but all of these are substantially reversed after insulin therapy (Figure 7). By lineage tracing analysis, we can examine the possibility of transdifferentiation of EGFP-positive β cells to glucagon-producing α cells via Ngn3-positive progenitors. Pancreatic sections from control and K_{ATP}-GOF mice were double stained for glucagon and EGFP (which will indicate K_{ATP}-GOF former β cells) (Figure 7A). The number of glucagon-positive cells increases in the diabetic state, but only a very small number of cells costain for both glucagon and EGFP in untreated or treated islets (Figures 7A and 7B), indicating that the increase in the number of α cells in these islets is not substantially attributable to conversion from β cells.

Rip-K_{ATP}-GOF Mice Also Develop Severe Diabetes and Show Similar Dedifferentiation of β Cells and Redifferentiation after Insulin Therapy

Pdx1 is also expressed in ductal and even acinar cells, raising the formal possibility that newly formed β cells might derive from non-β cells that express Pdx1 during tamoxifen treatment. We therefore performed similar experiments to those described earlier on mice in which the K_{ATP}-GOF transgene (Kir6.2 [K185Q,ΔN30]) was expressed under the control of the rat insulin promoter (Rip) in Rip-Cre-expressing mice (Rip-K_{ATP}-GOF). Rip-K_{ATP}-GOF mice show hyperglycemia immediately after birth, with development of severe diabetes over time (Remedi et al., 2009), similar to Pdx K_{ATP}-GOF mice. Severely diabetic, 75-day-old Rip-K_{ATP}-GOF mice were implanted with slow-release

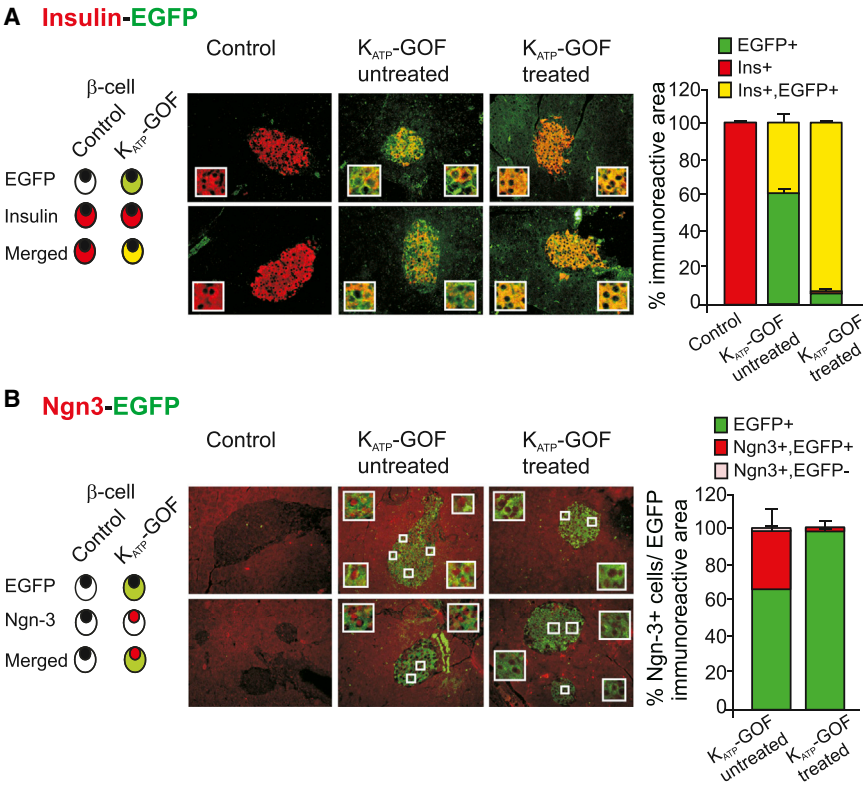

Figure 5. Dedifferentiated and Redifferentiated Islet Cells Are Originated from Former Adult β Cells

(A and B) Left panels show representative images of double immunostaining for EGFP (indicating transgene expression in pancreatic β cells) and insulin (A) or Ngn3 (B) on pancreatic sections from control and both untreated and insulin-treated K_{ATP}-GOF mice. Insets show representative cells. Right panels indicate the percentage of the immunopositive area of the islet insulin and EGFP (A) or of Ngn3 and EGFP (B) (mean ± SEM). Data represent n = 5 mice per group, five pancreatic sections per mouse.

insulin pellets, and blood glucose was followed over a period of 40 days (Figure S3A). Islet insulin content, which was markedly reduced compared to that in age-matched littermates, was also dramatically restored following insulin treatment (Figures S3B and S3C). There was again a striking increase in Ngn3-positive/insulin-negative cells in untreated Rip-K_{ATP}-GOF mice that disappeared following treatment (Figures S3D and S3E). Again, Ngn3-positive cells and insulin-positive cells both costained with EGFP-positive cells, again providing compelling evidence that dedifferentiated as well as redifferentiated cells originated from former β cells (Figure S3E). Glucagon immunostaining also demonstrated a significant increase in α cell population in diabetic Rip-K_{ATP}-GOF mice (Figure S3F).

DISCUSSION

This study shows that (1) loss of insulin content during the progression of diabetes that results from β cell secretory failure occurs primarily through glucotoxic β cell dedifferentiation rather than apoptosis and that (2) restoration of normoglycemia results in redifferentiation to insulin-positive β cells.

Controlling Blood Glucose Is Enough to Reverse Glucotoxicity in Diabetes

It is generally well established that hyperglycemia negatively affects β cell secretory capacity, but the underlying mechanisms and treatability are not completely understood. A progressive deterioration in β cell function is a common finding in patients with both type 2 diabetes (Matthews et al., 1998; Nolan et al., 2011; Rhodes, 2005; Sakuraba et al., 2002; UK Prospective Diabetes Study Group, 1998a, 1998b) and K_{ATP}-dependent monogenic diabetes (Pearson et al., 2006). We previously demonstrated that chronic hyperglycemia gradually leads to a dramatic loss of insulin-positive cells and insulin content in our K_{ATP}-GOF mouse model of neonatal diabetes (Remedi et al., 2009), correlating with the findings obtained in other rodent models of diabetes (Girard et al., 2009; Jonas et al., 2009; Laybutt et al., 2003; Porat et al., 2011; Talchai et al., 2012b). In this article, we demonstrate that chronic insulin treatment not only normalizes blood glucose in diabetic K_{ATP}-GOF mice but also dramatically restores insulin content and insulin-positive β cells. The secondary consequences of diabetes and their reversal were strikingly reflected in vivo by loss, and reversal, of insulin and C-peptide release in response to injected glibenclamide and in isolated islets by restored glibenclamide- and KCl-dependent insulin secretion. These results suggest that relieving β cells from systemic hyperglycemia may actually restore insulin content and thereby improve β cell drug responsiveness.

There has been much interest in the notion that β cells become "exhausted" due to the excess demands of secretion induced by hyperglycemia in the diabetic state and that β cell "rest" via exogenous insulin treatment permits restoration of β cell function in type 2 diabetic patients (Alvarsson et al., 2008; Greenwood et al., 1976; Qvigstad et al., 2004; Torella et al., 1991; Weng et al., 2008). The present results show that insulin therapy, by correcting the systemic diabetes, leads to restoration of endogenous islet insulin content and antidiabetic drug responsivity in islets that are intrinsically inexcitable and are, therefore, chronically low in $[Ca^{2+}]_i$ and do not secrete insulin in response to glucose (Benninger et al., 2011; Remedi et al., 2009). Thus, it must be that somewhere upstream of excitability and secretion is being affected. As we showed previously (Benninger et al., 2011), glucose-dependent metabolism, as assessed by NAD(P)H autofluorescence, is actually elevated in diabetic K_{ATP}-GOF islets, potentially a consequence of chronic in vivo hyperglycemia. Thus, we suggest that it is "rest" from hyperstimulation of metabolism, rather than "rest" from hyperexcitability and secretion, that permits β cell recovery of insulin-positive cells and insulin

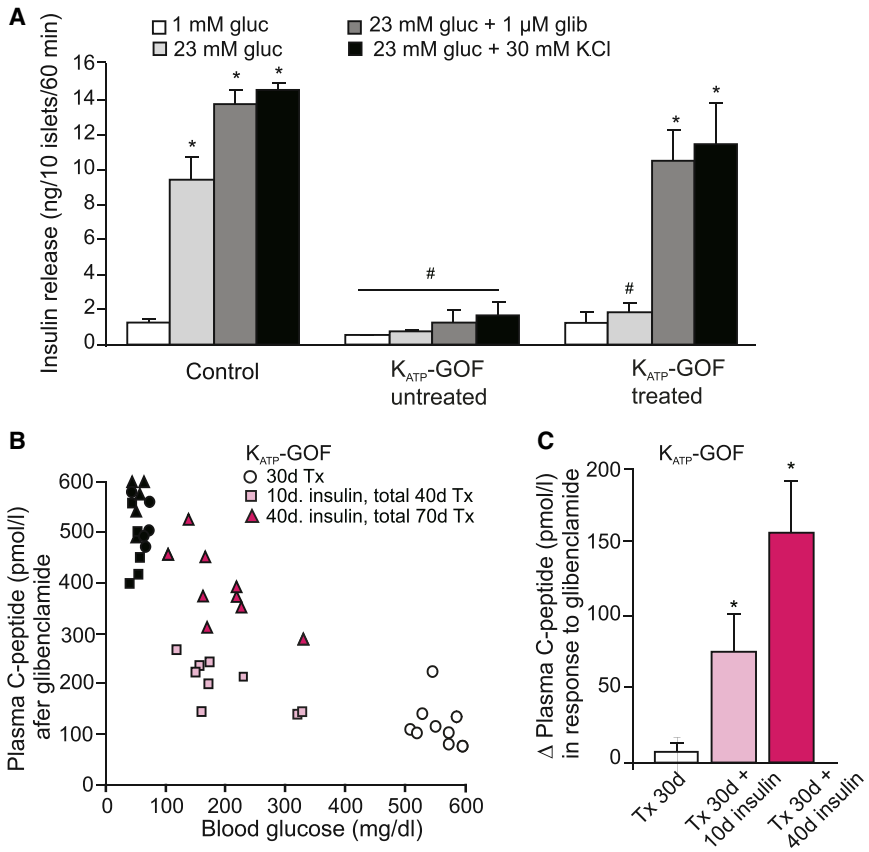

Figure 6. Glibenclamide-Dependent C-Peptide Release Is Restored Only in K_{ATP}-GOF Insulin-Treated Mice

(A) Insulin secretion from islets isolated from control and K_{ATP}-GOF untreated and insulin-treated mice (70 days after tamoxifen, ~40 days on insulin therapy). Islets were incubated in low (1 mM) and high (23 mM) glucose (gluc), and in the presence of the sulfonylurea glibenclamide (glib) (1 μM) or the depolarizing agent KCl (30 mM). Data represent mean ± SEM. Significant differences: $^{\#}p < 0.05$, with respect to control under the same condition, and $^{*}p < 0.05$, with respect to 1 mM glucose within the same group.

(B) Plasma C-peptide (individual values) 30 min after glibenclamide stimulation in control mice (black circles, 30 days after treatment [Tx]; black squares, 40 days after Tx; and black triangles, 70 days after Tx), K_{ATP}-GOF mice untreated 30 days after tamoxifen injection (white, 30 days after Tx [30d]) and K_{ATP}-GOF mice treated with insulin for 10 days ([10d insulin] total, 40 days after Tx; light pink) and 40 days ([40d insulin] total, 70 days after Tx; dark pink).

(C) Delta plasma C-peptide in response to glibenclamide in K_{ATP}-GOF untreated (white) and K_{ATP}-GOF insulin-treated (10 and 40 days, light pink and dark pink, respectively) mice (n = 5–9 mice per group, experiments made in triplicates, mean ± SEM). Significant differences: $^{*}p < 0.05$ with respect to K_{ATP}-GOF untreated mice.

content (Nichols and Remedi, 2012). The underlying mechanism of this improvement might also be key to the beneficial effects of insulin therapy and tight glycemic control in type 2 diabetic patients (Alvarsson et al., 2003; Ilkova et al., 1997; UK Prospective Diabetes Study Group, 1998a, 1998b; Wajchenberg, 2007; Weng et al., 2008).

Apoptosis versus Dedifferentiation as a Response to Hyperglycemia

In this article, we demonstrate that hyperglycemia-induced glucotoxicity, with marked loss of islet insulin content and insulin-positive β cells, is accompanied by a small increase of apoptosis (Figure 3), similar to the levels of apoptosis detected in human diabetic islets (Butler et al., 2007; Rahier et al., 2008). However, the number of cells exhibiting positive TUNEL staining or cleaved caspase-3 is markedly less than the number of insulin-negative cells (Figure 3), suggesting that β cell dedifferentiation, rather than apoptosis, may be the major mechanism underlying the loss of insulin positivity. Ngn3 expression is essentially undetectable in islets from control mice, reportedly present at very low levels in adult mouse pancreases (Wang et al., 2009), but it is clearly elevated in insulin-negative cells of untreated K_{ATP}-GOF islets (Figures 4 and S2A); and, at least at the messenger level, Ngn3 expression as well as that of other progenitor markers, Nanog and L-Myc, is elevated in diabetic islets form K_{ATP}-GOF diabetic mice (Figure S2D). These results are very comparable to the finding of marked increase in the number of dedifferentiated cells within stressed FoxO1-deficient and insulin-resistant GIRKO and db/db models of type 2 diabetes (Talchai et al., 2012b). As Talchai et al. suggested, such dedifferentiation, rather than a truly degenerative state, may provide an advantage to the islet and, as we show later, a potential route to the ultimate reversibility of islet demise. The underlying mechanism for the dedifferentiation is still not clear but might be linked to the down-regulation of FoxO1 following hyperglycemia-induced oxidative stress (Benninger et al., 2011; Kitamura et al., 2005; Talchai et al., 2012a). The phenomenon may provide an explanation for the slow decline of β cell mass in human diabetes (Rahier et al., 2008), although no changes in mRNA expression levels of Ngn3 or other stem cell markers were detected in a recent study (Guo et al., 2013) of human type 2 diabetic islets (with the exception of Oct4, which increased significantly). The dramatic decrease in mRNA levels of the β cell transcription factors Pdx1, MafA, and Nkx6.1 in our diabetic K_{ATP}-GOF mice (Figure S2C) correlates well with the marked reduction in these transcription factors in both human and mouse type 2 diabetic islets (Guo et al., 2013; Talchai et al., 2012b), reflecting loss of β cell identity in glucotoxic conditions in vivo.

Redifferentiation as a Response to Normalization of Glycemia

We demonstrate here a dramatic recovery of insulin-positive β cells and islet insulin content—and, hence, sulfonylurea responsivity—after insulin therapy in K_{ATP}-GOF mice (Figures 2, 5, and 6). The level of this recovery is proportional to the level of rescue of glycemia, pointing to hyperglycemia as the controlling feature.

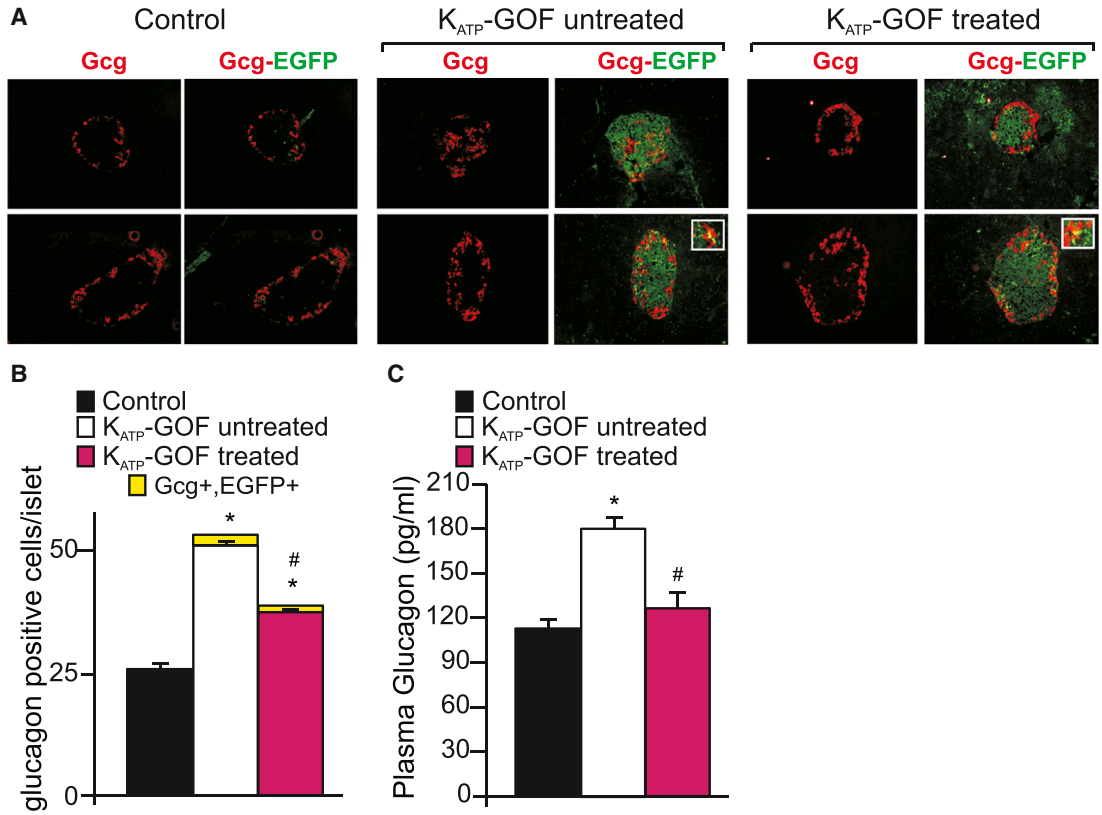

Figure 7. Increased α Cell Number and Plasma Glucagon in K$_{ATP}$-GOF Mice

(A) Representative images of double immunostaining for EGFP (indicating transgene expression in pancreatic β cells) and glucagon (Gcg) on pancreatic sections from control and both untreated and insulin-treated K$_{ATP}$-GOF mice. White-bordered insets show costaining for both glucagon and EGFP.

(B and C) Quantification of glucagon-positive cells (B) and plasma glucagon (C) from control (black), K$_{ATP}$-GOF untreated (white), and K$_{ATP}$-GOF insulin-treated (pink) mice (mean ± SEM). Data represent n = 5 mice per group, five pancreatic sections per mouse. Significant differences: *p < 0.05, with respect to control, and $^{\#}$p < 0.05, with respect to untreated K$_{ATP}$-GOF mice.

The recovery of insulin content (Figure 2C) is paralleled by loss of Ngn3 staining (Figures 4 and 5), with maintenance of EGFP staining in the recovered islet cells (Figure 5), indicating that recovery involves redifferentiation of the same former β cells within the islet and with no evidence for transdifferentiation of dedifferentiated β cells to α cells (Figure 7). This conclusion is supported by the finding that, while multiple markers of cell dedifferentiation are upregulated and specific β cell markers are reduced, in diabetic islets, all marker expression levels are restored to normal after intensive insulin therapy (Figures 4 and S2). Essentially, the same pattern of dedifferentiation and redifferentiation after insulin therapy is demonstrated in diabetes driven by Rip-Cre-induced expression of the same K$_{ATP}$ GOF transgene (Figure S3), providing further evidence that resurgent β cells are derived from the previously dedifferentiated β cells and excluding the possibility that these cells might be derived from ductal or acinar Pdx1-positive cells in the Pdx-Cre-driven model. More generally, these findings provide a potential explanation for the reversibility of β cell dysfunction and restoration of drug responsivity that is seen in human type 2 diabetics following β cell "rest" with intensive insulin therapy (Alvarsson et al., 2008; Ilkova et al., 1997; Torella et al., 1991; UK Prospective Diabetes Study Group, 1998b; Wajchenberg, 2007; Weng et al., 2008). They also raise the exciting possibility that the gradual decrease in antidiabetic drug responsivity that is frequently observed in diabetic patients may similarly be reversed by intensive normalization of blood glucose levels.

Conclusions

Our findings reveal a mechanism underlying loss of islet insulin content in response to hyperglycemia in a mouse model of K$_{ATP}$-dependent diabetes. These results might explain the decrease in β cell mass in multiple forms of long-standing or poorly controlled diabetes. They suggest that targeting glucotoxic β cell dysfunction and strictly normalizing blood glucose and systemic diabetes could induce cell redifferentiation to mature β cells and, hence, restoration of drug responsivity, providing an approach to rescuing "exhausted" β cells in diabetes.

EXPERIMENTAL PROCEDURES

Chronic Insulin Therapy in a Mouse Model of Neonatal Diabetes

All experiments were performed in compliance with institutional guidelines and approved by the Washington University Animal Studies Committee. Rip and tamoxifen-inducible Pdx1^{PB}Cre^{ER}TM β cell-specific K$_{ATP}$ gain-of-function (Kir6.2 [K185Q,ΔN30]) mutant mice were previously generated. In the case of inducible mice, transgene expression was induced by five consecutive

injections of tamoxifen as described elsewhere (Remedi et al., 2009). Littermate controls were used in all experiments. K_{ATP}-GOF transgenic mice with severe diabetes (blood glucose >500 mg/dl) for several weeks were anesthetized and implanted with time-release insulin pellets (release, 0.1 U/day per implant for 60 days; Linbit, Linshin Canada Inc.), and blood glucose was followed over time.

Blood Glucose, Plasma Insulin, Glucagon, and C-Peptide Levels

Tail blood was assayed for glucose content using the Glucometer Elite-XI (Bayer Corporation). The limit of detection was 600 mg/dl, and glucose at or above this level was recorded as 600 mg/dl but considered to be a lower limit of the true value. Plasma insulin was measured using the Singulex Erenna method (Washington University Immunoassay Core), and glucagon was measured by using radioimmunoassay (RIA, Millipore). C-peptide was measured using ELISA (Alpco Diagnostics, Washington University Immunoassay Core).

Pancreatic Islet Isolation

Mice were anesthetized with Isofluorane (0.2 ml) and killed by cervical dislocation; the bile duct was cannulated and perfused with Hank's solution (Sigma) containing collagenase (Collagenase Type XI, Sigma). Pancreases were removed and digested at 37°C, hand shaken, and washed in cold Hank's solution. Islets were isolated by hand under a dissecting microscope and maintained overnight in CMRL-1066 (5.6 mM glucose) culture medium (GIBCO) supplemented with fetal calf serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Remedi et al., 2009).

Insulin Secretion and Content

Following overnight incubation in low-glucose CMRL-1066 medium, islets (ten per well in 12-well plates) were preincubated in glucose-free CMRL-1066 plus 3 mM glucose (only for insulin secretion) and then incubated for 60 min at 37°C in CMRL-1066 plus different glucose concentrations, 1 μM glibenclamide, or 30 mM KCl, as indicated. After the incubation period, the medium was removed and assayed for insulin release. Experiments were repeated in triplicate. For islet insulin content, groups of five islets were disrupted using ethanol-HCl extraction and sonicated on ice for estimation of insulin content. Whole pancreas was removed and disrupted in homogenization buffer using a polytron homogenizer. Samples were centrifuged, and supernatant was used for measurement of total insulin content per pancreas. Insulin secretion and content were measured using a rat insulin radioimmunoassay according to manufacturer's procedure (RIA, Millipore) (Remedi et al., 2009).

Immunohistochemical and Morphometric Analysis

Pancreases from control, untreated K_{ATP}-GOF (day 30 after tamoxifen induction) and insulin-treated K_{ATP}-GOF mice (day 70 after tamoxifen induction, ~40 days after insulin pellet implantation) were fixed in 10% formalin and paraffin embedded for sectioning. Four to eight mice from each genotype were sampled on 5-μm-thick sections 150 μm apart and used for immunohistochemical and morphometric analysis. For morphometric analysis, at least five pancreatic sections from 3–5 mice from each genotype were covered systematically by accumulating images from nonoverlapping fields on an inverted fluorescence Zeiss microscope or on a Zeiss LSM 510 laser confocal microscope. Hematoxylin and eosin staining was carried out as described elsewhere (Remedi and Nichols, 2008). Antigen retrieval was performed for nuclear transcription factor detection (Nacalai USA) (Talchai et al., 2012b). For insulin positivity determination, islet insulin immunoreative cross-sectional area and total pancreatic area were measured and calculated using MetaMorph imaging software (Universal Imaging Corporation) and expressed as percentage of β cell area relative to the total pancreatic area. Apoptosis was determined on pancreatic paraffin sections by using TUNEL (ApopTag Plus Fluorescein In Situ Apoptosis, Chemicon International, Inc.) and cleaved caspase-3 staining techniques. Cleaved caspase-3 primary antibody (Cell Signaling) was detected by using secondary antibody conjugated with Alexa 488 (green, Molecular Probes). For apoptosis quantification, the number of TUNEL-positive cells per total islet nuclei (counterstained with DAPI) or intra-islet caspase-3 intensity per total islet area was determined. Quantification of Ngn3 was performed in pancreatic sections double stained for insulin and Ngn3 by counting the number of Ngn3-positive, insulin-negative, or insulin-positive cells in a merged red and green image. The presence of EGFP (indicating K_{ATP}-GOF transgene expression on pancreatic β cells) was assessed in pancreatic sections using EGFP primary antibody. Quantification of lineage tracing experiments in K_{ATP}-GOF mice was performed by determining EGFP, insulin, and glucagon immunoreactive areas and the ratio of overlapping area/total immunoreactive area. Ngn3-positive cells were counted and expressed as a percentage of Ngn3-positive cells per total EGFP immunoreactive area. To estimate α cell population, the total number of glucagon-positive cells was counted from several pancreatic sections immunostained for glucagon. For proliferation analysis, Ki67-positive β cells were counted in pancreatic sections costained for insulin and expressed as a percentage of Ki67-positive cells per total β cell area. Primary antibodies used were as follows: insulin (rabbit monoclonal, Cell Signaling; or guinea pig polyclonal, Abcam), neurogenin3 (goat polyclonal for the N terminus of Ngn3, Santa Cruz; Figures 4 and 5), Beta Cell Biology Consortium (BCBC) (mouse BCBC F25A1B3; Figure S2A), EGFP (rabbit polyclonal, Molecular Probes, Invitrogen), glucagon (guinea pig polyclonal, Millipore), Ki67 (rabbit polyclonal, Abcam), Nanog (rabbit polyclonal, Abcam), Oct4 (rabbit polyclonal, Stemgent), and Nestin (mouse monoclonal, Abcam). Detection was performed by using secondary antibody conjugated with Alexa 488 (green) or Alexa 594 (red) fluorescent dyes (Molecular Probes) (Remedi et al., 2009).

Quantitative RT-PCR Analysis

Islets were isolated 30 days after tamoxifen injection and 70 days after tamoxifen (40 days after insulin pellet implantation) for K_{ATP}-GOF-untreated and insulin-treated mice, respectively, and immediately processed for RNA isolation. Cellular RNA was isolated using the RNeasy Mini Kit (QIAGEN), and DNA was removed using DNase1 RNase-Free solution (QIAGEN). cDNA was prepared from RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and quantitative RT-PCR was performed using the One-Step system and Taq-Man primers (Applied Byosystems). The experimental data were normalized using two reference genes: β-actin for high abundant genes and β-actin and TATA binding protein for low abundant genes. mRNA changes were calculated by the comparative ΔCt method.

Statistics

Data are presented as means ± SEM. Differences between two groups were tested using t test and differences among several groups were tested using an ANOVA and post hoc Duncan's test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.03.010.

AUTHOR CONTRIBUTIONS

M.S.R. and C.G.N. designed the study. Z.W., N.Y., and M.S.R. carried out the experiments. M.S.R. and C.G.N. wrote the article.

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