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Research Article

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Recovery from diabetes in mice by β cell regeneration

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Introduction

Our understanding of the determinants of tissue mass during adult life is still rudimentary. Insights into this problem may suggest novel approaches for the treatment of neoplastic as well as degenerative diseases. In the case of the pancreas, elucidating the mechanisms that govern β cell mass will be important for the design of regenerative therapy for both type 1 and type 2 diabetes, diseases characterized by an insufficient mass of β cells (1). It is clear that β cell mass increases during pregnancy (2–4) and in insulin-resistant states (5–8), but evidence on the ability of β cells to regenerate from a severe, diabetogenic injury is conflicting.

Whereas autoimmune diabetes is normally irreversible, recent evidence from both humans (9–12) and rodents (13–18) suggests that β cell function (i.e., insulin production and the maintenance of glucose homeostasis) can partly recover if autoimmunity is blocked. However, most studies have not directly assessed the regeneration of β cell mass (as opposed to recovery of dysfunctional cells). Another outstanding issue is the cellular origins of β cells in adults (19, 20). We previously employed genetic lineage tracing in mice to demonstrate that proliferation of differentiated β cells, rather than differentiation of stem cells, is the major mechanism for maintenance of adult β cell mass (21). That study did not eliminate the possibility that under certain conditions, yet to be identified, facultative stem cells might exist and give rise to new β cells (19, 20). In any case, the mechanisms that sense β cell mass and operate to achieve homeostasis are largely unknown (22). Here we describe a transgenic mouse system for the specific and conditional ablation of β cells that allows us to address some of these issues without the complications of an ongoing autoimmune attack. We found that mice spontaneously recovered from diabetes by β cell regeneration, mediated by the enhanced proliferation of surviving β cells. The standard immune suppression drugs Sirolimus and Tacrolimus abolished β cell regeneration, indicating that regeneration-compatible immune suppression drugs will be required for successful regenerative therapy for type 1 diabetes.

Results

Conditional ablation of pancreatic β cells. We reasoned that a useful model to study β cell regeneration would permit: (a) specific killing of β cells; (b) temporal control over the onset of cell killing; (c) absolute ability to terminate injury; and (d) a binary mode of β cell injury (e.g., β cells will be either killed or unharmed). Using such a system, one should be able to eliminate a significant fraction of β cells at a desired time and then characterize regeneration in the absence of the confounding effects of autoimmunity, accompanying damage to other cell types, or recovery of dysfunctional β cells. Our strategy was based on doxycycline-induced expression of diphtheria toxin in β cells. We combined a transgenic mouse strain that expresses the reverse tetracycline-dependent transactivator in β cells (Insulin-rtTA; in which rtTA expression is driven by 9.5 kb of the 5′ flanking region of the rat insulin II gene) (23, 24) with a strain that expresses the diphtheria toxin A (DTA) subunit under a rtTA-responsive promoter (TET-DTA) (25) (Figure 1A). In the presence of doxycycline, rtTA induces the expression of DTA, causing β cell apoptosis. Because a single molecule of DTA suffices to kill a cell (26), sublethal β cell damage was not expected. Control experiments showed that in the absence of doxycycline, double-transgenic Insulin-rtTA;TET-DTA mice were indistinguishable from wild-type mice in terms of glucose homeostasis as well as islet histology (Figure 1, B–D). As early as 48 hours after the administration of doxycycline to adult double-transgenic mice, widespread apoptosis restricted to β cells was observed. No apoptosis was seen in islets of cotreated single-transgenic littermates (Figure 1, E and F, and Supplemental Figure 1; supplemental material available online

Nonstandard abbreviations used: DTA, diphtheria toxin A; HPAP, human placental alkaline phosphatase; SirTac, Sirolimus and Tacrolimus.

Conflict of interest: The authors have declared that no conflict of interest exists.

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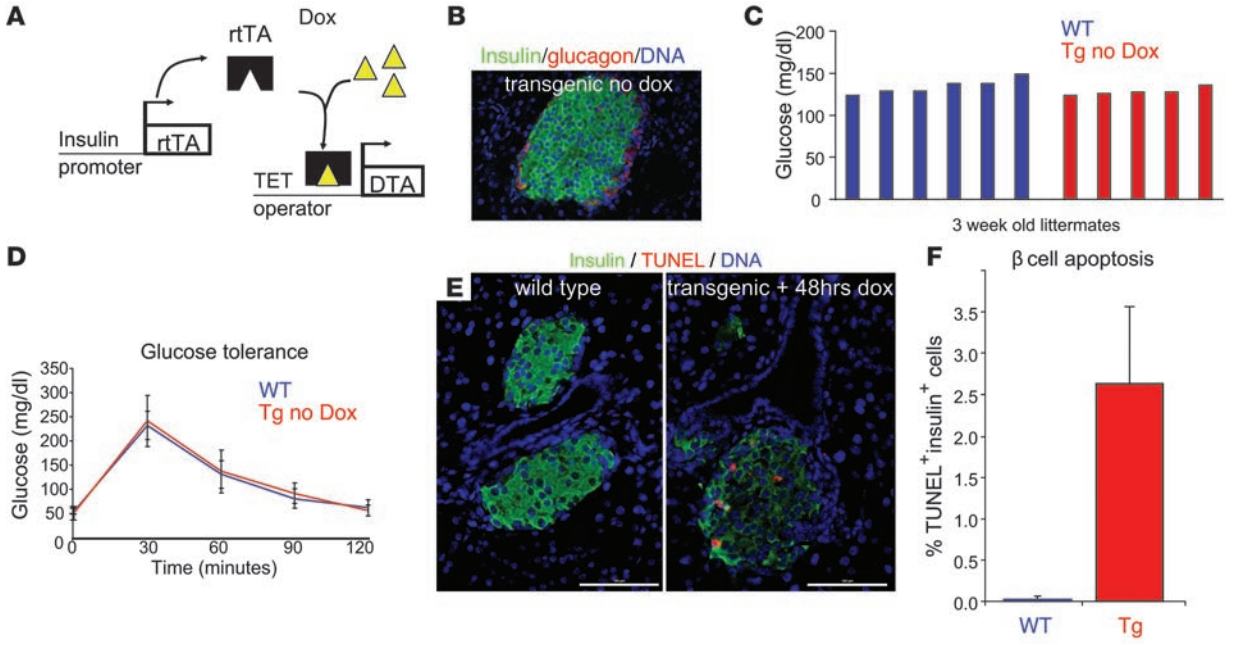

Figure 1

A transgenic mouse system for specific and conditional ablation of pancreatic β cells. (**A**) Application of doxycycline (Dox) to the drinking water of Insulin-rtTA;TET-DTA mice induces the expression of DTA specifically in β cells, causing apoptotic β cell death. (**B**) Normal islet morphology in a 4-week-old double-transgenic mouse untreated with doxycycline. (**C**) Normal blood glucose levels (measured during the day) in 3-week-old mice in the absence of doxycycline. Untreated transgenic mice at 1.5 years had blood glucose levels indistinguishable from those of age-matched wild-type mice (not shown). (**D**) In the absence of doxycycline, 4-week-old double-transgenic mice had normal fasting blood glucose levels and glucose tolerance. Values are mean ± SD (n = 5–7). Single-transgenic TET-DTA littermates are referred to as wild-type. (**E**) β Cell specificity of ablation. Four-week-old mice were treated with doxycycline for 48 hours, then sacrificed and assessed for apoptotic cell death. Extensive β cell apoptosis was detected by TUNEL staining in double-transgenic mice. No apoptosis was seen in non-β cells in islets or in the exocrine pancreas. Doxycycline-treated single-transgenic littermates showed no sign of apoptosis. (**F**) Quantification of β cell apoptosis detected 48 hours after the administration of doxycycline to 4-week-old mice. Values are mean ± SD from 5-week-old mice exposed to doxycycline for 48 hours before sacrifice (n = 3 per group). For each mouse, about 2,000 β cells were counted. Scale bars: 100 μm.

with this article; doi:10.1172/JCI32959DS1). These results confirm that the ablation system was tightly controlled by doxycycline and was β cell specific.

We assessed the histological and physiological consequences of β cell killing. Four-week-old mice were treated with doxycycline for 1 week and sacrificed at 5 weeks of age. Islet architecture in such transgenic mice was severely disrupted, with non-β cells located at the core of shriveled islets, whereas the islet core was normally occupied by β cells (Figure 2, A and B). Morphometric quantification of β cell mass revealed the loss of 70%–80% of β cells compared with control littermates (Figure 2, D and F). Pancreatic insulin content was reduced by about 85% (Figure 2G), and blood glucose levels were elevated to 300–600 mg/dl, rendering the mice overtly diabetic (Figure 2H). A slight, statistically insignificant, increase in α cell mass was noted (Supplemental Figure 4E).

Spontaneous regeneration of β *cells*. Surprisingly, following withdrawal of doxycycline the mice regained normal blood glucose levels. A spontaneous normalization of islet architecture occurred in more than 90% of islets (Figure 2, A–C, and Supplemental Figure 4C), and β cell mass increased to levels comparable to those of wild-type littermates (Figure 2, D–F), as did pancreatic insulin levels (Figure 2G). The distribution of islet sizes also returned to normal (Supplemental Figure 2I). These changes accompanied a spontaneous remission of hyperglycemia, such that fed and fasting blood glucose levels normalized (Figure 2H). With very long recovery periods (more than 8 months), glucose tolerance of transgenic mice began to recover (Figure 2I). Similar results were obtained when β cells were ablated between birth and 5 weeks of age; in this case, β cell mass normalized within about 15 weeks of doxycycline withdrawal (Supplemental Figure 2). Even mice that had severe (greater than 450 mg/dl), chronic (lasting more than 4 months), or adult-onset (starting at 4 months of age) hyperglycemia spontaneously normalized their blood glucose levels and β cell mass after doxycycline withdrawal (Supplemental Figure 3 and data not shown). The average size of individual β cells did not change after regeneration, nor did peripheral insulin sensitivity (Supplemental Figure 4, B and D). These results provide direct evidence that there is a significant capacity for the regeneration of pancreatic β cells following a diabetogenic injury.

Origin of new β *cells*. We sought to identify the cellular source responsible for β cell regeneration in this model. The rate of β cell apoptosis was not reduced in recovering mice compared with controls (measured by TUNEL staining; data not shown), suggesting that regeneration results from accelerated formation, rather than extended life span, of β cells.

Staining for Ki67 and insulin revealed that the β cell proliferation rate increased 2- to 3-fold as early as 48 hours after the onset of β cell ablation, even before hyperglycemia was detected (Figure 3, A and B). Proliferation of β cells persisted at this higher level for several weeks. Similar results were obtained with BrdU staining

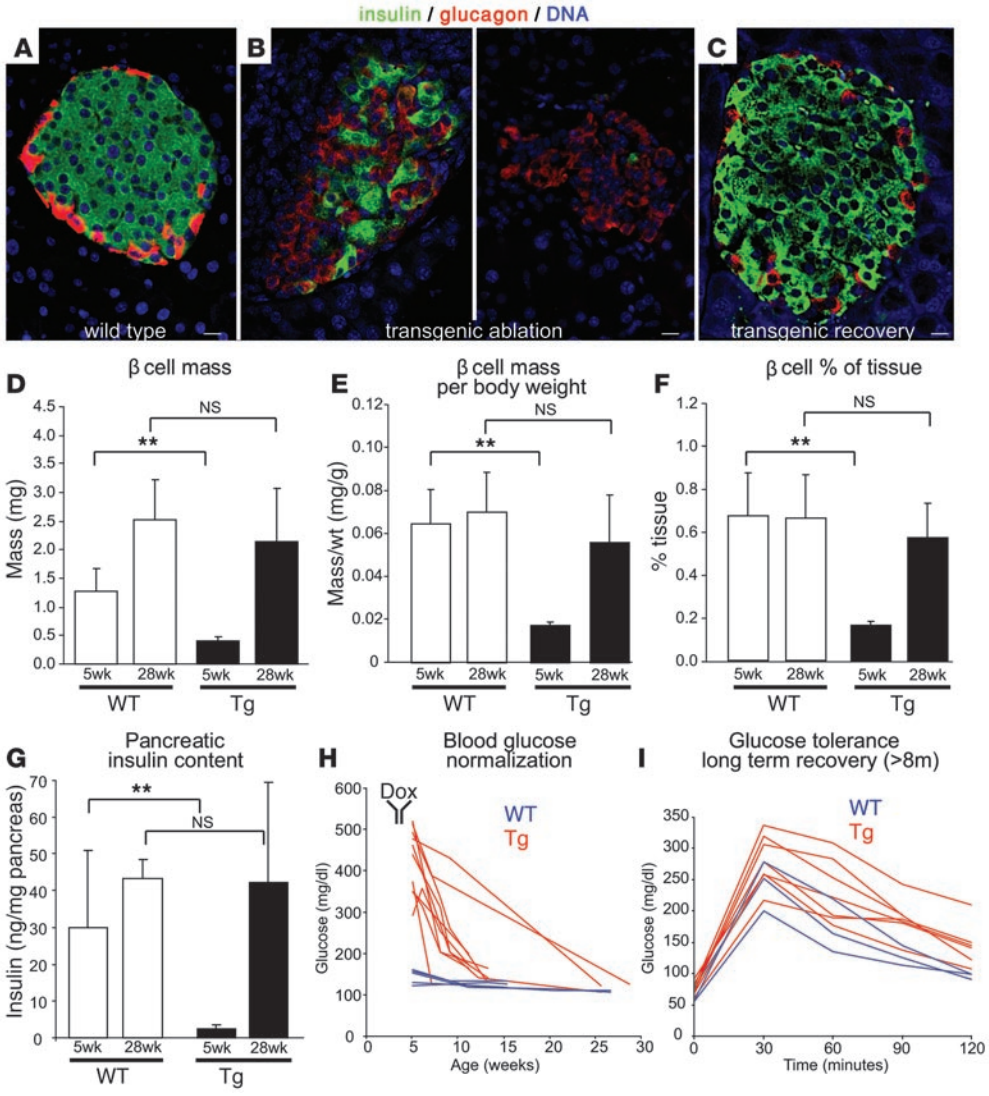

Figure 2

Ablation and regeneration of β cells. (**A**–**C**) Morphology of islets. Wild-type (**A**) and transgenic islets in the absence of doxycycline (Figure 1B) showed the typical organization of central β cells and peripheral α cells. Islets from 5-week-old transgenic mice treated with doxycycline for 7 days (i.e., from 4 weeks of age) showed a decrease in the abundance of β cells and a mixed-islet phenotype with multiple non-β cells at the center (**B**). Twenty-three weeks after the withdrawal of doxycycline (about 28 weeks of age), transgenic islets had a near-perfect architecture (**C**). Scale bars: 10 μm. (**D**–**F**) Morphometric assessment of β cell mass (**D**), β cell mass normalized to body weight (**E**), and the fraction of pancreas tissue area covered by β cells (**F**) in 5- and 28-week-old mice that received doxycycline between weeks 4 and 5 (n = 5–11 per group). Note a significant spontaneous normalization of β cell mass in transgenic mice relative to wild-type littermates undergoing the same treatment. $^{**}P < 0.01$. (**G**) Pancreatic insulin content in 5- and 28-week-old mice that received doxycycline between weeks 4 and 5 ($n > 3$ per group). Values are mean ± SD. (**H**) Fed blood glucose levels following doxycycline withdrawal in mice treated with doxycycline between 4 and 5 weeks. (**I**) Glucose tolerance following long-term (>8 months) recovery from diabetes. Some transgenic mice regained not only normal fed and fasting blood glucose levels, but also normal glucose tolerance.

in mice injected with BrdU 3 hours prior to sacrifice (data not shown). Interestingly, cells in the exocrine pancreas, but not in the main ducts, had a transient increase in proliferation rate during the peak of β cell ablation (Figure 3C and data not shown). Consistent with the transient nature of this wave of proliferation, overall pancreas weight did not change during ablation and regeneration. The global and transient proliferative response in the pancreas may be related to inflammation that develops as large numbers of dead β cells are removed. In agreement with this idea, β cell ablation led to the accumulation of F4/80$^+$ macrophages in the pancreas as early as 48 hours after the addition of doxycycline (Supplemental Figure 5).

We assayed for putative pancreatic progenitor cells during β cell regeneration. Immunostaining and RT-PCR revealed no upregulation of the embryonic endocrine progenitor cell marker neurogenin 3 (Ngn3) in regenerating pancreata, although Ngn3 expression was readily detected in the embryonic pancreas (Supplemental Figure 6). In addition, the expression pattern of the transcription factor Pdx1, known to be expressed in progenitor cells of the embryonic pancreas, did not differ between wild-type and transgenic mice (Supplemental Figure 6). We also searched for cells that coexpress insulin and other endocrine hormones, proposed by some to represent endocrine progenitor cells (27). The frequency of insulin$^+$/glucagon$^+$ cells increased from 1:5,500 β cells in wild-type mice to 1:1,000 β cells in diabetic mice (Supplemental Figure 6). If these double-hormone-positive cells are progenitor or precursor cells, as opposed to dead-end pathways (27), their numbers in this context appear too low to make a significant contribution to β cell regeneration. These results indicate that increased self-duplication of surviving β cells is the simplest explanation for β cell regeneration. Nonetheless, the relative importance of β cell proliferation versus differentiation or transdifferentiation of other cell types is difficult to discern from histological analysis and gene expression patterns. We therefore used genetic lineage tracing as a rigorous test for the cellular origins of new β cells in this model.

As described previously (21), we used Insulin-CreER™ transgenic mice, in which the insulin promoter drives the expression of tamoxifen-dependent Cre recombinase (CreER™) in β cells.

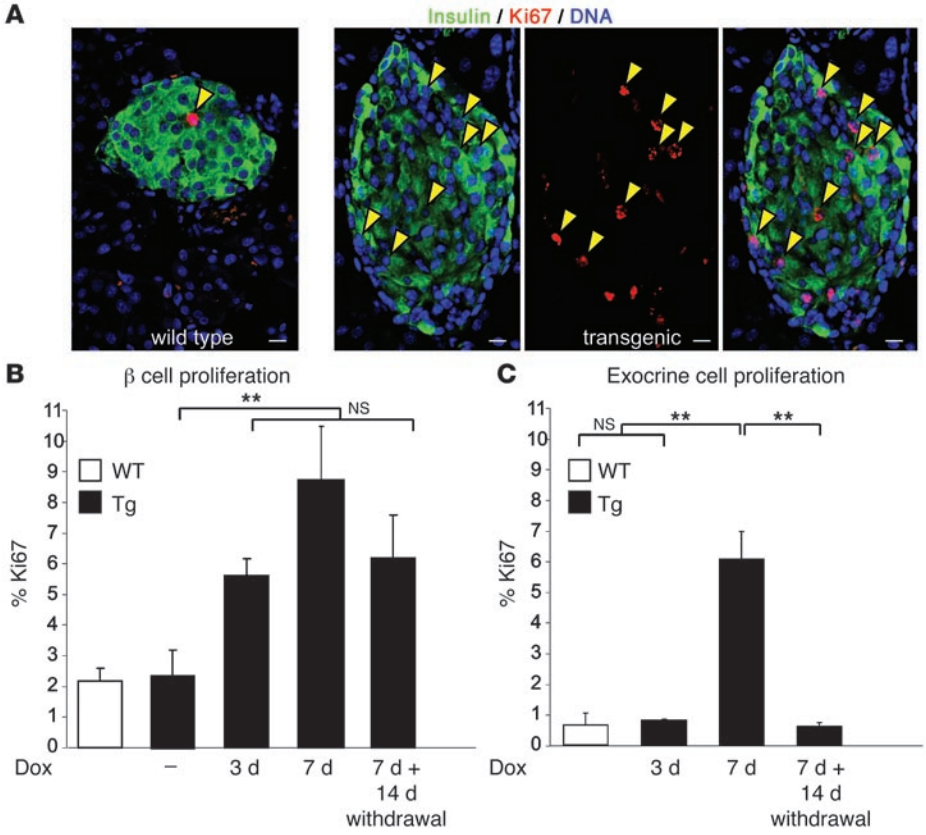

Figure 3
Cell proliferation during regeneration. (**A**) Proliferation of differentiated (insulin$^+$) β cells in 5-week-old mice treated with doxycycline between 4 and 5 weeks of age. Arrowheads denote proliferating β cells. (**B**) Proliferation of β cells following cell ablation and regeneration. Three days after the addition of doxycycline to the drinking water of 4-week-old mice, while fed and fasting glucose levels as well as glucose tolerance were still normal (not shown), the rate of β cell proliferation increased about 2.5-fold. The high rate of β cell proliferation was also seen after 7 days of doxycycline treatment and persisted 2 weeks after doxycycline withdrawal (i.e., 7-week-old mice, treated between weeks 4 and 5). (**C**) Exocrine cell proliferation during β cell ablation and regeneration. At the peak of β cell ablation (i.e., in 5-week-old mice, treated with doxycycline between weeks 4 and 5), a transient increase in exocrine cell proliferation was observed, possibly the result of an inflammatory process. Values are mean ± SD from 3 mice per time point. More than 1,000 β cells and 5,000 exocrine cells were counted per mouse. $^{**}P < 0.01$.

To allow for pulse labeling of differentiated β cells, we combined this strain with the Z/AP Cre reporter strain (21) to generate Insulin-CreER^{TM};Z/AP mice. The injection of tamoxifen into such mice leads to the transient activation of Cre recombinase exclusively in insulin-expressing cells. This results in removal of a transcriptional stop sequence from the Z/AP transgene and constitutive expression of the human placental alkaline phosphatase (HPAP) reporter gene in β cells and their progeny (Figure 4A). Thus, HPAP expression in β cells born at any time after tamoxifen injection identifies these cells as the progeny of preexisting Cre-expressing cells. We bred compound transgenic mice containing both the ablation system (Insulin-rtTA;TET-DTA) and the lineage tracing system (Insulin-CreER^{TM};Z/AP). To identify the source of new β cells during regeneration, β cells were ablated by treating newborn mice with doxycycline for 45 days, which rendered them diabetic. During the last 10 days of doxycycline treatment (age 35–45 days), surviving β cells were labeled by a series of 5 tamoxifen injections, after which doxycycline was withdrawn to allow for regeneration. During the course of regeneration, mice received BrdU in the drinking water to label new β cells, born by replication from any cellular source (Figure 4A). After 2 weeks mice were sacrificed, and the proportion of HPAP$^+$ β cells among all β cells and among the new (i.e., BrdU$^+$) β cells was determined. As shown in Figure 4B, a lower proportion of HPAP$^+$ cells among new β cells would indicate that non-β cells are contributing to β cell regeneration. Figure 4C shows a representative confocal image of an islet stained for insulin, HPAP, and BrdU. Note that only about 20% of β cells were labeled by HPAP (as a result of Cre activity), because of the inefficiency of the tamoxifen-induced Cre-mediated recombination. It should be emphasized that this recombination rate is sufficient to be informative, because for this analysis the ratio of HPAP$^+$ cells in all β cells to that in new β cells is the key parameter.

The results of the experiment are summarized in Figure 4D. In 5 independent Insulin-rtTA;TET-DTA;Insulin-CreER^{TM};Z/AP compound transgenic mice, the proportion of HPAP$^+$ cells among new β cells was similar to the overall efficiency of recombination in that mouse. Stated another way, if the recombination efficiency were 100%, nearly all new β cells would be HPAP$^+$, the progeny of surviving β cells that proliferated. The proportion of new β cells among the HPAP$^+$ population was similar to the proportion of new β cells among the general β cell population (BrdU incorporation in 21%–24% of both recombined HPAP$^+$ and unrecombined β cells). As shown in Figure 4B, this result indicates that new β cells in regenerating islets are largely, and perhaps exclusively, derived from β cells that survived ablation. There is no evidence for new β cell differentiation from a stem cell, as this would be associated with a lower proportion of HPAP expression in the BrdU$^+$ β cell population. When the experimental protocol was modified such that β cells were heritably labeled prior to the onset of β cell ablation, similar results were obtained (data not shown). We conclude that β cell proliferation is the major source of new β cells and can account for the regeneration observed in this model.

Immune suppressants abolish β cell regeneration. This β cell ablation system provides a screening platform for factors and drugs that affect β cell regeneration. The Edmonton protocol for islet transplantation greatly improved the success of human islet transplants, as a result of better islet preparation techniques and the use of the nonsteroidal immune suppressants Sirolimus (also known as rapamycin) and Tacrolimus (also known as FK-506) (28). However, most patients

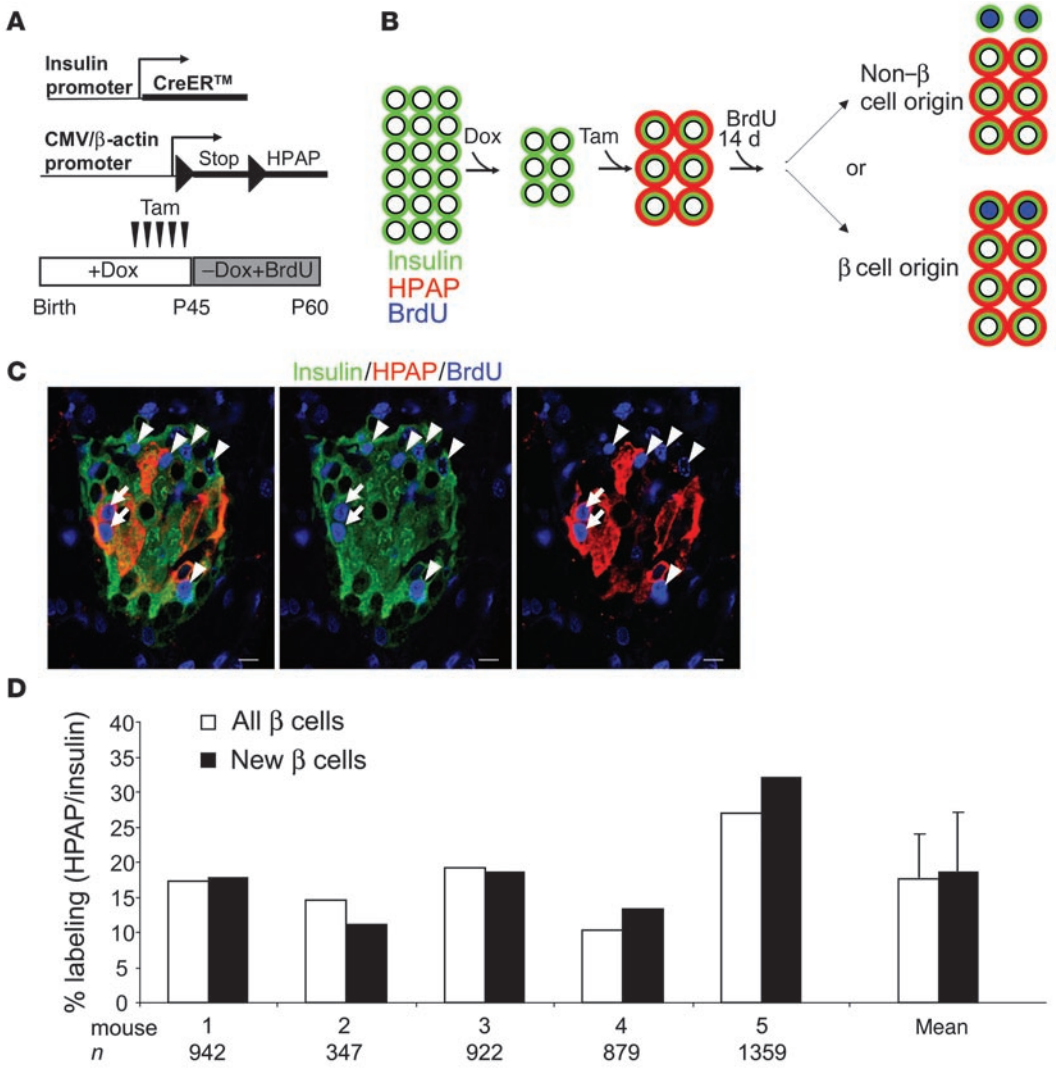

Figure 4

New β cells come predominantly from preexisting β cells. (**A**) Genetic lineage tracing system, used in conjunction with the ablation system, to determine the cellular origin of new β cells. The experimental protocol for the ablation-lineage tracing experiment is shown below. Tam, tamoxifen. (**B**) Experiment design and possible interpretations of lineage tracing results. For simplicity, the β cell recombination rate (labeling cells by HPAP expression) is presented as 100% and the rate of new β cell accumulation as 25%. (**C**) Representative confocal image of an islet from a 2-month-old Insulin-rtTA;TET-DTA;Insulin-CreER™;Z/AP transgenic mouse exposed to doxycycline to ablate β cells, injected with tamoxifen to label surviving β cells, and treated with BrdU for 2 weeks to label new cells as shown in **A**. Arrows denote HPAP$^+$BrdU$^+$ β cells, the progeny of surviving and proliferating β cells; arrowheads mark HPAP$^-$BrdU$^+$ β cells, derived from either non-β cells or nonlabeled β cells. Because the Insulin-rtTA and Insulin-CreER™ driver strains are distinct transgenes, their efficiency of β cell killing and recombination, respectively, was not expected to be identical. (**D**) Quantification of the fraction of labeled (HPAP$^+$) β cells compared with the fraction of labeled cells among newly born (BrdU$^+$) β cells in 5 mice (*n* denotes number of β cells counted per mouse). The similar percentages of labeled cells indicates that new β cells are derived primarily by proliferation of surviving β cells.

lose graft function over time, a phenomenon usually ascribed to either allo- or autoimmunity (29–31). We used our conditional ablation-regeneration system to ask whether these drugs might additionally interfere with β cell regeneration. Transgenic mice were rendered diabetic by the administration of doxycycline from birth to 1 month and were then allowed to recover in the absence or presence of clinically relevant doses of Sirolimus and Tacrolimus (SirTac; see Methods) (32, 33). Mice were sacrificed either 2 weeks after doxycycline withdrawal to observe β cell proliferation, or 2 months after doxycycline withdrawal to observe changes in blood glucose and β cell mass. In the former experiment, BrdU was added to the drinking water for 2 weeks to document the accumulation of new β cells. As shown in Figure 5, A–C, SirTac drastically reduced the β cell proliferation rate (the fraction of Ki67$^+$ β cells) as well as β cell accumulation (the fraction of BrdU$^+$ β cells) in diabetic mice and to a lesser extent in wild-type mice. The rate of β cell apoptosis did not increase following SirTac administration (data not shown), suggesting that at the doses used, the effect of these drugs on β cell number is primarily via reduced proliferation. Figure 5D shows that while wild-type islets appeared grossly normal after 2 months' treatment with

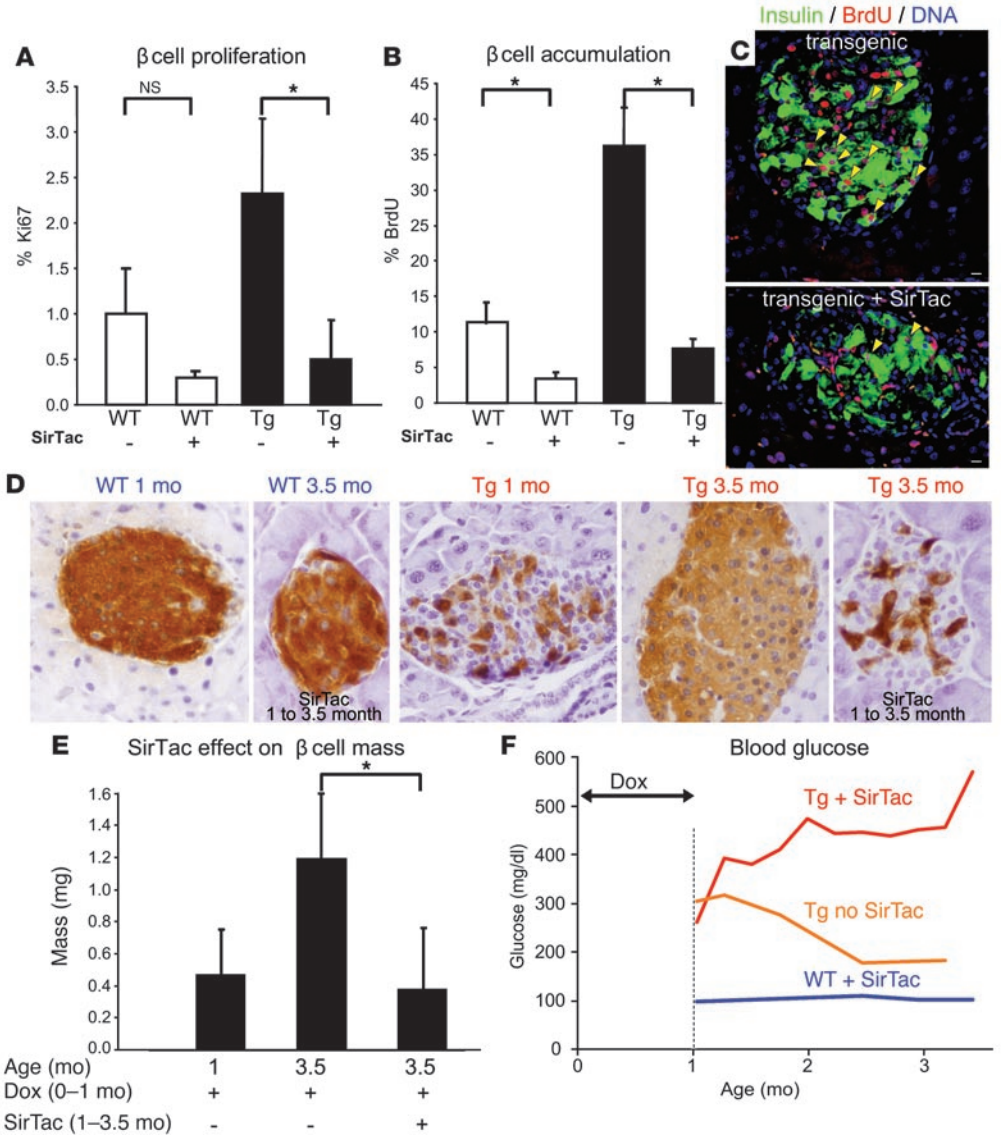

Figure 5

SirTac inhibits β cell regeneration. (**A**) Treatment with SirTac for 14 days immediately after doxycycline withdrawal caused an approximately 80% decrease in β cell proliferation in both wild-type and diabetic mice. Mice were treated with doxycycline from birth to 1 month of age, and sacrificed 2 weeks later. (**B**) Accumulation of β cells during SirTac treatment, as assessed by administering BrdU to the drinking water between doxycycline withdrawal and sacrifice, concomitant with SirTac administration as in **A**. Values are mean ± SD (n = 3–6). More than 500 β cells were counted per mouse. *P < 0.05. (**C**) Representative images of new β cell accumulation in transgenic mice recovering from diabetes in the absence or presence of SirTac. Arrowheads denote BrdU$^+$Insulin$^+$ cells. (**D**) Islet morphology at 1 and 3.5 months of age with or without SirTac treatment for months 1–3.5. Note the persistent ablated islet phenotype following SirTac treatment, in contrast to the spontaneous recovery of islet morphology in its absence. Slides were stained for insulin (brown) and hematoxylin (blue). Original magnification, ×350. (**E**) Abrogation of β cell regeneration in SirTac-treated transgenic mice. Doxycycline was administered from birth to 1 month, after which mice were sacrificed (n = 13) or allowed to recover for 2.5 months in the absence (n = 4) or presence (n = 6) of SirTac. (**F**) Blood glucose levels of mice treated as in **D** and **E**. n = 5 (SirTac-treated wild-type); 4 (untreated transgenic); 9 (SirTac-treated transgenic).

SirTac, islet architecture in diabetic mice treated with SirTac failed to normalize. Accordingly, β cell mass in SirTac-treated mice did not increase upon doxycycline withdrawal (Figure 5E). As a result, diabetic mice treated with SirTac failed to normalize blood glucose during a period in which untreated diabetic littermates recovered spontaneously (Figure 5F). Notably, at the dose and time used, wild-type mice receiving SirTac remained normoglycemic. The results indicate that this commonly used immune suppression protocol prevents β cell regeneration. This may provide a partial alternative explanation for islet graft failure in the Edmonton protocol and for posttransplantation diabetes.

Discussion

Spontaneous regeneration of pancreatic β cells. The results presented here show that pancreatic β cells have a significant capacity for spontaneous regeneration, sufficient for recovery from overt dia-

betes. This contrasts with the failure of β cell regeneration in both autoimmune and pharmacological models of diabetes, which has led to the common perception that β cells have a poor or nonexistent regenerative capacity. We suggest that in existing mouse models for, and human patients with, type 1 diabetes confounding factors mask the innate regenerative response of β cells. In autoimmune diabetes, persistent autoreactive T cells may continually eliminate regenerating β cells. Consistent with this proposition, recently reported results from an autopsy provided evidence for ongoing, albeit futile, attempts for β cell regeneration in a type 1 diabetes patient (34), as well as for β cell formation even in longtime diabetics, formerly believed to be devoid of β cells (35). In addition, protocols for blocking autoimmunity in humans with type 1 diabetes (9–11) and in NOD mice (13–18, 36) result in some degree of remission from diabetes, although most of these studies did not distinguish between an increase in β cell mass (true regeneration) and physiological recovery of dysfunctional β cells (36). Finally, diabetes induced by injection of β cell toxins, including streptozotocin and alloxan, is also largely irreversible. In these cases, we suggest that all β cells, including any that survive the toxic insult, may have suffered irreversible damage that prevents subsequent divisions needed for regeneration (37). This proposition is consistent with the conclusion that surviving β cells represent the major cellular pool responsible for regeneration. Our DTA-mediated β cell ablation system may allow for β cell regeneration as a result of the tight control over the duration of insult and the fact that surviving β cells do not suffer sublethal damage (26).

Cellular origins of new β cells. The identity of the cellular origins for new β cells has been controversial (19, 20). We previously used genetic lineage tracing to show that proliferation of differentiated β cells is the major mechanism for β cell formation during adult life in the mouse (21). More recent studies further concluded that all β cells have an equal potential for proliferation (38–41). However, it remains possible that rare facultative stem cells, yet to be identified, are activated in the pancreas following specific injury conditions (19, 20). The ablation system described here provides a particularly useful setting, in which the magnitude of β cell regeneration sufficed to cure diabetes. Several lines of evidence support the conclusion that in this model, proliferation of differentiated β cells is the predominant mechanism of regeneration. First, we observed that β cell ablation led to a rapid increase in β cell proliferation rate, preceding any change in the proliferation of non-β cells. Second, markers of embryonic-type pancreatic progenitor cells (Ngn3 and Pdx1) were not activated during the course of β cell ablation and regeneration. Third, genetic lineage tracing showed that most new β cells formed during regeneration were the progeny of differentiated β cells that survived ablation. Nevertheless, we cannot exclude the possibility of a small contribution of non-β cells to β cell regeneration in this model. It is also possible that under other settings, perhaps involving near-total ablation of β cells, stem cells or other non-β cell types can contribute to β cell regeneration. In addition, regeneration could involve the transient dedifferentiation of β cells followed by proliferation and redifferentiation (42, 43), although our observation of multiple proliferating insulin$^+$ β cells argues against this interpretation. Notably, recent lineage tracing studies of cultured β cells found little evidence for the proliferation of dedifferentiated β cells (44–47).

Determinants of β cell regeneration. The ablation system described here allows for studying signals that trigger and control β cell regeneration. The results provide some interesting hints regarding this challenging problem. First, the β cell proliferation rate increased rapidly after β cell killing, prior to any observed systemic perturbation of glucose physiology. This suggests that local processes within damaged islets play a role in β cell regeneration. We speculate that the disruption of islet architecture, that is, the apposition of β cells to blood vessels or to other endocrine cells, can trigger a regenerative response. Alternatively, dying β cells could provide a mitogenic signal to adjacent β cells, analogous to the induction of mitosis in the embryonic *Drosophila* wing by apoptotic cells (48). Such a mechanism could be acting normally to maintain the correct number of β cells independent of fluctuations in blood glucose levels. Second, the spontaneous recovery from severe hyperglycemia (often greater than 500 mg/dl for several weeks) challenges the widely held view that glucotoxicity is a major barrier for β cell proliferation in vivo and a cause for β cell failure in type 2 diabetes (49). Third, the observed recovery of adult mice (up to 4 months of age) from diabetes argues against an age-associated decline in the regenerative capacity of β cells (50). More experiments are needed to determine whether older mice retain the ability to regenerate their β cells. Fourth, the slow kinetics of β cell regeneration (several months) distinguish this mode of regeneration from other settings of mammalian solid tissue regeneration such as posthepatectomy liver regeneration (51) or postcaerulein regeneration of the exocrine pancreas (52), both of which occur in a few days. This might be related to the metabolic burden on surviving β cells or to antiproliferative mechanisms permitting the cell-cycle entry of a small proportion of β cells at a given time (40). Reducing hyperglycemia in DTA mice by the transplantation of exogenous islets could provide insights into the impact of metabolic stress and glucose physiology on β cell regeneration. Finally, the prevention of regeneration by SirTac suggests a key role for the targets of these drugs, namely mammalian target of rapamycin and calcineurin pathways, in β cell proliferation and regeneration. This is consistent with results of studies in rodents and humans that documented a negative effect of the Edmonton protocol on glucose physiology and β cell function (33, 53) and showed a role for calcineurin in β cell proliferation (54). Nonetheless, to our knowledge, the effect of these drugs on β cell regeneration after injury was not addressed before. More experiments are needed to determine the toxicity of rapamycin and FK-506 given individually and to establish whether they affect regeneration by acting directly on β cells or via a non–cell autonomous mechanism (e.g., inhibition of islet angiogenesis).

Clinical implications. Our results have a number of implications for regenerative therapy in diabetes. Most importantly, the demonstrated regenerative capacity of β cells provides an encouraging proof of concept for regenerative medicine approaches to diabetes treatment. The finding that recovery from a diabetogenic injury occurred spontaneously in both adult and neonatal mice and that it was not prevented by glucotoxicity further indicates that β cell regeneration is a robust biological response that can potentially be used therapeutically. The reliance of regeneration on proliferation of surviving β cells underscores the importance of diagnosis and intervention early in the course of autoimmune diabetes, before β cells are totally eliminated. These experiments also uncover an unanticipated toxic effect of the common nonsteroidal immunosuppressants Sirolimus and Tacrolimus on β cell regeneration. This begs the question of whether one can find other immunosuppressants that are compatible with β cell regeneration. Furthermore, the results raise the interesting possibility that, in addition to allo- and autorejection, current long-

term islet transplantation failure has an iatrogenic component of compromised β cell turnover in the graft. Altering the immune suppression component of the Edmonton protocol may significantly prolong graft survival and function.

Methods

Mice. Animal care and experiments were approved by the Institutional Animal Care and Use Committee of the Hebrew University. Transgenic mice were genotyped by PCR on ear punches using the following primers: TET-DTA (25), 5′-TTTTGACCTCCATAGAAGAC-3′ and 5′-GGCATTATCCACTTTTAGTGC-3′; Insulin-rtTA (23), 5′-TAGATGTGCTTTACTAAGTCATCGCG-3′ and 5′-GAGATCGAGCAGGCCCTCGATGGTAG-3′; Insulin-CreER™ (21), 5′-TGCCACGACCAAGTGACAGC-3′ and 5′-CCAGGTTACGGATATAGTTCATG-3′. Z/AP mice (55) were identified by performing X-gal staining on tail biopsies. Doxycycline (Dexon) was administered in the drinking water (200 μg/ml doxycycline, 2% w/v sucrose). Water was changed every 5 days. In our routine protocol, 4-week-old mice were treated with doxycycline for 7 days and then sacrificed or allowed to recover, in the absence of doxycycline, for 5–7 months. In some experiments, mice received doxycycline (via the drinking water of the mother) between birth and 4 weeks of age (Figure 5), between birth and 5 weeks of age (Supplemental Figure 2), or between birth and 45 days of age (Figure 4). Tamoxifen (Sigma-Aldrich) was dissolved in corn oil to 20 mg/ml and injected subcutaneously over 10 days in 5 doses of 8 mg each. Glucose tolerance tests were performed by injecting glucose (2 mg/kg) intraperitoneally after overnight fasting. Insulin sensitivity was determined by injecting insulin (Humalog, 0.75 U/kg; Eli Lilly). For the SirTac experiments we followed an earlier study that adapted the human protocol to mice (32). Briefly, Rapamune (LC Laboratories) was loaded at 0.3 mg/kg on day 1, followed by 0.15 mg/kg every other day until day 26, then reduced to 0.1 mg/kg for subsequent loading. Tacrolimus (Prograf; Astellas) was injected daily at 1 mg/kg. BrdU (Sigma-Aldrich) was either injected intraperitoneally (100 mg/kg) 3 hours prior to sacrifice or administered in the drinking water (0.8 mg/ml). Water was changed every 5 days.

Analysis. Paraffin sections (5 μm thick) were rehydrated, and antigen retrieval was performed using a PickCell pressure cooker. The following primary antibodies were used: guinea pig anti-insulin (1:200; DAKO), rabbit anti-glucagon (1:200; DAKO), rabbit anti-somatostatin (1:200; Zymed), rabbit anti–pancreatic polypeptide (1:200; Zymed), mouse anti-BrdU (cell proliferation kit, 1:300; Amersham), rabbit anti-ki67 (1:200; Neo markers), mouse anti–E-cadherin (1:50; BD), rabbit anti-HPAP (1:100; Zymed), guinea pig anti-Pdx1 (1:2,500; a generous gift of Chris Wright), mouse anti-Ngn3 (1:2,000; a generous gift of Ole Madsen, Beta Cell Biology Consortium), and rat anti-F4/80 (1:50; Serotec). For DNA counterstain we used either propidium iodide (2 μg/ml) or Toto3 (1:500; Molecular Probes). TUNEL staining was performed using the Roche cell death detection kit. Secondary antibodies were all from Jackson Immunoresearch Laboratories. For double staining we used only affinity-purified secondary antibodies, suitable for multiple labeling. As an alternative method to detect HPAP$^+$ cells we applied the alkaline phosphatase chromogenic substrate BCIP/NBT as previously described (21). All immunofluorescence images were captured on a Nikon C1 confocal microscope. To determine β cell mass, consecutive paraffin sections 75 μm apart spanning the entire pancreas (approximately 9 sections/pancreas) were stained for insulin and hematoxylin. Digital images of sections at a magnification of ×40 were obtained and stitched using NIS-Elements software, and the fraction of tissue covered by insulin staining was determined. The mass of β cells was calculated as the product of pancreas weight and the fraction of tissue covered by β cells. Control experiments showed that interindividual variation in β cell mass (normalized to body weight) was about 10% in C57BL/6 mice and 25% in ICR mice (Supplemental Figure 4F). Insulin levels in the pancreas and in the serum were determined using an ultrasensitive insulin ELISA kit (Chrystal Chem). The degree of islet organization was quantified by applying a MATLAB algorithm to digital fluorescent images of islets stained for insulin and glucagon. Briefly, the algorithm determines the probability that the spatial distribution of glucagon$^+$ cells within the islet is random. The more restricted α cells are to the periphery of an islet, the lower the probability that this islet is a random mix of endocrine cells. RNA was prepared from whole adult pancreata using the Qiagen RNEasy kit. Taqman analysis was used to determine the levels of Ngn3 normalized to mTBP.

Statistics. Statistical analyses were performed using 2-tailed Student's *t* test. A *P* value less than 0.05 was considered significant. Data are presented as mean ± SD.

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