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Adult Duct-Lining Cells Can Reprogram into β-like Cells Able to Counter Repeated Cycles of Toxin-Induced Diabetes

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SUMMARY

It was recently demonstrated that embryonic glucagon-producing cells in the pancreas can regenerate and convert into insulin-producing β-like cells through the constitutive/ectopic expression of the *Pax4* gene. However, whether α cells in adult mice display the same plasticity is unknown. Similarly, the mechanisms underlying such reprogramming remain unclear. We now demonstrate that the misexpression of *Pax4* in glucagon$^+$ cells age-independently induces their conversion into β-like cells and their glucagon shortage-mediated replacement, resulting in islet hypertrophy and in an unexpected islet neogenesis. Combining several lineage-tracing approaches, we show that, upon Pax4-mediated α-to-β-like cell conversion, pancreatic duct-lining precursor cells are continuously mobilized, re-express the developmental gene *Ngn3*, and successively adopt a glucagon$^+$ and a β-like cell identity through a mechanism involving the reawakening of the epithelial-to-mesenchymal transition. Importantly, these processes can repeatedly regenerate the whole β cell mass and thereby reverse several rounds of toxin-induced diabetes, providing perspectives to design therapeutic regenerative strategies.

INTRODUCTION

The endocrine pancreas is organized into micro-organs termed islets of Langerhans comprising α, β, δ, ε, and pancreatic polypeptide (PP) cells secreting glucagon, insulin, somatostatin, ghrelin, and PP, respectively. Both types I and II diabetes conditions may ultimately result in pancreatic β cell loss and chronic hyperglycemia. Although current therapies provide a measure of control of the glycemia, treated diabetic patients still display a dramatically shortened life expectancy as compared to their healthy counterparts (Diabetes in the UK 2010: Key statistics on diabetes online, http://www.diabetes.org.uk/Documents/Reports/Diabetes_in_the_UK_2010.pdf). In this context, deciphering the mechanisms underlying β cell genesis/regeneration may uncover avenues toward alternative therapies. Thus, it was previously demonstrated that, during pancreas morphogenesis, the activation of a transcription factor network successively specifies progenitor cells toward the pancreatic, endocrine, and ultimately islet cell fates (Habener et al., 2005). Hence, Pdx1 determines the pancreatic epithelium (Ahlgren et al., 1996; Grapin-Botton et al., 2001; Jonsson et al., 1994; Offield et al., 1996), whereas Ngn3 specifies the endocrine cell lineage (Gradwohl et al., 2000; Gu et al., 2002; Johansson et al., 2007).

Subsequently, additional transcription factors were identified through their involvement in islet cell lineage allocation. Among these, *Arx* and *Pax4* are instrumental for the specification toward the α/PP and β/δ cell fates, respectively (Collombat et al., 2003, 2005, 2007; Sosa-Pineda et al., 1997). Importantly, the forced expression of *Pax4* in embryonic glucagon$^+$ cells was found to induce their neogenesis and subsequent conversion into cells displaying a β cell phenotype (Collombat et al., 2009). Interestingly, the overexpression of *Pax4* in β cells was also shown to prevent their toxin-mediated loss (Hu He et al., 2011).

Although these findings allowed us to gain insight into the regenerative potential and plasticity of embryonic/newborn glucagon$^+$ cells misexpressing *Pax4*, several issues of fundamental importance to type I diabetes remain hitherto unresolved: (1) Do adult α cells that have been subjected to environmental signaling, maturation, and aging retain the ability to be converted into β-like cells upon *Pax4* expression? (2) Could these glucagon$^+$ cells restore a functional beta-like cell mass after beta cell ablation? (3) Is the number of precursor cells limited and does it restrict β-like cell regeneration in aging mice? (4) Can β cell neogenesis be controlled? (5) What are the mechanisms underlying glucagon$^+$ cell-mediated β-like cell neogenesis? To address these pivotal questions and thereby potentially open avenues in the context of type I diabetes research, we generated double-transgenic mice allowing the inducible, ectopic, and reversible expression of *Pax4* in glucagon-producing cells. Our results provide conclusive evidence that adult α cells can also be converted into functional β-like cells at any age upon *Pax4* misexpression, and that a cycle of endocrine cell/islet neogenesis is subsequently activated. Specifically, a *Ngn3*-dependent reawakening of the epithelial-to-mesenchymal transition in this adult context promotes duct-lining precursor cells to adopt a glucagon$^+$ cell identity and, subsequently, a β-like cell phenotype, these being responsive to physiological stimuli. Importantly, such controllable regeneration processes allow for several cycles of replenishment of a complete β-like cell mass that counters toxin-induced diabetes.

RESULTS

Adult α Cells Can Be Converted into β-like Cells upon *Pax4* Misexpression

Taking advantage of the Tet-ON system (Clontech Laboratories), we generated two mouse lines using pronuclear injection of recombinant transgenes (Figure S1 available online): Glu-rtTA contained the rat *glucagon* promoter (Herrera et al., 1994) upstream of the *reverse tetracycline-dependent transactivator* and TetO-Pax4 included the *Tet operator* upstream of *Pax4* cDNA. For each, four or more founder lines were generated. To assess the specificity of the regulatory sequences and any putative leakiness of transgene expression, Glu-rtTA mice were crossed with the well-established TetO-β-gal mouse line (Hennighausen et al., 1995). Pancreata of 4-week-old Glu-rtTA::TetO-β-gal mice were assayed for *β-galactosidase* expression in glucagon$^+$ cells after 2 weeks of doxycycline (Dox) treatment (Figure S2A). Transgene expression was found specifically regulated by Glu-rtTA as β-galactosidase activity was detected in 80%–90% of glucagon$^+$ cells but not in any other cell type of the pancreas.

Next, Glu-rtTA::TetO-Pax4 animals were generated, treated with Dox at 4 weeks of age, and assayed for *Pax4* mRNA at 12 weeks of age. From here on, mice treated with doxycycline/tamoxifen for *x* months will be referred to as *x*mDox+/*x*mTam+. In all cases, untreated animals were found phenotypically similar to their wild-type (WT) counterparts, and they will be referred to as Dox$^-$/Tam$^-$ or controls. Accordingly, wild-type and Dox$^-$ Glu-rtTA::TetO-Pax4 control mice displayed similarly low levels of *Pax4* mRNA (WT not shown and Figure S2B). However, in pancreata of 2mDox+ Glu-rtTA::TetO-Pax4 mice, the content in *Pax4* transcripts was found considerably increased from 2.6- to 27-fold as compared to control mice, depending on the mouse line analyzed (and most likely on the loci of integration of the transgenes). In the following studies, the mouse line with 2.6-fold increase (line m2, Figure S2B) was used.

Bitransgenic Glu-rtTA::TetO-Pax4 mice were found to be viable, fertile, and healthy. Their basal glycemia and life span were within normal range, independent of Dox administration (Table S1). Importantly, a thorough investigation of islet size in Glu-rtTA::TetO-Pax4 mice of 1–11 months of age, treated with Dox from 10 days up to 20 months, outlined a massive increase in average islet size in all cases (Table S1; Figures 1A and 1B). This hypertrophy was associated with a massive insulin$^+$ cell hyperplasia and was found not to depend on the age at Dox administration (Table S1) but rather on its duration (Table S2). For instance, 1.5-, 7-, or 11-month-old Glu-rtTA::TetO-Pax4 mice treated for 5 months with Dox all displayed an approximate 3.5-fold increase in average islet size compared to their untreated counterparts (Table S2). As remarkable was the 2.7-fold increase in the overall islet number after only 10 days of Dox administration (Table S2). Such neo-formed islets were initially found relatively smaller in size (Figures S3A and S3B). However, unlike the aforementioned continuous expansion of islet size, the increase in islet number plateaued at approximately 2.8 times the numbers observed in controls (Table S2). Islet hypertrophy/multiplication were further demonstrated by means of optical projection tomography allowing visualization of the entire pancreatic insulin$^+$ cell content (Figures S3C–S3J; Movie S1). Both head and tail of the pancreas were thereby found to contain significantly more islets, a majority being much larger than their control counterparts. Altogether, our data indicate that the conditional misexpression of *Pax4* in α cells of any age results in a progressive islet hypertrophy as well as in the generation of islets that are mainly composed of insulin-expressing cells.

Phenotypic Analysis of Mice Conditionally Misexpressing *Pax4* in Adult α Cells

To determine whether the supplementary insulin-labeled cells displayed a β cell signature, the expression of endocrine labels was determined in pancreata of 4-, 6-, and 10-month-old Glu-rtTA::TetO-Pax4 mice treated (or not) with Dox for 2–8 months (Figure 1 displaying 6-month-old 4mDox+ pancreata; data not shown). In all cases, insulin-expressing cells uniformly expressed the bona fide β cell markers, including Pdx1 (Figures 1C–1F), Nkx6.1 (Figures 1G–1J), Pax4 (Figures 1K–1N), PC1/PC3 (Figures 1O–1R), Glut-2 (Figures 1S–1V), MafA (Figures S4A–S4D), and NeuroD1 (Figures S4E–S4H).

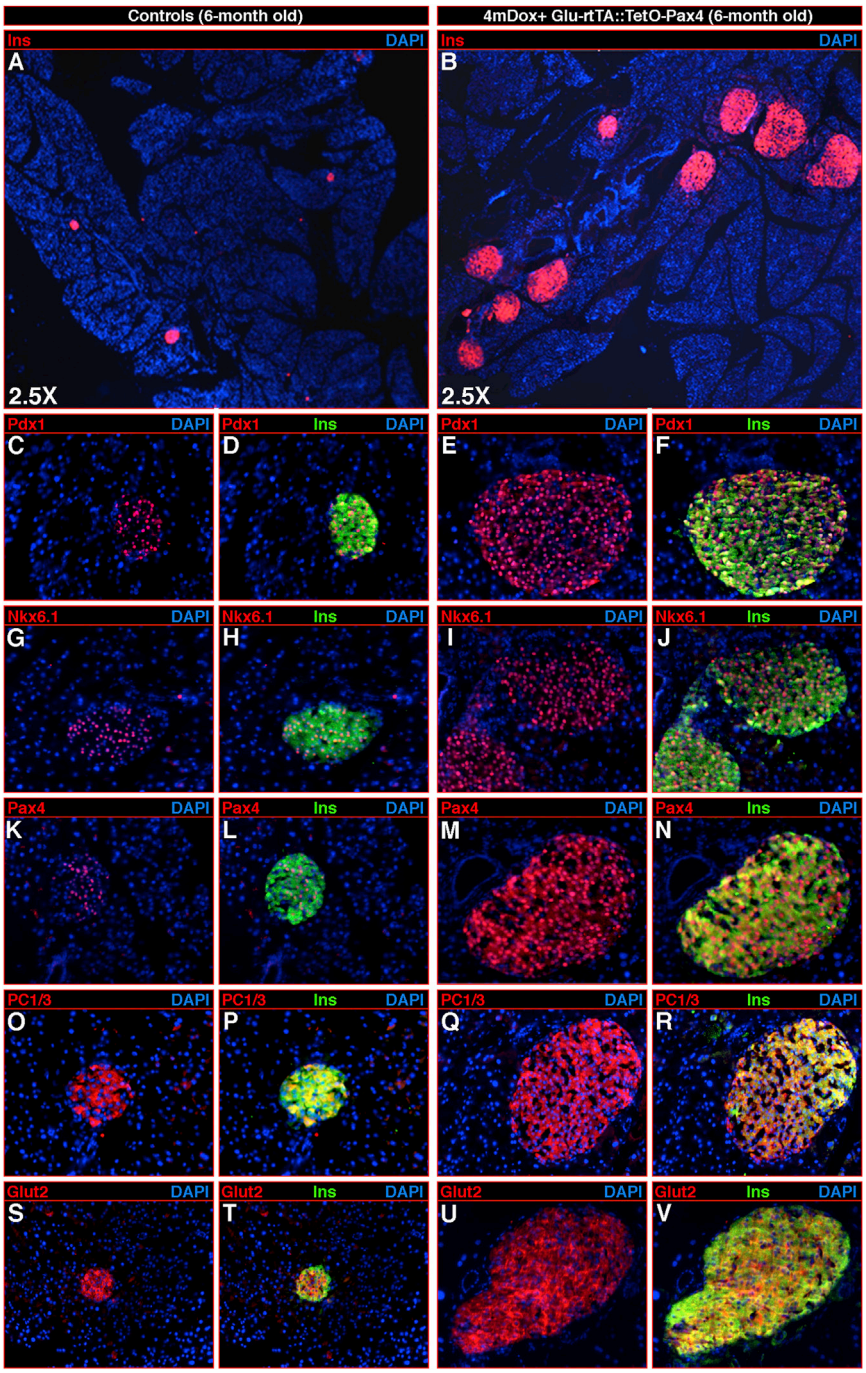


Figure 1. The Ectopic Expression of *Pax4* in Adult α Cells Induces an Insulin$^+$ β-like Cell Hyperplasia, Resulting in Hypertrophic Islets and Also in Increased Islet Numbers

Representative pictures of immunohistochemical analyses performed on pancreas sections of 4-, 6-, and 10-month-old WT/Dox− (controls, A, C and D, G and H, K and L, O and P, S and T) and on age-/sex-matched Glu-rtTA::TetO-Pax4 mice treated with Dox for 2, 4, or 8 months (Dox+ Glu-rtTA::TetO-Pax4, B, E and F, I and J, M and N, Q and R, U and V). Represented 6-month-old 4mDox+ pancreas sections outline a dramatic islet hypertrophy caused by an insulin$^+$ cell hyperplasia as well as a massive increase in islet numbers (A and B). Importantly, insulin$^+$ cells uniformly express the bona fide β cell markers Pdx1 (C–F), Nkx6.1 (G–J), Pax4 (K–N), PC1/3 (O–R), Glut2 (S–V), but also MafA, NeuroD1, and Pax6 (Figure S4).

See also Tables S1 and S2, Figures S1–S4, and Movie S1.

These cells also expressed the pan-endocrine marker, Pax6 (Figures S4I–S4L), but were predominantly negative for non-β cell determinants, such as Arx, Brn4, glucagon, somatostatin, and PP (Figures S4M–S4P; data not shown). To further ascertain the identity of the insulin-producing cells observed in Dox+ Glu-rtTA::TetO-Pax4 pancreata, electron microscopy examination was combined with insulin detection by immunogold labeling: a thorough analyses of more than 200 photographs per pancreas (n = 3) indicated that the ultrastructure of insulin-producing cells found in *Pax4*-misexpressing pancreata was comparable to that of WT β cells (Figures S3K–S3N).

In addition to the increased number of islets and insulin$^+$ cells, Dox+ Glu-rtTA::TetO-Pax4 mice also showed augmented contents of glucagon$^+$ (Figures 2A–2C and S4Q), PP$^+$ (Figures 2A, 2D, and S4T), and somatostatin$^+$ (Figures 2E, 2F, and S4S) cells (5.5, 2.7, or 14.6 times, respectively, in 7-month-old 5mDox+ Glu-rtTA::TetO-Pax4 animals as compared to their Dox$^-$ counterparts). Interestingly, these were not found uniformly distributed within the islet mantle but clustered at one pole of the islet, adjacent to neighboring ducts (Figures 2A–2F; Movie S2). Intriguingly, and solely in Dox+ Glu-rtTA::TetO-Pax4 pancreata, ducts appeared consistently surrounded by a cell-dense zone reminiscent of the mesenchyme phenotype (Figure S5 and below). Altogether, these data indicate that induced misexpression of *Pax4* in α cells of any age results in progressive β-like cell hyperplasia and islet number increase. An atypical location and augmented numbers of non-β cells are outlined near the ducts that are surrounded by a cell-dense area.

Controlling Insulin-Producing Cell Regeneration

To determine whether the misexpression of *Pax4* in adult α cells and the resulting hyperplasia of insulin$^+$ cells could be controlled, 1.5-month-old controls or Glu-rtTA::TetO-Pax4 mice were administered (or not) with Dox for 1 month, Dox treatment was subsequently continued (or not) for 3 additional months. Quantitative analyses revealed a 2.1-fold increase in

average islet size in 1mDox+ Glu-rtTA::TetO-Pax4 animals, as compared to their untreated counterparts (Figure 2G). Islet size and insulin$^+$ cell counts were found further increased in 4mDox+ mice (up to 6.3 times). Importantly, in 1mDox+ animals examined 3 months after the interruption of Dox treatment, islets did not continue to expand but they did remain enlarged as compared to controls (2.3 times), indicating that the supplementary insulin$^+$ cells generated between 1.5 and 2.5 months of age were maintained after the arrest of *Pax4* misexpression (Figure 2G). Interestingly, compared to controls, the amount of *Pax4* transcripts was 6.7-fold increased in 4mDox+ Glu-rtTA::TetO-Pax4 mice and only 2.5-fold in 1mDox+ 3mDox$^-$ mice (Figure 2G). Concerning the latter, it is important to note that *Pax4* is normally expressed in adult β cells (Figures 1K and 1L; Collombat et al., 2009; Lu et al., 2010): our results therefore confirm an arrest of *Pax4* misexpression, the 2.5-fold increase in the *Pax4* transcripts corresponding to the 2.3-fold augmentation in islet size (and thereby in Pax4$^+$ insulin$^+$ cells), as compared to controls. The ectopic expression of *Pax4* in adult glucagon$^+$ cells and the subsequent islet hypertrophy can thus be tightly controlled in this experimental model.

Mobilization of *Ngn3*-Re-expressing Duct-Lining Precursor Cells to Adopt a Glucagon$^+$ Cell Identity and, Subsequently, a β Cell Phenotype

To determine the origin of newly formed insulin$^+$ cells in Glu-rtTA::TetO-Pax4 mice, further immunohistochemical analyses and lineage-tracing experiments were undertaken. A small number of insulin$^+$/glucagon$^+$ cells were consistently observed (Figures 3A–3D), suggestive for a putative transitional stage during β-like cell neogenesis. To validate this hypothesis, Glu-rtTA::TetO-Pax4 mice were crossed with Glu-Cre::ROSA26-β-gal animals allowing permanent labeling of ~78% ± 8% of cells that had ever expressed *glucagon* (Figures S6A–S6C and S6G). Using a quantitative analysis of 4-month-old 2mDox+ Glu-rtTA::TetO-Pax4::Glu-Cre::ROSA26-β-gal pancreata, insulin$^+$ β-galactosidase$^+$ cells (formerly glucagon$^+$ cells) were found to represent ~45% ± 11% of the total insulin$^+$ cell count, that is ~74% ± 9% of the calculated supplementary insulin$^+$ cell count (Figures 3E–3I and S6D–S6G). These results suggest that adult glucagon$^+$ cells can be regenerated and converted into insulin$^+$ β-like cells upon *Pax4* induction. Indeed, given the limitation in labeling of glucagon$^+$ cells (~78% ± 8%), these data indicate that most of the supplementary insulin$^+$ cells derived from glucagon$^+$ cells (~74% ± 9% of these insulin$^+$ cells were found labeled).

In Glu-rtTA::TetO-Pax4::Glu-Cre::ROSA26-β-gal animals, glucagon$^+$ cells are labeled independently of *Pax4* misexpression. Therefore, since *Pax4* is not induced in all glucagon$^+$ cells, due to the inherent imperfection of these transgenic systems, it could have a paracrine effect on Pax4$^-$ β-galactosidase$^+$ glucagon$^+$ cells and induce their conversion into β-galactosidase$^+$ β-like cells. To exclude this possibility, Glu-rtTA::TetO-Pax4 mice were also crossed to TetO-Cre::ROSA26-β-gal animals, the resulting quadruple transgenics allowing the sole labeling of glucagon$^+$ cells having misexpressed *Pax4*. Two-month-old animals were also treated with Dox for 2 months (or 4 months), and results similar to that found in Glu-rtTA::TetO-Pax4::Glu-Cre::ROSA26-β-gal mice were obtained with a labeling of ~74%–76% of neogenerated β-like cells (Figure S6G). Thus, the proportion of β-galactosidase$^+$ β-like cells was found unchanged across models or with different induction times, indicating that Pax4 does not have a paracrine activity on Pax4$^-$ glucagon$^+$ cells.

Of further interest was the detection of very few cells simultaneously expressing endocrine hormones and the duct-specific biomarker osteopontin in islets and/or adjacent to ducts (Figures 3J–3L). To examine a putative ductal ontogeny of these cells, HNF1β-CreER mice (Solar et al., 2009), allowing the labeling and tracing of ~40% ± 8% of HNF1β$^+$ duct cells (Figures S6H–S6K), were crossed with Glu-rtTA::TetO-Pax4::ROSA26-β-gal mice. When Glu-rtTA::TetO-Pax4::HNF1β-CreER::ROSA26-β-gal mice were treated with Dox+Tam, ~42% ± 8% of duct cells showed β-galactosidase activity (Figures 3M–3U and S6H–S6K). Importantly, ~38% ± 7% of neogenerated (or newly formed) endocrine cells were found to be β-galactosidase$^+$, supporting the concept that a majority of these endocrine cells passed through a HNF1β$^+$ transitional phase. Thus, tracing of different cell lineages provide conclusive evidence that the ectopic expression of *Pax4* in adult α cells can stimulate a subset of HNF1β$^+$-duct-lining cells to adopt an endocrine cell identity and that, at least the glucagon$^+$ cells thereby generated, can subsequently be converted into β-like cells.

To gain further insight into the mechanisms underlying the insulin$^+$ cell hyperplasia observed in this model, a 10 day bromodeoxyuridine (BrdU) pulse-chase was performed. A 5.2-fold increase in BrdU-labeled cell number was thereby observed (Figures 4A–4D and S4V). Surprisingly, most BrdU$^+$ cells were found predominantly localized in the ductal lining (Figures 4B–4D). Under these conditions, the expression of the developmental proendocrine gene, *Ngn3*, was investigated. *Ngn3* has previously been found re-expressed in duct-lining cells after severe injury (Pan et al., 2013; Xu et al., 2008) or constitutive, ectopic expression of *Pax4* (Collombat et al., 2009). *Ngn3* expression in adult islet cells was previously reported by others (Wang et al., 2009), but it appeared extremely weak in our control mice (Figure S7A) due to a very low expression level. Nevertheless, in 12-month-old Dox+ Glu-rtTA::TetO-Pax4 mice, *Ngn3* expression was found robustly reactivated within the ductal lining (Figures 4D, 4G, 4H, and S7B) and, transitorily, in a subset of islet cells located adjacent to ducts (Figures 4E–4H). The contribution of *Ngn3*-re-expressing duct-lining cells to the islet hypertrophy was determined by lineage tracing in Glu-rtTA::TetO-Pax4::ROSA26-β-gal mice crossed with Ngn3-CreER animals (Gu et al., 2002). Although islet cells of Tam-treated quadruple transgenics or of Ngn3-CreER::Rosa26-β-gal controls appeared mostly negative for β-galactosidase (~2.3% of endocrine cells labeled after 2 months of Tam treatment, suggestive of scattered/low expression of *Ngn3* in adult cells; Figures 4I and 4J), ~35% ± 7% of periductal cells were found to be positive for β-galactosidase in Tam+Dox-treated animals (Figures 4I–4U). Importantly, a similar proportion, ~28% ± 7%, of neogenerated endocrine cells appeared to be β-galactosidase$^+$, with such cells expressing insulin, glucagon, or somatostatin (Figures 4Q–4T). Hence, *Pax4* misexpression in adult glucagon$^+$ cells leads to their conversion into β-like cells. Subsequently, our data indicate a transient re-expression of *Ngn3* in duct-lining cells (labeled and traced

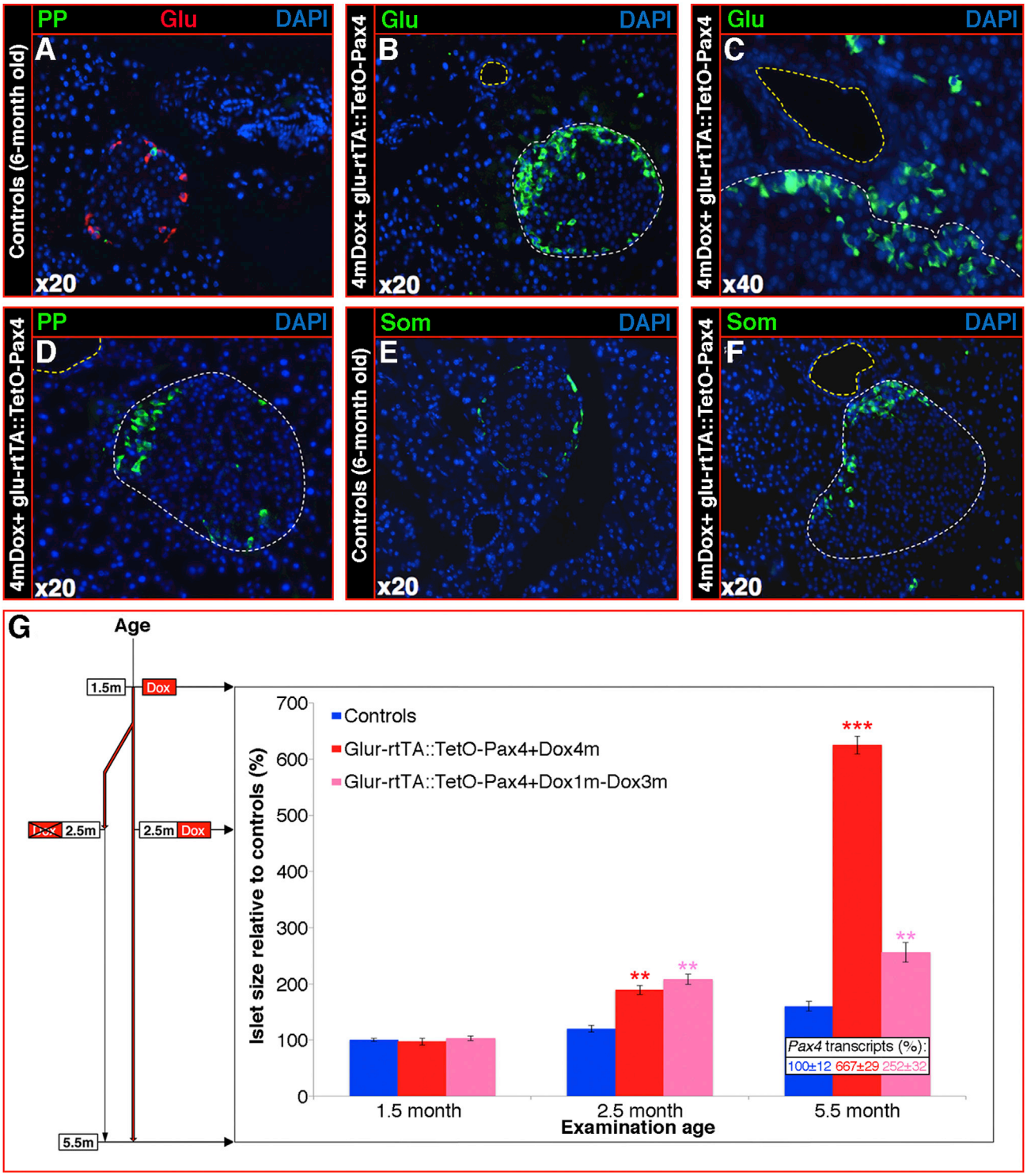


Figure 2. *Pax4* Misexpression in Adult Glucagon$^+$ Cells Can Be Controlled and Results in Islet Hypertrophy and in the Misallocation of Non-β Cells within the Islet

(A–F) Not only do 6-month-old 4mDox+ pancreata display a substantial β-like cell hyperplasia (islets outlined in white), an abnormal localization of glucagon$^+$ (A–C), PP (A and D), and δ cells (E and F) is also noted, these being mostly detected at a pole of the islet adjacent to neighboring ducts (lumen outlined in yellow) or within the ductal lining. In subsequent figures, non-β cells may not appear at a pole of the islet because ducts can be located above/below the section: only a three-dimensional examination allows proper visualization (Movie S2).

(G) To determine whether *Pax4* misexpression could be arrested, 1.5-month-old WT and Glu-rtTA::TetO-Pax4 animals were initially treated (or not) with Dox for 1 month. Subsequently, Dox administration was either discontinued or prolonged for 3 additional months. Examination and islet size quantification were

(legend continued on next page)

through *HNF1β* expression), most of these cells being, in turn, converted into endocrine cells.

Ngn3-Mediated EMT in Adult Dox+ Glu-rtTA::TetO-Pax4 Mice

To gain a deeper insight into the molecular mechanisms underlying Pax4-mediated regeneration, transcriptome analyses were performed on pancreata of Glu-rtTA::TetO-Pax4 mice that received Dox for increasing periods of time compared to age-/sex-matched Dox- controls. Interestingly, whereas the expression of the stem cell marker genes *Oct4* and *Nanog* was unaltered (Table S3), the expression of *Snail2*, *Vimentin*, *Ngn3*, and *Sox11*, transcriptional regulators of the developmental epithelial-mesenchymal transition (EMT) (Hargrave et al., 1997; Lioubinski et al., 2003; Rukstalis and Habener, 2007), was found dramatically increased (Table S3). These results were confirmed by real-time quantitative PCR (qPCR) (Table S3) and immunohistochemistry (Figure 5). Indeed, Vimentin, the canonical mesenchymal marker, was found widely re-expressed in the cell-dense clusters surrounding ducts (Figures 5A–5F, 5I, S5, S7C, and S7D). Nestin$^+$ cells were also found in the same location (Figures 5G and 5H). It is important to note that the expression of Vimentin is normally absent in this location in WT/Dox$^-$ pancreas (Figures 5E and S7C). In fact, further examination outlined a few E-cadherin$^+$ Vimentin$^+$ duct-lining cells (Figures 5A–5D); these cells most likely acquired a migratory phenotype as seen during embryonic EMT. Conversion from Vimentin$^+$ to insulin$^+$ cells was suggested by the presence of rare double-positive cells (Figure 5I). Due to technical limitations, we could not codetect β-gal$^+$ and Vimentin$^+$ cells in Glu-rtTA::TetO-Pax4::HNF1β-CreER::ROSA26-β-gal pancreata; however, numerous β-gal$^+$ cells were found within the ductal lining where most Vimentin$^+$ cells were detected (Figure 5J). Sox11 and Snail2 also appeared ectopically expressed in cells clustered around the ductal epithelium of Dox+ Glu-rtTA::TetO-Pax4 pancreata (Figures 5L, 5N, and S7F), in contrast to control mice (Figures 5K and 5M). Of note, Pan et al. (2013) recently found that, upon pancreatic duct ligation (PDL), acinar cells could be converted into duct-like cells ectopically expressing *Ptf1a*, a fraction of which being further converted into endocrine cells. Interestingly, Ptf1a was not detected in ductal cells of Glu-rtTA::TetO-Pax4 pancreata (Figures S7G and S7H), indicating that acinar cells do not seem to contribute to the neogenerated endocrine cell population in our model.

Since Ngn3 can induce the EMT during pancreas morphogenesis through the stabilization/activation of Snail2 (Gouzi et al., 2011), *Ngn3* expression was inhibited by knockdown to assess its role in EMT reinduction and islet overgrowth upon *Pax4* misexpression. Glu-rtTA::TetO-Pax4 animals were therefore injected with GFP-encoding lentiviruses containing either a small hairpin RNA (shRNA) targeting *Ngn3* or a scrambled sequence (Collombat et al., 2009; Xu et al., 2008), subsequently treated with Dox, and examined 3 weeks later. Immunohistochemical analyses using antibodies raised against GFP, CK19, and synaptophysin confirmed previously published results (Collombat et al., 2009; Xu et al., 2008) by demonstrating a targeting of 53% ± 8% of CK19$^+$ ductal cells and of 4% ± 3% of synaptophysin$^+$ endocrine cells (Figure 6C). Similarly, using qPCR, we noted a 45% ± 9% decrease in *Ngn3* transcript contents in pancreata infected with the lentiviruses containing shRNAs targeting *Ngn3,* as compared to scrambled-infected pancreata (Figure 6C). Both islet size and number were found significantly decreased in *Ngn3* knockdown pancreata compared to controls (Figures 6A–6C). Similarly, Ngn3$^+$ and Snail2$^+$ cell contents were also found diminished (Figures 6D and 6E), suggesting a causative role of Ngn3 in EMT. Interestingly, a similar prevention of islet hypertrophy was noted when Dox+ Glu-rtTA::TetO-Pax4 animals were treated for 1 month with exogenous glucagon (Figures 6F–6K), suggesting that the shortage in the glucagon hormone caused by the Pax4-mediated glucagon$^+$-to-β-like conversion promotes such β-like cell neogenesis. Thus, in animals misexpressing *Pax4* in adult glucagon$^+$ cells, our results support the notion of a glucagon shortage-dependent mobilization of duct-lining cells that re-express *Ngn3* and adopt an endocrine cell identity through processes involving the reactivation of embryonic EMT. To our knowledge, this corresponds to the first demonstration of EMT reactivation in the adult context outside of pathological conditions.

Controlled *Pax4* Misexpression in Adult Glucagon$^+$ Cells Can Induce Multiple Cycles of β Cell-Mass Regeneration

The β cell phenotype and glucose responsiveness of Dox+ Glu-rtTA::TetO-Pax4 mice were examined in vivo. In addition to a normal basal glycemia (Table S1), these animals responded better to glucose challenges performed at different ages or after various durations of Dox administration, with a lower peak in glycemia and a faster normalization (Figure 7A; data not shown). As expected, circulating insulin levels were found normal under physiological conditions but dramatically increased in challenged Dox+ mice compared to controls (Figure 7A), suggestive of an increased β-like cell mass. However, upon insulin challenge, the glycemic variations in induced Glu-rtTA::TetO-Pax4 mice remained similar to that of controls, indicating an unaltered response to insulin (data not shown).

To determine whether neogenerated insulin$^+$ cells in Dox+ Glu-rtTA::TetO-Pax4 mice were functional, 4-month-old 1mDox+ Glu-rtTA::TetO-Pax4 mice were injected with a high

performed at 1.5, 2.5, and 5.5 months of age. Although islet size appears similar in all conditions at the time of Dox induction (1.5 month of age), a significant increase is noted at 2.5 months of age solely in Dox-treated mice. Islet size is found to be further increased in Glu-rtTA::TetO-Pax4 mice treated with Dox for 4 months (in red). Importantly, such reincrease is not seen in 5.5-month-old Glu-rtTA::TetO-Pax4 mice for which Dox administration has been stopped at 2.5 months of age (in pink). An interesting observation is that there is no return to a normal size once Dox treatment is arrested, suggesting that the neo-generated β-like cells are maintained even when *Pax4* misexpression has been discontinued. The amount of *Pax4* transcripts, quantified in 5.5-month-old animals, is found to match the increase in insulin$^+$/Pax4$^+$ cell numbers: a drastic increase is noted in 4mDox+ mice (as compared to controls, $p < 0.001$), which is not seen in animals for which Dox administration was interrupted. The latter still display a slightly elevated *Pax4* transcript content reflecting the increased insulin$^+$/Pax4$^+$ cell population. $n > 3$.

All values are depicted as mean ± SEM. ***$p < 0.001$, **$p < 0.01$ using ANOVA comparison. See also Figure S5 and Movie S2.

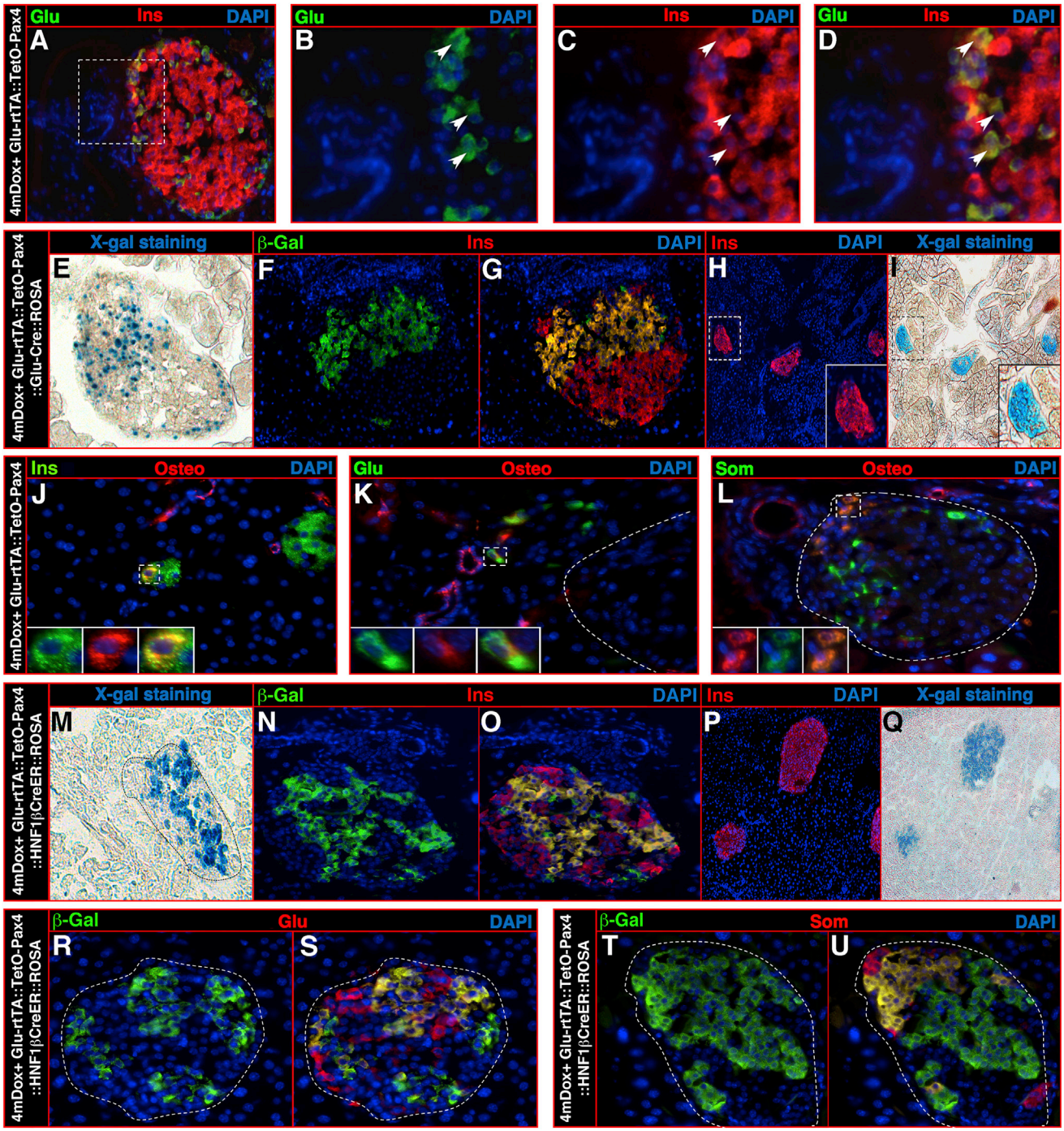
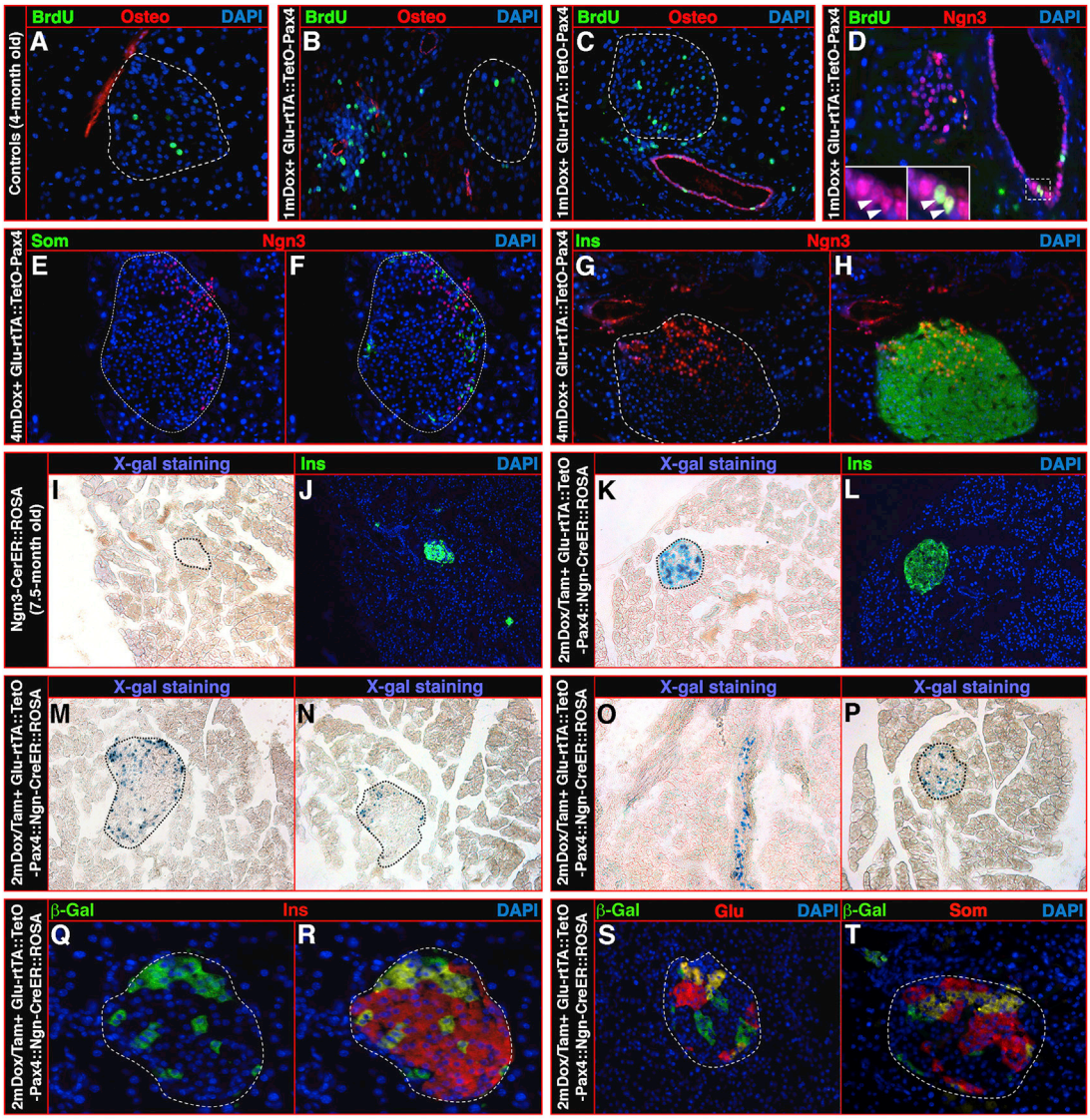


Figure 3. Upon *Pax4* Misexpression in Adult α Cells, Glucagon-Expressing Cells and HNF1β$^+$ Duct Cells Can Adopt an Insulin$^+$ Cell Identity

(A–I) Immunofluorescence of pancreata of 6-month-old 4mDox+ Glu-rtTA::TetO-Pax4 mice outlines few cells positive for both insulin and glucagon (A–D), suggesting a putative glucagon$^+$-to-β-like cell conversion. Glucagon$^+$ cell lineage tracing on 6-month-old 4mDox+ Glu-rtTA::TetO-Pax4::Glu-Cre::ROSA26-β-gal pancreata shows X-gal staining in a number of cells located within the islet core where insulin$^+$ cells are normally located (E). Immunohistochemistry reveals that most β-gal$^+$ cells are, in fact, insulin$^+$, indicating a conversion of glucagon$^+$ cells into insulin$^+$ cells (F and G). Smaller islets, most likely newly formed, are found almost uniformly positive for β-gal (H and I).

(J–U) The detection of cells expressing the ductal marker osteopontin together with insulin (J), glucagon (K), or somatostatin (L) suggests a potential involvement of duct-lining cells in endocrine cell neogenesis. Importantly, after their outcome using lineage tracing, we demonstrate the conversion of HNF1β$^+$ duct-lining cells into endocrine cells and more specifically, insulin-producing cells (M–U).

See also Figure S6.



U

Treatment	Labeled duct cells (%)	Labeled islet cells (%)	Relative endocrine cell count (%)	Labeled Supplementary endocrine cells (%)
-Tam -Dox	0.12±0.05	2.14±0.84	100±3.5	-
+Tam -Dox	0.18±0.10	2.34±0.21	102.23±5.35	-
+Tam +Dox 1m	34.01±5.61	12.33±3.02	170.12±11.33	29.91±5.19
+Tam +Dox 4m	35.80±9.01	18.02±5.12	307.10±22.89	26.72±9.02

Figure 4. Assessment of Cell Proliferation and of the Contribution of *Ngn3*-Re-expressing Cells to the Supplementary Endocrine Cell Population of Induced Glu-rtTA::TetO-Pax4 Mice

(A–H) The detection of proliferating cells in 4-month-old 1mDox+ Glu-rtTA::TetO-Pax4 animals, using a 10 day pulse of BrdU, outlines a 5.2-fold increase in BrdU-labeled cell numbers (B–D), as compared to their nontreated counterparts (A). Importantly, these are mostly found outside of the islets, in the adjacent ductal lining. Ngn3 detection reveals a reactivation of this developmental factor in the ductal lining, its expression persisting in a few insulin-labeled cells for a limited amount of time (D–H).

(I–T) Lineage tracing experiments were undertaken by following the fate of adult Ngn3$^+$ cells in 7.5-month-old 2mDox+/2mTam+ Glu-rtTA::TetO-Pax4::Ngn3-CreER::ROSA26-β-gal. β-galactosidase activity assayed using X-gal staining and immunohistochemistry is found in very few islet cells of 2mTam+ Ngn3-CreER::ROSA26-β-gal controls (I and J), but present in Dox+/Tam+ quadruple transgenic islets (K–N and P), ductal lining (O), and a number of insulin$^+$ (K and L, and Q and R), glucagon$^+$ (S), or somatostatin$^+$ (T) cells, indicating that cells re-expressing *Ngn3* eventually adopt an endocrine cell identity.

(U) In fact, quantitative analyses demonstrate a β-gal labeling of ~35% of duct cells. Importantly, ~16% of endocrine cells in induced Glu-rtTA::TetO-Pax4::Ngn3-CreER::ROSA26-β-gal pancreata also appear to be β-gal$^+$. By factoring in the islet hypertrophy, these are found to represent ~28% of supplementary endocrine cells, a percentage in line with the proportion of labeled duct cells. The indicated percentages (±SEM) correspond to quantification of Pax6$^+$ (endocrine cell quantification) or osteospontin$^+$ (duct cell assessment) cells expressing β-galactosidase using cell counts (every tenth section) on at least three animals per genotype. For X-gal staining, the counting of β-galactosidase$^+$ cells was reported to the number of islet cells assessed with DAPI staining.

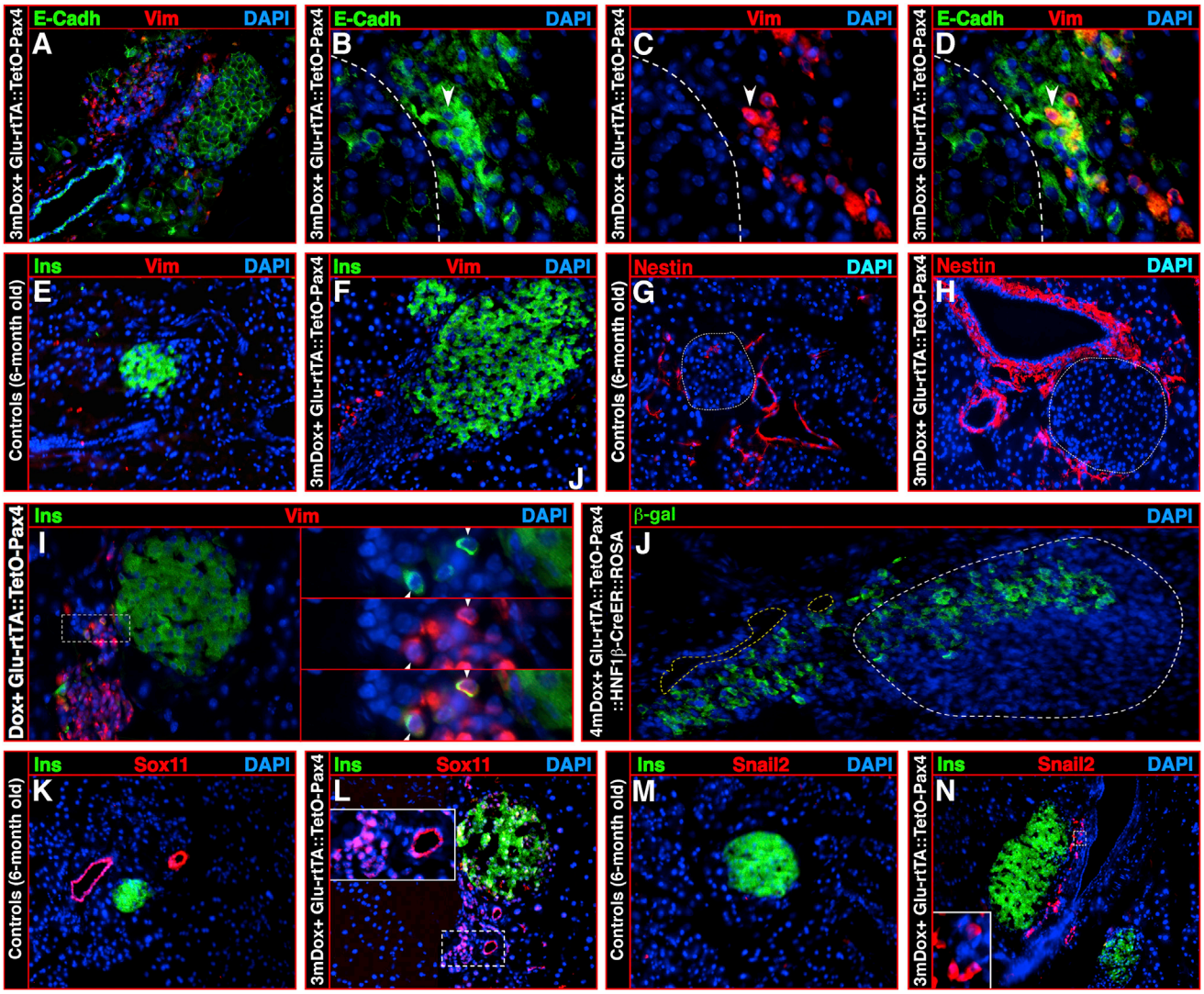


Figure 5. Reawakening of the EMT in Glu-rtTA::TetO-Pax4 Pancreata

Immunohistochemical analyses reveal that key players of the developmental EMT are re-expressed in Dox+ Glu-rtTA::TetO-Pax4 pancreata. In 6-month-old 3mDox+ animals, the mesenchymal markers Vimentin (A–F and I), Nestin (G and H), Sox11 (K and L), and Snail2 (M and N) are all found re-/ectopically expressed in the previously noted mesenchyme-like structures that surround a number of ducts (Figure S5). A coexpression of Vimentin and E-cadherin is also observed in scattered cells (A–D); similarly, a number of cells are found to be positive for both insulin and Vimentin (I): these results suggest a putative transition from an epithelial to mesenchymal phenotype and, subsequently, from a mesenchymal to an endocrine identity. Due to technical limitations, we could not perform β-gal$^+$ and Vimentin$^+$ cell codetection in Glu-rtTA::TetO-Pax4::HNF1β-CreER::ROSA26-β-gal pancreata. Despite this issue, numerous β-gal$^+$ cells were found located in the ductal lining (J) where most Vimentin$^+$ cells are observed, supporting the notion that duct-lining cells pass through a Vimentin-expressing cell stage/EMT cycle prior to the acquisition of an endocrine cell identity. Note that, due to the strength of E-cadherin labeling in ducts, the overall brightness of E-cadherin staining was digitally diminished to allow for a clearer visualization. In selected photographs, islets are outlined in white dashed lines whereas the ductal lumen is outlined in yellow dashed lines. See also Figure S7.

dose of streptozotocin (STZ) to eliminate endogenous insulin-producing cells (Mansford and Opie, 1968). Although control mice quickly became diabetic and died from massive hyperglycemia, all Dox+ Glu-rtTA::TetO-Pax4 mice displayed a short period of hyperglycemia but then steadily restored normoglycemia and survived (Figures 7B and 7C). Immunohistochemical analysis at different time points demonstrated that insulin$^+$ cells were progressively regenerated after β cell loss (Figure 7D). Of note, in pancreata of 1mDox+ Glu-rtTA::TetO-Pax4::Glu-Cre::ROSA26-β-gal animals treated with STZ and examined 2 months later, the majority of insulin$^+$ cells were found to be β-galactosidase$^+$ (Figure 7E), further documenting that numerous newly generated β-like cells were derived from glucagon-expressing cells.

The surviving mice were then maintained on Dox for 1 month, and, subsequently, Dox treatment was either discontinued or prolonged. Five weeks later, another glucose challenge showed that solely the mice that were maintained on Dox during the

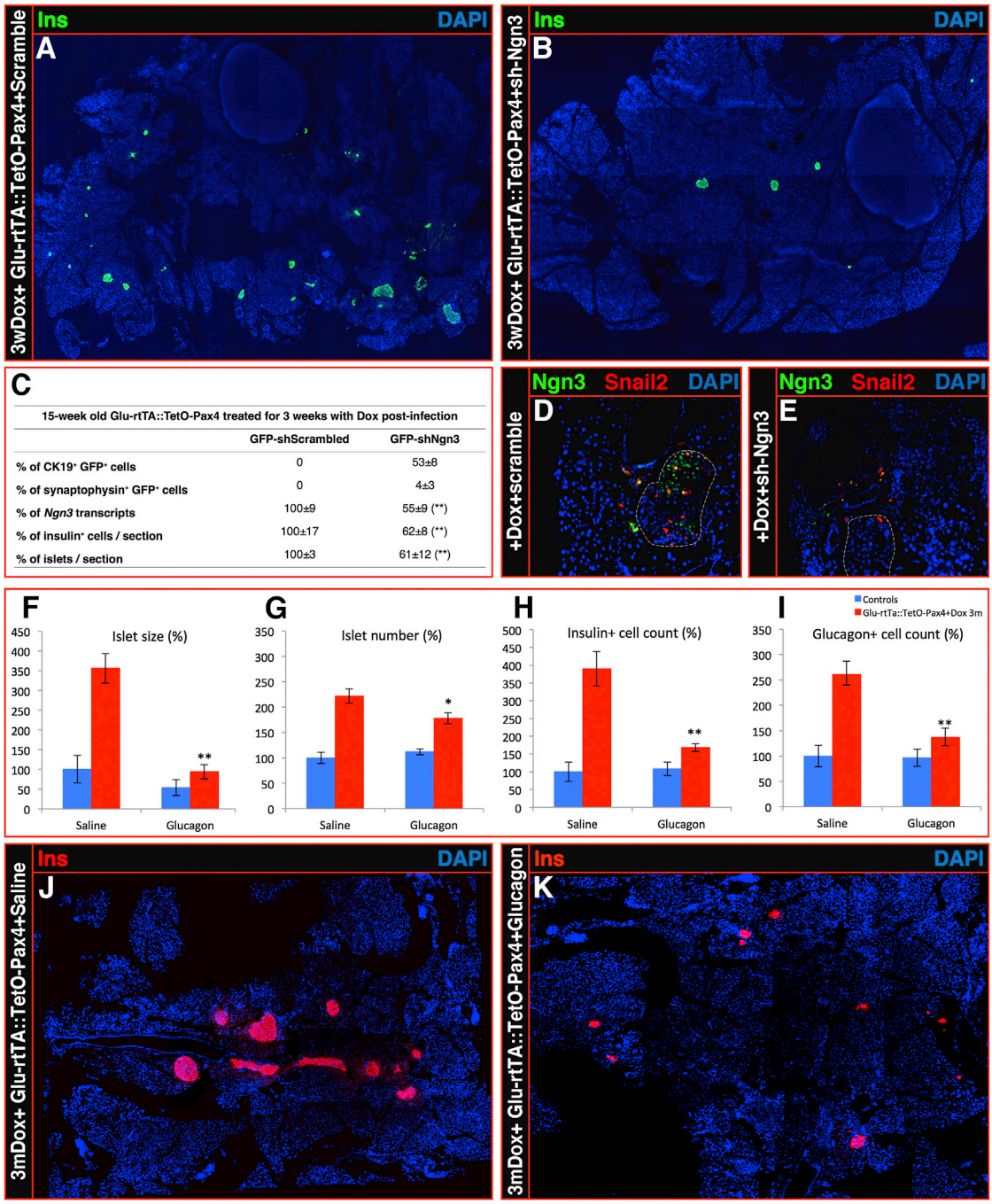


Figure 6. *Ngn3* Knockdown or Glucagon Supplementation Prevents EMT-Driven Islet Hyperplasia/Neogenesis

(A–E) Infection of 3-month-old Glu-rtTA::TetO-Pax4 animals using GFP-encoding lentiviruses producing either a shRNA targeting *Ngn3* transcripts or a scrambled shRNA. After infection, the animals were administered Dox for 3 weeks and sacrificed for examination: *Ngn3* knockdown pancreata exhibit a 45% decrease in *Ngn3* transcript content (C), but also a significant 38% diminution in insulin$^+$ cell counts and a 39% decrease in islet numbers, as compared to their scramble-infected counterparts (A–C). A drastic reduction in Snail2-re-expressing cells is also noted in knockdown animals (D–E). For a better overview, photographs in (A) and (B) correspond to a composite regrouping 64 pictures.

(F–K) Examination of 5-month-old Glu-rtTA::TetO-Pax4 animals, injected with either a saline solution or glucagon, and concomitantly treated with Dox for 3 months. Although their glycemia was found within normal ranges (data not shown), note the diminished size (F, J, and K) and number of islets (G, J, and K), as well as decreased insulin- (H, J, and K), and glucagon- (I) producing cell counts in animals supplemented with glucagon as compared to genotype-matched Dox-treated animals injected with saline. n > 3. All values are depicted as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ using ANOVA comparison.

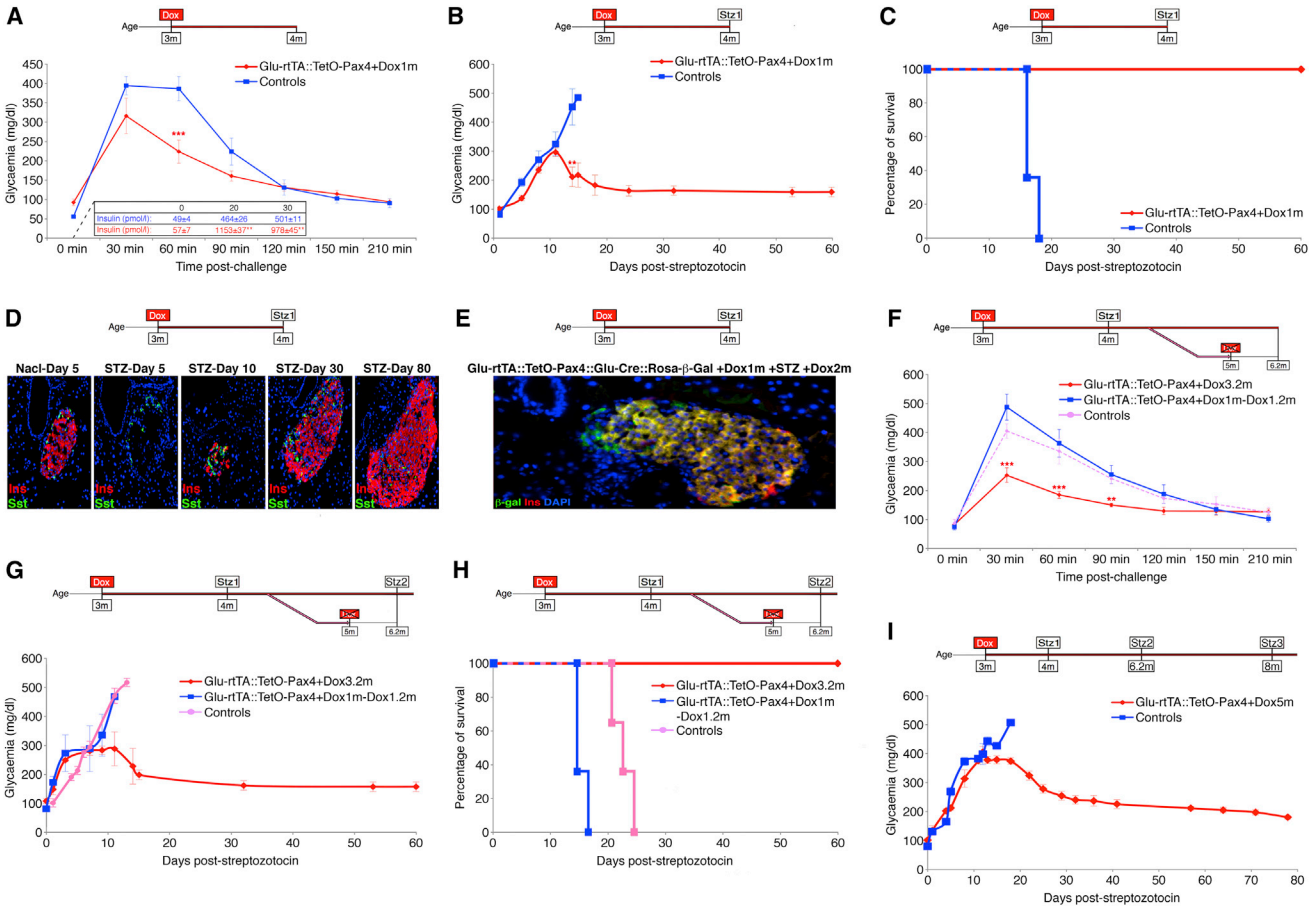


Figure 7. The Whole β Cell Mass Can Be Replenished Repeatedly in Dox-Treated Glu-rtTA::TetO-Pax4 Animals

(A) Three-month-old 1mDox Glu-rtTA::TetO-Pax4 (and controls corresponding to WT/Dox$^-$ age-/sex-matched animals) were challenged with glucose. Induced animals perform better than controls with a lower peak in glycemia and a faster return to euglycemia, suggestive of an increased β-like cell mass as outlined by circulating insulin measurements.

(B–E) One month after Dox administration, mice were subjected to streptozotocin treatment. Their glycemia (B), survival (C), and pancreatic islet composition (D) were monitored. After a peak in glycemia, a steady recovery is noted for induced mice (B), whereas controls die from massive hyperglycemia (C). Such recovery is associated with a clear regeneration of their β cell mass monitored 5, 10, 30, and 80 days poststreptozotocin administration (D). The same experiments performed in Glu-rtTA::TetO-Pax4::Glu-Cre::Rosa26-β-gal animals reveals that most regenerated β-like cells derive from cells that expressed the glucagon hormone (E).

(F–H) At 5 months of age, Dox administration was either discontinued or prolonged for the surviving animals. Four weeks later, another glucose challenge was initiated and yet again, Dox-treated animals performed better than controls (F). Interestingly, Glu-rtTA::TetO-Pax4 animals for which Dox administration has been arrested react in a similar fashion to controls. Subsequently, these "survivors" were reinjected with streptozotocin resulting in Dox-treated mice seeing another increase in their glycemia followed by a progressive return to normal ranges whereas all controls died displaying a massive hyperglycemia (G and H). Importantly, animals for which Dox treatment has been stopped also died presenting a massive increase in blood glucose levels (G and H).

(I) In addition, a third streptozotocin treatment leads to the same outcome, indicating that the β cell mass can be replaced at least three times.

For all experiments, n > 3; all values are depicted as mean ± SEM. ***p < 0.001, **p < 0.01 using ANOVA comparison (A, B, and F). (C and H) Kaplan-Meier survival curve with ***p < 0.001 by log-rank test.

entire procedure displayed an improved response (Figure 7F). When these mice were subjected to another round of STZ, again only those that were kept on Dox during the whole process survived and showed a progressive normalization of their glycemia (Figures 7G and 7H). This indicates that the newly formed β-like cells are sensitive to STZ but also that a second cycle of β-like cell regeneration can be induced (data similar to Figure 7D). In contrast, mice with discontinued Dox supply died of hyperglycemia (Figures 7G and 7H). To further test the robust capacity of this regeneration process, a third dose of STZ was administered to the surviving mice and resulted in a similar outcome (Figure 7I). The latter animals survived more than 7 months after the third injection of STZ (data not shown). These results provide evidence (1) that Pax4 is instrumental to glucagon$^+$ cell-mediated β-like cell regeneration, (2) that the newly formed β-like cells are functional and can counter chemically induced diabetes, and (3) that the functional β cell mass can be repetitively replenished.

DISCUSSION

Our study demonstrates that the misexpression of *Pax4* in adult α cells promotes their conversion into β-like cells, but

also induces a cycle of β-like cell neogenesis, resulting in an increase in islet size and number. Importantly, these processes can be regulated and depend on the duration of *Pax4* misexpression, but not on the age of induction. After the initial conversion of α cells into β-like cells, increased proliferation and ectopic expression of *Ngn3* are noted in the ductal lining, a subset of duct-lining/*Ngn3*-re-expressing cells eventually adopting a glucagon$^+$ cell identity and, subsequently, a β-like cell phenotype. Further analyses demonstrate that such endocrine cell neogenesis involves a Ngn3-mediated reactivation of the developmental EMT. Last, we provide evidence that *Pax4* misexpression in adult glucagon$^+$ cells is able to promote several cycles of replenishment of the β-like cell mass, and thereby to reverse several rounds of toxin-induced diabetes.

Pax4 Promotes Glucagon$^+$ Cell-Mediated β-like Cell Neogenesis

Our analysis indicates that, upon *Pax4* misexpression, adult α cells can be converted into cells displaying most features of true β cells independently of their age, maturation state, and microenvironment. Subsequently, the consistent detection of glucagon$^+$ cells combined to lineage-tracing experiments show a cycle of glucagon$^+$ cell neogenesis and conversion into β-like cells, resulting in a limited increase in islet number and in a progressive, continuous, and controllable β-like cell hyperplasia. Interestingly, the continuous generation of β-like cells observed in these animals suggests an inherent capability of the adult pancreas to generate glucagon-producing cells in great numbers, these being turned into β-like cells upon *Pax4* misexpression and islets reaching up to ten times the size of control islets. As important was the observation of a limited increase in the number of islets: understanding the mechanisms involved in this islet neogenesis, but also in its restriction, would be of interest for diabetes research.

Concomitantly to β-like cell neogenesis, an increase in the number of proliferating cells is noted in the ductal epithelium/lining where *Ngn3* expression is reactivated. These results are in agreement with previous reports suggesting that, under specific conditions, facultative precursor cells may be induced to re-express *Ngn3* and eventually adopt an endocrine cell phenotype (Collombat et al., 2009; Pan and Wright, 2011; Xu et al., 2008). Of interest was the recent detection of low levels of *Ngn3* expression in adult endocrine cells but not in the ductal compartment (Wang et al., 2009). Under our experimental conditions, whereas we detected very low expression levels of Ngn3 in control islets by immunohistochemistry, a robust re-expression was noted in duct-lining cells of Dox+ Glu-rtTA::TetO-Pax4 mouse pancreas. Lineage-tracing experiments indicated that a number of adult Ngn3$^+$ or HNF1β$^+$ duct-lining cells could eventually adopt a β-like cell identity. Interestingly, combining these quantitative analyses with glucagon$^+$ cell lineage tracing, our data suggest that: (1) the large proportion of labeled glucagon$^+$ cells matched the proportion of newly formed marked β-like cells, suggesting that, upon initial α-to-β-like cell conversion, glucagon$^+$ cells are regenerated and further converted into β-like cells as long as *Pax4* is misexpressed; (2) the proportion of labeled Ngn3$^+$ or HNF1β$^+$ cells also nearly matched the proportion of marked newly formed β-like cells, indicating that a majority of these β-like cells derived from *Ngn3* re-expressing or HNF1β-labeled cells. However, due to the incomplete labeling of these different cell subtypes (inherent to lineage tracing approaches), one cannot exclude that a fraction of neo-generated β-like cells could have an alternative origin. A possibility could be an acinar origin: indeed, a recent report from Pan et al. (2013) suggests that, upon PDL, Ptf1a$^+$ acinar cells could adopt a Ptf1a$^+$ HNF1β$^+$ ductal cell identity, re-express *Ngn3*, and be further converted into endocrine cells. However, in *Pax4*-misexpressing animals, *Ptf1a* was not found ectopically produced in ductal or endocrine cells, and no evidence of acinar-to-duct or acinar-to-endocrine cell conversion was noted, suggesting that two different mechanisms operate. A likely explanation could lie in the differences between the approaches used. Indeed, with PDL corresponding to a fairly drastic model of acute pancreatitis involving massive inflammation and cell death, it is therefore conceivable that additional/facultative cell-replacement mechanisms are activated as compared to *Pax4*-misexpressing animals, which are mostly characterized by a glucagon$^+$-to-β-like cell conversion. Thus, our data demonstrate that *Pax4* misexpression can single-handedly promote several cycles of regeneration of the β cell mass without requiring PDL. Additionally, these results also suggest that Ngn3$^+$ or HNF1β$^+$ cells are initially converted into glucagon$^+$ cells and subsequently into β-like cells. The key role exerted by *Ngn3* in these processes was further confirmed in knockdown experiments showing a strongly diminished β-like cell hyperplasia.

Our analyses indicate a recapitulation of the Ngn3-mediated endocrine developmental program in this specific model of *Pax4* misexpression in adult glucagon$^+$ cells. However, unlike during pancreas morphogenesis, the proportion of the generated endocrine cell subtypes is clearly in favor of the glucagon$^+$ cell lineage. One likely explanation could be the glucagon depletion provoked by the glucagon$^+$-to-β-cell conversion. Indeed, it was previously found that alterations in glucagon signaling or glucagon shortage could trigger an α-like cell neogenesis (Furuta et al., 1997; Gelling et al., 2003; Longuet et al., 2013). It is conceivable that, in the present case, a similar α-like cell hyperplasia is induced, such cells subsequently misexpressing *Pax4* and being turned into β-like cells. The decreased β-like cell hyperplasia observed after glucagon supplementation supports this notion. It should be noted that, in *Pax4*-misexpressing animals, the neo-generated glucagon$^+$ cells detected prior to their conversion into β-like cells display a mature PC1/3$^-$/GLP-1R$^-$ α-like cell phenotype (and thereby not a pro-α cell identity). However, due to the lack of well-characterized marker genes and in the absence of a more in-depth analysis, one cannot ascertain that these correspond to true α cells.

Although the glucagon$^+$ cell lineage is clearly favored in these regeneration processes, a few somatostatin$^+$ or PP$^+$ cells were also detected. However, technical limitations (lack of appropriate CreER mouse lines) prevented the tracing of their lineage. Given the systematic polarity of their distribution within islets and the fact that their number does not increase over time, it is conceivable that such cells correspond to Ngn3-derived cells (as demonstrated using lineage tracing) that have escaped the α-cell-lineage favoring. One could also postulate that such cells could also acquire a β-like cell identity. However, the definitive validation of this postulate will require the generation of proper mouse lines.

Ngn3 Re-expression Reinduces the EMT in the Adult Pancreas

The exploration of the molecular mechanisms underlying these endocrine cell regeneration processes outlined a *Ngn3*-mediated reawakening of the developmental EMT (Chiang and Melton, 2003; Cole et al., 2009; Gouzi et al., 2011; Rukstalis and Habener, 2007). Indeed, besides *Ngn3* re-expression, the ectopic expression of epithelial/mesenchymal markers, such as *Vimentin*, *Nestin*, *Snail2*, and *Sox11*, is noted in our adult mice, with the knockdown of *Ngn3* preventing their reactivation.

Of note, the acquisition of a mesenchymal, migratory phenotype implies the subsequent and opposite conversion of mesenchyme-like cells to epithelial/endocrine ones. Despite an apparently rapid transition, we did indeed observe a few cells expressing Vimentin and insulin/glucagon. Together, our results thus support the concept that precursor cells localized in the ductal lining are mobilized and re-express *Ngn3* prior to embarking on the EMT pathway leading to endocrine cell genesis and, at least, glucagon$^+$-to-β-like cell conversion (see model in Figure S7I). Whether all neo-generated islet cells pass through this cycle or whether a subset of duct-derived cells can expand prior to hormone expression or transactivate the dedifferentiation/proliferation/redifferentiation of endogenous islet cells remains to be determined.

Age-Independent Reversion of Several Rounds of Toxin-Induced Diabetes

In Dox-treated Glu-rtTA::TetO-Pax4 animals, hyperplastic insulin$^+$ cells display a β cell phenotype and are fully functional upon challenge. Interestingly, the newly formed β-like cells can repopulate the islets of STZ-treated animals and restore euglycemia. Importantly, these regenerative processes were responsive to at least two additional rounds of STZ injections. The capacity to repeatedly activate duct-to-β-like cell regeneration may therefore offer new therapeutic strategies for type 1, but also type 2, diabetes where putatively regenerated β-like cells could, with time, be killed by the immune system. Taken together, our data indicate that (1) *Pax4* misexpression is both necessary and sufficient for β-like cell neogenesis, (2) the newly formed β-like cells are functional, (3) *Pax4* expression can be regulated, (4) the complete β cell mass can be replaced at least three times, and (5) that several cycles of glucagon$^+$ cell-mediated β-like cell regeneration can be successfully triggered in an age-independent manner. Based on these findings, we suggest that strategies controlling the expression of *Pax4*, *Ngn3*, or EMT induction, or of their molecular targets/cofactors, may pave new avenues for the treatment of diabetes.

EXPERIMENTAL PROCEDURES

Mouse Manipulations

The strategy used to generate the Glu-rtTA::TetO-Pax4 mouse line is depicted in Figures S1A and S1B and detailed in the corresponding figure legends. These mice were also crossed with the Glucagon-Cre (Herrera, 2000), Ngn3-CreER (Gu et al., 2002), HNF-CreER (Gu et al., 2002), TetO-Cre (Perl et al., 2002), and/or ROSA-β-gal (Soriano, 1999) mouse lines. Doxycycline (Sigma) was administered via the drinking water prepared freshly once a week at a concentration of 2 g/l. Tamoxifen (Sigma) was administered by gavage at a concentration of 20 mg/ml. To assess the cellular proliferation upon doxycycline-mediated induction of *Pax4*, Glu-rtTA::TetO-Pax4 mice were treated with doxycycline and subsequently with BrdU (in drinking water) for 10 days prior to examination (1 mg/ml solution). To assess the effects of glucagon on islet size, mice were injected intraperitoneally twice daily (every 12 hr) with 6 μg of glucagon (Sigma) and sacrificed after 3 weeks of treatment. Last, lentivirus production and injection were performed as previously described (Xu et al., 2008, Collombat et al., 2009). All animal experiments were validated by our local ethical committees.

Immunohistochemistry

Tissues were fixed for 30 min in 4% paraformaldehyde at 4°C and embedded in paraffin, and 8 μm sections were applied to slides. These sections were assayed as previously described (Collombat et al., 2003) using DAPI or propidium iodine as counterstain. The primary antibodies used were the following: mouse monoclonal anti-insulin (1/500; Sigma), anti-glucagon (1/500; Sigma), anti-E-cadherin (1/200; BD Transduction Lab), anti-Nestin (1/1,000; Millipore), and anti-SNAI2 (1/100; Santa Cruz Biotechnology); guinea pig anti-insulin (1/500; Linco), anti-glucagon (1/500; Millipore); rat monoclonal anti-somatostatin (1/250; Chemical International); mouse anti-Ngn3 (1/10,000; Novo Nordisk), anti-BrdU (1/40; Roche); rabbit anti-Nkx6.1 (1/3,000; Novo Nordisk), anti-Pax4 (1/4,000 kindly provided by B. Sosa-Pineda), anti-Arx (1/500), anti-Pdx1 (1/1,000; kindly provided by C. Wright), anti-NeuroD1 (1/500; Millipore), anti-MafA (1/500; Abcam), anti-PC1/3 (1/500; Milipore), anti-Sox11 (1/200; Santa Cruz), anti-GLP1R (1/1,000; kindly provided by J. Habener) and anti-Glut2 (1/5,000; Chemical International); goat anti-osteopontin (1/100; R&D Systems); and anti-Vimentin (1/50; Santa Cruz). The secondary antibodies (1/1,000; Molecular Probes) used were Alexa Fluor 594 and 488 anti-mouse; Alexa Fluor 594 and 488 anti-rabbit; Alexa Fluor 594 and 488 anti-guinea pig; Alexa Fluor 594 and 488 anti-goat. Pictures were processed using ZEISS Axioimager Z1 and LEICA DM 6000 B. For quantification purposes, stained cells were counted manually on every tenth section.

β-Galactosidase-Based Lineage-Tracing Experiments

Pancreatic tissues were isolated and fixed for 30 min at 4°C in a solution containing 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% NP40. The tissues were dehydrated in 25% sucrose overnight at 4°C. Prior to sectioning, tissues were embedded in freezing medium. For β-galactosidase activity assessment, the tissues were washed in PBS and then incubated overnight in staining solution (500 mM $K_3Fe[CN]_6$, 250 mM $K_4Fe[CN]_6$, 0.5 M $MgCl_2$, 40 mg/ml X-gal in dimethylformamide).

Cell Counts

Quantitative analyses were performed by counting of colored pixels on (immuno-) stained sections of pancreas using the Photoshop software. Specifically, every tenth section was processed using the same settings for all animals and genotypes.

Challenges and Blood Glucose Level Measurement

For challenge purposes, animals were fasted for 16 hr and injected intraperitoneally with glucose (2 g/kg of bodyweight) or insulin (0.75 U/kg). Blood glucose levels were measured at the indicated time points postinjection with a ONETOUCH Vita glucometer (Life Scan).

Induction of Streptozotocin-Mediated Diabetes

To induce hyperglycemia, STZ (Sigma) was dissolved in 0.1 M sodium citrate buffer (pH 4.5), and a single dose was administered intraperitoneally (100–200 mg/kg) within 10 min of dissolution. Diabetes progression was assessed by monitoring the blood glucose levels and/or survival rates of mice.

Data Analysis

All values are depicted as mean ± SEM and considered significant if $p < 0.05$. Data were statistically analyzed by ANOVA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, three tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.05.018.

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