Regulated β-cell regeneration in the adult mouse pancreas

David A. Cano,1* Ingrid C. Rulifson,1* Patrick W. Heiser,1* Lamorna B. Swigart,2 Stella Pelengaris,3 Mike German1, Gerard I. Evan,2 Jeffrey A. Bluestone,1 and Matthias Hebrok1

1Diabetes Center, 2Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA 94143, 3Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

*These authors contributed equally
Co-senior authors: J.A.B and M.H.

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Correspondence:
Jeffrey A. Bluestone
jbluest@diabetes.ucsf.edu
Matthias Hebrok
mhebrok@diabetes.ucsf.edu

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ABSTRACT

Objective: Several studies have shown that the adult pancreas possesses a limited potential for β–cell regeneration upon tissue injury. One of the difficulties in studying β-cell regeneration has been the lack of a robust, synchronized animal model system that would allow controlled regulation of β–cell loss and subsequent proliferation in adult pancreas.

Research Design and Methods: Here we present a transgenic mouse regeneration model in which the c–Myc transcription factor/mutant estrogen receptor (cMycERTAM) fusion protein can be specifically activated in mature β–cells. We have studied these transgenic mice by immunohistochemical and biochemical methods to assess the ablation and posterior regeneration of β-cells.

Results: Activation of the cMycERTAM fusion protein results in synchronous and selective β–cell apoptosis followed by the onset of acute diabetes. Inactivation of c-Myc leads to gradual regeneration of insulin-expressing cells and reversal of diabetes.

Conclusions: Our results demonstrate that the mature pancreas has the ability to fully recover from almost complete ablation of all existing β–cells. Our results also suggest the regeneration of β-cells is mediated by replication of β-cells rather than neogenesis from pancreatic ducts. Keywords: Islet, pancreas, regeneration, insulin
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The recent success of islet transplantation using purified islets from cadaveric donors presents new opportunities for the treatment of Type 1 Diabetes Mellitus (T1DM) (1). However, the severe shortage of donor pancreata limits the procedure to less than 0.2% of potential recipients. Developing in vitro models of β-cell expansion provide potentially powerful therapeutics for T1DM. One promising approach is to direct differentiation of pluripotent embryonic stem cells (ES) towards insulin-producing cells. While significant advances have recently been made, the generation of fully matured and functional insulin-producing cells has not been achieved as of yet (2-6).

An alternative approach would be to harness the regenerative capacity of pancreatic endocrine cells. In the adult pancreas, β-cells progressively lose their proliferative capacity. However, β-cells can enter the cell cycle during organ regeneration upon tissue injury and several recent studies have analyzed the molecular mechanisms underlying β-cell proliferation (7-10). Importantly, these studies have demonstrated increased proliferation of existing β-cells after birth rather than neogenesis from preexisting precursors. Current knowledge also indicates that β-cell regeneration occurs via increased proliferation of the remaining β-cell pool (11-13). Alternatively, β-cell regeneration may derive from latent progenitor cells residing within the ductal and islet compartment (14-16). One of the difficulties in studying β-cell regeneration has been the lack of a robust, synchronized animal model system that would allow controlled regulation of β-cell loss and subsequent proliferation in adult pancreas. A transgenic model developed by Pelengaris et al. to determine the role of c–Myc in cancer formation fulfills this role. In pINS-cMycERTAM transgenic mice the basic helix-loop-helix transcription factor c–Myc has been fused to a mutant form of the estrogen receptor (ER-TAM) (17). The cMycERTAM transgene is regulated by the insulin promoter (pINS) and its expression is specifically targeted to insulin-producing β-cells. However, c–Myc activity is suppressed in the absence of the synthetic reagent tamoxifen (TAM) and under these conditions adult pINS-cMycERTAM mice are indistinguishable from non-transgenic littermates. In contrast, activation of cMycERTAM, following administration of tamoxifen, results in β-cell apoptosis after a short burst of proliferation. Moreover, prolonged tamoxifen treatment ablates mature β-cells, resulting in acute onset of diabetes. Thus, regulated expression and activation of c–Myc in pINS-cMycERTAM mice allows for controlled temporal loss of β-cells without the general cellular toxicity caused by chemicals such as alloxan or streptozotocin. Here, we demonstrate that withdrawal of tamoxifen leads to gradual regeneration of β-cell numbers and function with progressive normalization of blood glucose levels.

RESEARCH DESIGN AND METHODS

Mice. pINS-cMycERTAM transgenic mice were maintained in a conventional pathogen-free facility at the University of California San Francisco (San Francisco, CA) according to the National Institutes of Health guidelines. The mice {originally out-bred from a (CBA x C57BL/6) F1 colony} were backcrossed 2 times to the C57BL/6 strain (Jackson Laboratories, Bar Harbor, ME). Mice were screened for the c–MycERTAM transgene by polymerase chain reaction, as previously described (17). The results are representative of pancreas tissue from 4-10 mice examined for every time point indicated in the figures.

Administration of tamoxifen (TAM). To activate cMycERTAM in the pancreatic β-cells of adult transgenic tamoxifen (TAM: Sigma-Aldrich, St. Louis, MO) was dissolved in corn
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oil (10 mg/ml) and administered intraperitoneally at 1mg/mouse/day for 6 continual days, except where noted. Non-fasting blood glucose levels were measured throughout the course of the experiments as an assessment of normal β-cell function using a Lifescan Glucometer (as per manufacturer’s specifications).

**Immunohistochemistry, Immunofluorocytochemistry and TUNEL assays.** Immunohistochemistry assays were performed on paraffin sections as described previously (18). The following primary antibodies were used: guinea pig anti-insulin 1:300; rabbit anti-glucagon 1:300 (Linco Research Inc., St. Charles, MO), (Sigma, St. Louis, MO), guinea pig anti-glucagon 1:1,000 (Linco), rabbit anti-amylin 1:1000 (Advanced Chem Tech, Louisville, KY), mouse anti-Ki67 1:100 (Novocastra, Burlingame, CA), mouse anti-Glut2 1:500 (ADI, San Antonio, TX), mouse anti-Is1 clone 39.4D5 1:25 and mouse anti-C-peptide 1:500 (O. Madsen, Hagedorn, Denmark), and Nkx6.1 1:1000 (19). Primary antibodies were detected with FITC- (1:200) and Cy3- (1:600) conjugated secondary antibodies (Jackson Immunoresearch Laboratory, West Grove, PA). The TUNEL assay was performed using the TACS*XL-Blue Label kit from Trevigen (Gaithersburg, MD). A Zeiss Axiophot2 plus fluorescent microscope was used for image acquisition.

**Histological analysis.** To measure beta cell mass, six insulin and glucagon-stained pancreas sections from each mouse (n=3), separated by at least 60µm, were imaged using a Zeiss Axiophot2 plus microscope. Pancreatic and islet areas were outlined and quantified using Open Lab software (Improvision, Lexington, MA) and are presented as a ratio.

To measure α-cells and PP-producing cells mass, six stained pancreas sections from each mouse (n=3), separated by at least 60µm, were imaged using a Zeiss Axiophot2 plus microscope. Cell number was counted manually and pancreatic area was outlined and quantified using Open Lab software (Improvision, Lexington, MA). The results are presented as the number of cells per mm² of total pancreas area.

**Glucose tolerance tests.** After a 16-18 hour fast, mice were weighed and a fasting blood glucose level was measured using the Lifescan Glucometer. Mice were then injected intraperitoneally with a 1M glucose solution at 10 µl per g body weight. Blood glucose levels were then measured every 20 minutes for 2 hours after injection. The results are shown as an average of 6 mice tested at day 0, 24, and 40, and 5 mice at day 90 of the experiment.

**Quantitative PCR analysis.** RNA isolation, cDNA preparation, and qPCR was performed as described previously (20). RNA expression of target genes was normalized based upon comparison to Cyclophilin or glucoronidase (GUS) expression. Primer sequences are included in Supplemental Table 1 (available at http://diabetes.diabetesjournals.org).

**RESULTS**

**Prolonged c-Myc activation induces hyperglycemia and β-cell apoptosis.** Activation of c-Myc induces expression of down-stream targets, resulting in either cell proliferation or cell death (21; 22). Pelengaris and colleagues demonstrated that intraperitoneal (IP) administration of tamoxifen in pINS-cMycER<sup>TAM</sup> transgenic mice resulted in a brief phase of β-cell proliferation, characterized by a rapid, ubiquitous, and synchronous entry of β-cells into cell cycle (17). However, within one day of tamoxifen treatment, β-cells die from apoptosis, indicating that in the context of the
β-cell, the pro-apoptotic activity of c-Myc is more prominent than its proliferative capacity.

Before tamoxifen administration, pINS-cMycER<sup>TAM</sup> mice display normal blood glucose levels of about 100 mg/dL and no detectable signs of apoptosis in pancreatic tissue (Fig. 1A, B). After a single dose of tamoxifen, pINS-cMycER<sup>TAM</sup> mice have a sudden episode of hypoglycemia that is most likely a result of the unregulated insulin release from apoptosing cells. However, continued administration of tamoxifen for six days results in significant apoptosis of β-cells, paralleled by extreme hyperglycemia with blood glucose levels increasing above 300 mg/dL (Fig. 1A; solid lines, and C). In addition, pINS-cMycER<sup>TAM</sup> mice suffer from other diabetes-related symptoms, including polyuria, glycosuria, and significant weight loss (data not shown). The specificity of tamoxifen for cMycER<sup>TAM</sup> activation was confirmed by the maintenance of normoglycemia in non-transgenic mice treated with tamoxifen (Fig. 1A; dotted lines). It is important to note that expression levels of cMycER<sup>TAM</sup> in the pINS-cMycER<sup>TAM</sup> transgenic mice are similar to c-Myc levels detected in normal mouse fibroblasts, indicating that c-Myc expression in β-cells remains within physiological levels (17). Thus, sustained administration of tamoxifen consistently triggers β-cell apoptosis and acute diabetes in pINS-cMycER<sup>TAM</sup> mice.

We note that the observed cell loss was specific to β-cells; representative serial sections from pINS-cMycER<sup>TAM</sup> mice that were sacrificed three days after a six-day period of continued tamoxifen administrations revealed that glucagon-, pancreatic polypeptide-, and somatostatin-positive cells were easily detected after tamoxifen treatment (data not shown). Quantification of the number of α- and PP-cells confirmed that these endocrine cell populations are not depleted upon tamoxifen treatment (Supplemental figure 1). Loss of central β-cells results in collapse of islet structures by clusters of non-β-cells that are now evenly distributed throughout the islet center rather than confined to the islet periphery (Fig. 2B). Thus, c-Myc activation under control of the insulin-promoter specifically abolishes β-cells while non-β endocrine cells are not affected.

**Regeneration of insulin<sup>+</sup> cells following targeted β-cell destruction.** To determine whether insulin<sup>+</sup> β-cells regenerate following targeted destruction, we collected pancreata from several mice at different time points for up to 90 days post tamoxifen treatment (Fig 2). Examination of pancreas tissue from untreated transgenic mice demonstrates that islet number, size and morphology were comparable to non-transgenic animals; well-rounded islets with strong insulin expression in the center and glucagon expression around the periphery were observed throughout the pancreas (Fig.2A and G). In contrast, dramatic changes in islet morphology were evident by day seven, one day post-withdrawal of tamoxifen (data not shown), when the remaining islets were involuted and significantly smaller due to c-Myc induced β-cell apoptosis. By day nine, three days post-withdrawal of tamoxifen, only small islet remnants are detectable throughout the pancreatic tissue (Fig. 2C), identified mostly by remaining glucagon<sup>+</sup> cells. One week post-withdrawal of tamoxifen, the number of insulin<sup>+</sup> cells increases throughout the tissue samples. However, islet size is still small, and organization with respect to insulin<sup>+</sup> and glucagon<sup>+</sup> cells, is atypical (Fig. 2D). In contrast, between day 40 and day 90, pINS-cMycER<sup>TAM</sup> islets regain their normal morphology in terms of size, number, and cell distribution (Fig. 2E and F). Morphometric analysis confirmed the immunohistochemical data and revealed a more than 90% reduction in β-cells at day 9 (Fig. 2G). Islet area is restored at day 90 when compared to wild type controls (Fig. 2H). However, a
comparison to age-matched, vehicle treated pINS-cMycERTAM mice shows that islet area continues to expand in these mice, possibly a consequence of increased proliferation due to low-level transgene expression even in the absence of tamoxifen. Together these results demonstrate that tamoxifen-induced c-Myc expression leads to targeted ablation of insulin-producing β–cells while inactivation of c–Myc via withdrawal of tamoxifen reveals an astounding ability for islet regeneration.

Bonner-Weir and colleagues have shown that c-Myc expression decreases insulin transcription (23). To confirm that we observe loss of β–cells rather than a transient reduction in insulin expression, we co-stained pancreatic tissue from tamoxifen-treated transgenic mice with additional β–cell markers: islet amyloid polypeptide (IAPP) and glucose transporter 2 (Glut2) (24). Untreated, adult pINS-cMycERTAM transgenic mice display typical co-expression of insulin, IAPP and Glut2 in islets throughout the pancreas (Fig. 3A and E). After two days of tamoxifen treatment, β–cell marker expression was significantly down regulated (Fig. 3B and F). By day nine, three days after tamoxifen withdrawal, almost no insulin/IAPP or insulin/Glut2 double positive cells were detected in the pancreas of pINS-cMycERTAM mice (Fig. 3C and G) while regenerated β–cells display normal expression of mature markers at day 90 (Fig. 3D and H). These findings demonstrate that c-Myc activation destroys mature β–cells and that de–activation of c–Myc results in significant regeneration of functional β–cells.

**Restoration of β–cell function is concomitant with β–cell regeneration.** To confirm that regeneration results in functional β–cells, insulin-positive cells were analyzed for expression of mature β–cell transcription factors (24) at day 90 (Fig. 4). Expression of all markers tested, including Isl1 (Fig. 4A and B), Nkx6.1 (Fig. 4C and D), and Pax6 (Fig. 4E and F), was comparable to untreated tissue, indicating that newly formed cells possess the proper repertoire of mature β–cell transcription factors (quantitative PCR for these markers is shown in Supplemental figure 2). In addition to morphologically assessing β–cell re-growth, we examined the physiological activity of the new β–cell population over the course of regeneration. Withdrawal of tamoxifen from pINS-cMycERTAM mice resulted in a gradual normalization in blood glucose levels (Fig. 5A). Around day 40, when few, but typical looking islets were detectable (Fig. 2E), blood glucose levels were recovering although they still were markedly above the normal level of ~100 dl/mg. In contrast, blood glucose levels of individual mice returned close to normal in the majority of mice (Fig. 5A) by day 90 when a significant number of islets were detectable (Fig. 2F).

To more vigorously test islet function, transgenic mice were challenged with a concentrated glucose solution and examined for their ability to metabolize glucose over time. By day 90, pINS-cMycERTAM mice exhibited glucose tolerance profile identical to that found in age-matched, vehicle treated pINS-cMycERTAM control mice (Fig. 5B). There was a slight decrease in the ability of the transgenic mice to regulate glucose levels when compared to age matched wild type littermates at the 30 min time point of the analysis, but no differences were observed at the end of the assay two hours after glucose injection. In contrast, by day 24, 18 days after tamoxifen withdrawal, as well day 40, 36 days after tamoxifen withdrawal, glucose tolerance in pINS-cMycERTAM mice was still severely affected, with animals remaining hyperglycemic even two hours after glucose administration (Supplemental Figure 3). Thus, tamoxifen-treated transgenic pINS-cMycERTAM mice progressively improved their response proportionally to the length of the regeneration period. To determine why glucose tolerance is still impaired at day 40, a
time point when substantial regeneration of β–cells has already occurred (Fig. 2E), we tested for a potential delay in insulin processing. The elevated levels of proinsulin, together with low levels of C-peptide, in day 40 treated mice suggests that newly formed β–cells still maturate after insulin expression is first observed (Fig. 5C, E). However, at day 90, β–cells seem to be fully functional as proinsulin levels are significantly reduced and all cells express high levels of C-peptide (Fig. 5D, F). Thus, based on measurements of blood glucose levels with and without glucose challenge, regenerating β–cells are functional and maintain normal metabolic function over time as soon as a critical mass of fully matured β–cells is reached.

Evidence for replication as the predominant mode of β–cell regeneration. The controlled onset of β–cell ablation followed by progressive restoration of glucose homeostasis in the pINS-cMycERTAM mice suggest that important insights into the process of mammalian β–cell regeneration can be gained from the study of these transgenic mice. Several hypotheses have been proposed to explain the regeneration of pancreatic β-cells, including neogenesis from progenitor cells located in ductal structures, spleen, bone marrow or islets. More recently, lineage-tracing experiments have found that pre-existing β-cells are the main source of new β-cells in adult mice (12). We performed several experiments designed to determine the origin of the regenerating β-cells in pINS-cMycERTAM mice.

The increase of insulin-expressing cells in pancreatic ducts in certain animal models of pancreas regeneration or injury has served as support for the existence of pancreatic progenitor cells in ducts. Similarly, an increase in Pdx1 expression has been observed in duct and duct-associated cells in models of pancreas regeneration (25-27). Extensive confocal microscopy analysis failed to reveal any cells displaying both insulin immunoreactivity and staining for the ductal marker lectin Dolichos biflorus agglutinin (DBA) at any of the stages analyzed in pINS-cMycERTAM mice (Fig. 6A, B). Similarly, no Pdx1 expression was found in ducts or in close association with ducts (Fig. 6C, D), suggesting little contribution of duct cells during β-cell regeneration in pINS-cMycERTAM mice.

To determine the extent of overall cell proliferation in pINS-cMycERTAM mice we performed immunohistochemical analysis for Ki-67, a proliferation marker. Foci of proliferating cells were present in islets, both in involuted and well-preserved islets, and ducts adjacent to them within 6 days after tamoxifen withdrawal (Fig. 6E, F). Active proliferating cells remained in islets at later stages during β-cell regeneration (Fig. 6G). By day 90, proliferation in islet and duct cells return to levels similar to those found in untreated pINS-cMycERTAM mice.

Summarily, these results indicate that β–cell regeneration appears to be mediated by replication of remaining β–cells rather than neogenesis from ductal cells. Formal proof of this hypothesis requires the use of lineage tracing analysis. An elegant genetic lineage tracing system has recently been developed that allows the irreversible labeling of β-cells. In these mice, the insulin promoter drives the expression of a tamoxifen-inducible form of Cre recombinase (Ins-CreER mice). Crossing these mice with a universal reporter line (Z/AP) allows the expression of a reporter gene encoding for the alkaline phosphatase protein (AP) upon tamoxifen treatment (12). We have used this genetic system to determine the cell of origin of regenerating β–cells in pINS-cMycERTAM mice. Unfortunately, we have not been able to achieve efficient β-cell labelling in triple transgenic InsCreER;Z/AP;pINS-cMycERTAM mice (data not shown), possibly due to the fact that c-Myc blocks the transcription from the insulin promoter (22; 23). In this
Regulated β-cell regeneration scenario, c-Myc induced inhibition of InsCreER;Z/AP impairs the expression of the alkaline phosphatase reporter gene in β-cells.

DISCUSSION

The initiation of in vivo β-cell regeneration to repair damaged pancreatic tissues in type 1 and type 2 diabetics remains a desirable and plausible, yet unrealized, therapeutic opportunity. Although growing evidence from numerous studies, including 90% pancreatectomy, pancreatic duct ligation or induced inflammation via ectopic expression of IFN-γ (14; 26; 28) suggests that endocrine cells can renew upon tissue damage, the full extent of β-cell regeneration has not been assessed previously. This is in part because these models rely on global pancreas injury or treatment with chemicals that also affect non-β-cells in other organs. Thus, finding a means to specifically deplete existing β-cells and to monitor subsequent β-cell regeneration in a controlled manner in vivo would be critical to elucidate a more detailed map of the latent β-cell replacement program in adult tissue.

Here we report that pINS-cMycERTAM transgenic mice offer such a model. Repeated administration of tamoxifen activates cMycERTAM and results in the synchronous ablation of β-cells in islets throughout the pancreas. As a consequence, treated animals rapidly lose their ability to maintain glucose tolerance and become diabetic. These physiological changes are accompanied by the collapse of normal islet structures, resulting in islet remnants that are outlined by non-β endocrine cell types unaffected by tamoxifen treatment. A previous report by Laybutt and colleagues supports the apoptosis-promoting role of c–Myc (23). Constitutive activation of c–Myc in mice under control of the insulin promoter results in β-cell loss and postnatal death due to hyperglycemia within three days after birth, a complication that prohibits the study of β-cell regeneration. In contrast, withdrawal of tamoxifen in pINS-cMycERTAM transgenic mice allows gradual recovery of normoglycemia through maturation of new β–cells and restoration of islet architecture, demonstrating that the mature pancreas has the ability to fully recover from almost complete ablation of all existing β–cells.

An unexpected finding is the observation that restoration of both islet architecture and normoglycemia in pINS-cMycERTAM transgenic mice treated with tamoxifen gradually improves over several months. However, prolonged increase of endocrine cell mass has also been observed in other models of regeneration. Removal of 90% of pancreas tissue results in regeneration of endocrine cells and 42% of the normal mass of β–cells are found within the pancreatic remnant eight weeks after surgery (29). While some of this regeneration might be due to rapid proliferation and differentiation of ductal cells, mitotic figures of β–cells are still significantly increased three weeks after pancreatectomy (14; 30), suggesting that regeneration occurs progressively over time. Additional support for this notion comes from another recently developed mouse model of β-cell regeneration. In these mice, the expression of diphtheria toxin, controlled by doxycycline administration, results in a 70-80% reduction in β-cells followed by severe hyperglycemia (31). Interestingly, withdrawal of doxycycline results in normalization of blood glucose levels over several months. These observations together with our results indicate that β-cell regeneration stimulated by extensive loss of existing insulin-producing cells is a relatively slow process. Furthermore, it is well known that hyperglycemia inhibits islet function and decreases expression of β–cell genes, including Pdx-1 and Glut2 (32; 33). Thus, the delayed regenerative response in pINS-cMycERTAM transgenic mice could be explained by the inhibitive effect of
hyperglycemia on the expression of genes essential for β-cell development.

Another unifying characteristic of islet regeneration is the apparent increase in general cell proliferation. Specific ablation of β-cells (27; 31) or broader pancreatic injury (14; 34) results in both β-cell and exocrine (including ductal tissue) cell proliferation. In agreement with these results, we also observed an increase in β-cell and duct cell proliferation in regenerating pINS-cMycERTAM transgenic mice.

The current gold standard for cell lineage tracing in regenerating tissues is the irreversible labeling of putative progenitor cell types. Increasing evidence points to the existing β-cell as the most likely progenitor for regenerating β-cells. We have put significant efforts in performing such cell lineage tracing experiments with existing transgenic mice that had previously been used for such purposes in pancreas. Unfortunately, the intrinsic mechanisms of β-cell destruction in our pINS-cMycERTAM mice depends on strong activation of cMyc activity that is known to effectively block the insulin promoter. By inhibiting the activity of the insulin promoter, expression of the CreER protein required for the activation of the AP-reporter is also impaired. The complete absence of AP activity is nonetheless surprising as both cMycER and CreER proteins are expressed before tamoxifen treatment and are only translocated into the nucleus upon tamoxifen binding. A possible explanation comes from the observation that even in the absence of tamoxifen treatment a small increase in β-cell proliferation is observed in pINS-cMycERTAM mice, suggesting precocious activity of the cMycER protein (data not shown). Thus, although our data support the notion that the major mechanism of β-cell regeneration involves expansion of the β-cells that have escaped apoptosis, we cannot unequivocally prove this point at the current time. Furthermore, the identity of putative β-cell progenitors has been controversial (35; 36). Several studies have pointed the existence of pancreatic progenitors in or associated with ducts (14; 28; 37). Additional pancreas cell type specific Cre lines are currently being generated by others, including duct cell specific Cre lines, and these could be used in future experiments to reveal potential contribution of these cell types to regenerating β-cells.

In the future, employing emerging technologies such as in vivo biophotonic imaging of cells expressing detectable markers, e.g. luciferase, may allow for more detailed monitoring of individual regenerating islets over time (38). Advances in high resolution electron tomography will provide detailed insights into the cellular architecture of the regenerating β-cells and islets (39; 40) while laser capture microscopy will provide the means for isolating and characterizing regenerating populations. From a clinical standpoint, the ultimate future success of in vivo β-cell regeneration will depend on unmasking the molecular mechanisms that underlie this process. The pINS-cMycERTAM transgenic mouse is a highly regulated model for β–cell death and subsequent regeneration that could provide means to isolate and fully characterize ‘early’ regenerating β-cells with the goal to understand the molecular signals that initiate and regulate β-cell expansion.

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FIGURE LEGENDS

Fig. 1. Response to c-Myc activation.  A, Blood glucose levels were measured in pINS-cMycER\textsuperscript{TAM} transgenic mice (solid lines), or non-transgenic mice (dotted line), treated with tamoxifen (1mg/mouse/day) for up to 6 days. Pancreas tissue from pINS-cMycER\textsuperscript{TAM} transgenic mice was assessed for β–cell apoptosis via TUNEL analysis before (B) and at day 4 of treatment with tamoxifen (C).

Fig. 2. Regeneration of insulin\textsuperscript{+} β cells. Pancreas tissue from pINS-cMycER\textsuperscript{TAM} transgenic mice were collected on A, day 0 (control, before tamoxifen treatment); B, day 3; C, day 9; D, day 13; E, day 40; F, day 90 and assessed by immunofluorescence staining for insulin (green) and glucagon (red). Morphometric analysis revealed a dramatic decrease in islet area in tamoxifen-treated pINS-cMycER\textsuperscript{TAM} mice compared to pINS-cMycER\textsuperscript{TAM} mice treated with vehicle (corn oil) or tamoxifen-treated wild type mice on day 9 (G, n=3). By day 90, islet area in tamoxifen-treated pINS-cMycER\textsuperscript{TAM} mice was similar to that found in wild type controls (H, n=3). Islet area is shown as islet:total pancreas area ratio. The average value is indicated as an horizontal line.

Fig. 3. Loss of mature β–cells. pINS-cMycER\textsuperscript{TAM} transgenic mice were treated with tamoxifen for up to 6 days. Pancreas tissue was collected on day 0, day 2, day 9 and day 90 and assessed by immunofluorescence staining for insulin (green) and IAPP (red, A-D), and Glut2 (red, E–H).

Fig. 4. Regenerated islets express mature β-cell-specific markers. Pancreas tissue from pINS-cMycER\textsuperscript{TAM} transgenic mice treated with vehicle (corn oil) (A, C, E) or tamoxifen (B, D, F) were harvested at day 90 and assessed by immunofluorescence staining for Isl1 (A and B), Nkx6.1 (C and D), and Pax 6 (E and F), all shown in red, and insulin (green).

Fig. 5. Restoration of glucose homeostasis and mature insulin processing during β–cell regeneration. A, Blood glucose levels of tamoxifen-treated (empty squares) and age-matched vehicle-treated (filled circles) pINS-cMycER\textsuperscript{TAM} transgenic mice following tamoxifen treatment. B, Glucose tolerance tests in tamoxifen-treated (squares) and age-matched vehicle-treated (diamonds) pINS-cMycER\textsuperscript{TAM} transgenic mice and tamoxifen-treated (triangles) and vehicle-treated (circles) wild type mice at day 90 (n=5). Pancreas tissue from pINS-cMycER\textsuperscript{TAM} transgenic mice were collected on day 40 (C and E) and day 90 (D and F) and assessed by immunofluorescence staining for insulin (green) and proinsulin (red, C and D) and c-peptide (red, E and F).

Fig. 6. Increased proliferation in duct cells in the absence of endocrine marker expression. No insulin-expressing cells were detected in ducts of untreated or regenerating pINS-cMycER\textsuperscript{TAM} transgenic mice (A, B). No increase in Pdx-1 expression was observed in ducts of pINS-cMycER\textsuperscript{TAM} transgenic mice, either untreated (C) or during regeneration (D). Proliferation was assessed by immunofluorescence staining for insulin (green) and Ki-67 (red). Increased proliferation was observed on day 13 in ducts (outlined in white), and in islets (F) compared to untreated mice (E). By day 40 some proliferating cells could still be found (G). Proliferation levels return to basal levels by day 90 (H).
FIGURE 2
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FIGURE 6