ARTICLES

Conversion of adult pancreatic α-cells to β-cells after extreme β-cell loss

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Pancreatic insulin-producing β-cells have a long lifespan, such that in healthy conditions they replicate little during a lifetime. Nevertheless, they show increased self-duplication after increased metabolic demand or after injury (that is, β-cell loss). It is not known whether adult mammals can differentiate (regenerate) new β-cells after extreme, total β-cell loss, as in diabetes. This would indicate differentiation from precursors or another heterologous (non-β-cell) source. Here we show β-cell regeneration in a transgenic model of diphtheria-toxin-induced acute selective near-total β-cell ablation. If given insulin, the mice survived and showed β-cell mass augmentation with time. Lineage-tracing to label the glucagon-producing α-cells before β-cell ablation tracked large fractions of regenerated β-cells as deriving from α-cells, revealing a previously disregarded degree of pancreatic cell plasticity. Such inter-endocrine spontaneous adult cell conversion could be harnessed towards methods of producing β-cells for diabetes therapies, either in differentiation settings *in vitro* or in induced regeneration.

In vivo adult lineage reprogramming (transdifferentiation)—the notion that adult differentiated cells can change fates from one cell type to another—has had little experimental support from mouse models[1,2]. In the pancreas, nevertheless, there is evidence of induced exocrine acinar cell reprogramming: ectopic expression of pro-endocrine factors resulted in conversion of acinar cells into insulin-producing β-cells[3]. In another study, the loss of c-Myc activity in pancreatic progenitor cells led to a progressive transdifferentiation of adult acinar cells into adipocytes[4].

The adult endocrine pancreas, that is, the islets of Langerhans, is made of four different hormone-producing cell types: β-cells producing insulin, α-cells producing glucagon, δ-cells producing somatostatin and PP cells producing pancreatic polypeptide. During development, a fifth cell type, ε, contains ghrelin. In normal conditions, β-cell maintenance relies on their long lifespan, as they proliferate little[5,6]. In response to increased physiological demand, there is increased β-cell proliferation[7,8]. If the β-cell mass decreases below certain critical levels (around 10% of the normal values[9]) there is diabetes onset; this is very clear for the juvenile form of the disease, known as type 1 diabetes (T1D), usually of autoimmune aetiology. There is evidence of β-cell regeneration in children with T1D and in young diabetic rats[10–12], as well as after experimental surgical or chemical pancreatic injury[13–16]. In these conditions, β-cell replication accounts for β-cell regeneration[13–16], although in the absence of appropriate cell lineage tracing studies other processes cannot be excluded[17–19]. In this respect, the formation of new β-cells from precursors expressing neurogenin 3, thus mimicking embryonic islet development, was reported in adult mice with acute pancreatitis induced by ductal ligation[20].

β-cell loss in all available experimental models of diabetes is partial, uncontrolled, or associated with inflammation or autoimmunity. Here we studied the inherent regenerative capacity of the adult pancreas to produce new β-cells after their near-total loss, a condition close to T1D, but without autoimmunity. Such an extreme situation allowed us to explore whether new insulin-producing cells can emerge from other sources than pre-existing β-cells, as these were almost totally depleted. For this purpose, we used two *in vivo* genetic approaches: cell ablation combined with cell lineage tracing[21,22]. We created a model of inducible, rapid β-cell removal (>99%) by administration of diphtheria toxin (DT)[22,23]. In mice, the transgenic expression of the DT receptor (DTR) followed by systemic administration of DT permits an exquisite, specific cell ablation by apoptosis[24,25]. We thus generated mice in which β-cells bore DTR. In this model, β-cell regeneration was monitored in combination with cell lineage tracing devised to investigate the origin of newly formed β-cells. We found that the adult pancreas can generate new β-cells after their near total loss, mainly by the spontaneous reprogramming of α-cells.

Ablation of β-cells

We generated mice bearing a transgene containing an insulin promoter and the diphtheria toxin receptor coding sequence (*RIP-DTR*). The transgene was targeted to the *Hprt* locus of the X chromosome. The aim was to ablate either 50% or 100% of the β-cell mass using hemizygous females (in which there is random X inactivation) or males, respectively (Fig. 1a). DTR expression per se did not cause any distinguishable phenotype. The administration of DT to hemizygous *RIP-DTR* females did not affect their basal glycaemia or life expectancy, whereas males and homozygous females became rapidly hyperglycaemic (Supplementary Fig. 1a).

All subsequent experiments were performed using two-month-old male mice. DT treatment triggered full-blown diabetes, with polyuria, polydipsia, polyphagia, ketoacidosis and weight loss, and, in the absence of insulin treatment, resulted in death (Supplementary Fig. 1b–d and not shown). Two weeks after DT treatment, the pancreatic insulin content (Supplementary Fig. 1e) and the insulin transcription level (Supplementary Fig. 1f) had dropped to 0.3% and 0.01% of the control value, respectively. β-cell loss was confirmed histologically (Fig. 1a and Supplementary Fig. 2a–c): the β-cell mass decreased from 1,594 μg to

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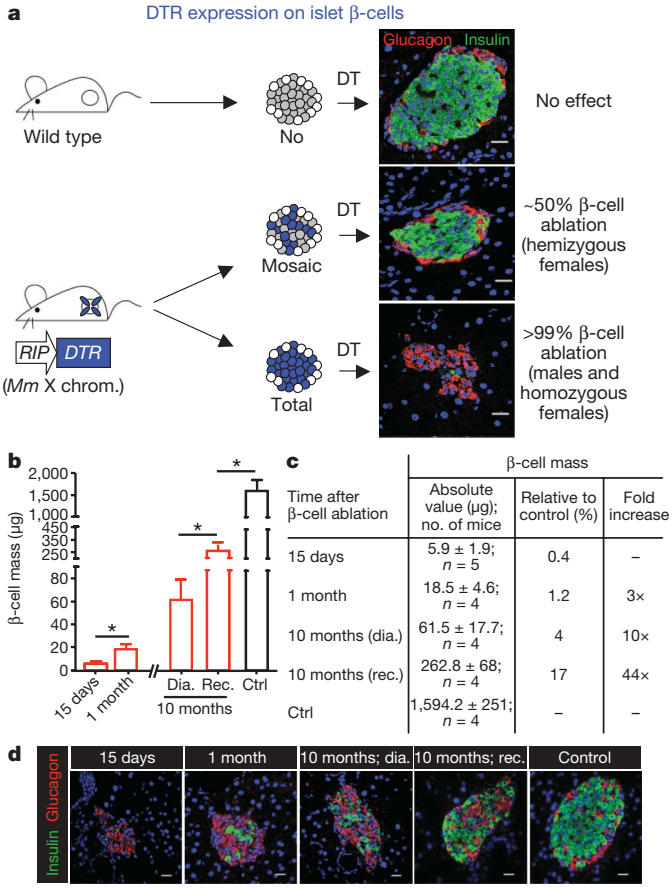

Figure 1 | β-cell ablation and regeneration. a, *RIP-DTR* mice express DTR on 100% or 50% of β-cells (blue cells in the cartoon denote DTR-bearing β-cells). **b**, **c**, Measurement of β-cell mass after ablation and regeneration. Ablation of 99.6% of the β-cell mass is followed by a threefold increase between 15 and 30 days (from 5.9 to 18.5 μg), and up to a 10–44-fold increase 10 months later. One-way analysis of variance (ANOVA) ($P = 0.0009$) and Mann–Whitney tests (*$P < 0.05$). Ctrl, control; dia., diabetic; rec., recovered. Error bars denote s.e.m. **d**, Representative islets at various moments after DT. Scale bars, 20 μm.

6 μg after 15 days of DT treatment (Fig. 1b, c), which corresponds to a disappearance of 99.6% of the β-cells. Apoptotic β-cells and mild islet fibrosis were apparent in the days after DT injections, but inflammation, insulitis or extra-insular cell death were not observed (Supplementary Fig. 2d and data not shown).

β-cell regeneration

To explore the possibility of β-cell regeneration and its kinetics, mice were euthanized at different time points after β-cell ablation, for a period of up to 10 months. Between 15 days and 1 month, β-cell mass and total pancreatic insulin content increased by a factor of 3 (from 5.9 ± 1.9 μg to 18.5 ± 4.6 μg; (all values are mean ± s.e.m.); Fig. 1b–d and data not shown). During this initial period, transcription of the two insulin genes increased by a factor of 10 (Supplementary Figs 1f and 10a).

In long-term experiments, mice were kept alive for up to 10 months after ablation. During the initial 5 months, animals were regularly given subcutaneous insulin implants whenever their glycaemia was above 20 mM (16 mice were studied in total). From the sixth month on, all mice survived without further insulin treatment, thus showing clear signs of recovery (Supplementary Fig. 3a). The β-cell mass was found to be increased in all animals: tenfold in mice that remained diabetic, and up to 44-fold in animals that showed improved glycaemic control. This increment corresponds on average to 10% of the normal β-cell mass (between 4% and 17%, respectively; Fig. 1b–d). About 10% of a normal β-cell mass is found in patients with recent-onset T1D, and represents the lowest amount of β-cells able to ensure a near normal basal glycaemia[9].

Almost all medium and large islets showed signs of β-cell regeneration. In fact, 60% of islets contained no or up to two β-cells per islet section 15 days after ablation, whereas 10 months later 96% of islet sections contained more than two β-cells (Supplementary Fig. 3b, c). This suggests that all islets in the adult pancreas can regenerate β-cells. No β-cells were found in extra-insular locations.

Spared β-cells do not increase replication

The first month after β-cell ablation is a period of intense regeneration in *RIP-DTR* mice: β-cell mass triplicates between 15 and 30 days after β-cell destruction (Fig. 1b, c). Therefore, the origin of β-cells that are found 1 month after β-cell killing was studied by lineage tracing. We used the tamoxifen-dependent Cre-*loxP* system ('pulse-chase' rationale) to label pre-existing β-cells and measure the contribution of the rare β-cells spared by DT treatment to regeneration. We generated transgenic mice bearing the transgenes *RIP-CreERT* (inducible tagger)[7], *R26-YFP* as reporter[26] and *RIP-DTR* (toxigene) (Fig. 2a and Supplementary Fig. 4a). The administration of tamoxifen induces the expression of the reporter marker yellow fluorescent protein (YFP) from the *Rosa26* locus exclusively in β-cells: roughly all of them (95.4 ± 0.5%) were YFP$^+$ (Fig. 2c–e). We measured the contribution of surviving β-cells or their progeny to regeneration as follows: animals were given tamoxifen and then DT 7 days later, and the proportion of YFP-tagged cells was determined after 15 and 30 days (Supplementary Fig. 4a). Fifteen days after ablation, only 80 ± 2.9% of the escaping remaining β-cells were YFP$^+$; this proportion dropped further to 7.6 ± 1.8% of the β-cells after 30 days of DT treatment (Fig. 2b–k). These results indicate that: (1) formation of new β-cells from non-β cell origins occurs very rapidly after ablation (about 20% and 90% of them were not labelled 2 weeks and 1 month after DT treatment, respectively), and (2) escaping β-cells probably do not contribute to the expansion of the β-cell mass, because the proportion of labelled β-cells decreased by tenfold, from 80% to 7.6%, whereas the β-cell mass tripled.

Contrary to previous models of β-cell ablation/regeneration[16,27,28], we noticed that as compared with untreated controls, β-cell proliferation in *RIP-DTR* mice was not increased during this period (15 and 30 days after DT treatment), independently of whether β-cells were labelled or not (0.96% β-cells were Ki67$^+$ versus 0.7% in healthy mice of the same age; 207 β-cells were scored out of 329 islets from 119 sections of five DT-treated mice, and 4,367 β-cells from five mice in controls; $P = 0.9$, non-significant; Fischer's test). The same pattern of low proliferation was observed long after ablation (at 10 months: 0.3% β-cells were Ki67$^+$; 869 β-cells from eight mice); this suggests that long-term regeneration (Fig. 1) does not rely on increased β-cell replication.

Together, the dilution of labelled β-cells while β-cell mass increases, and the absence of increased β-cell proliferation, is most consistent with a model of regeneration from heterologous, that is, non β-cell, origins.

Bihormonal cells arise after β-cell loss

Increased glucagon production and secretion are often associated with insulin deficiency in T1D and T2D humans[29]. In *RIP-DTR* mice, the total pancreatic glucagon content, glucagonemia, and glucagon gene expression were increased by twofold after β-cell loss (Supplementary Fig. 5a–c). Islets became prominently composed of α-cells, yet α-cell proliferation and mass remained unchanged during the entire analysis period (up to 10 months; Supplementary Fig. 5d, e). In the days after β-cell destruction, cells co-expressing glucagon and insulin became frequent: about one-third of the rare cells containing insulin (Supplementary Fig. 5f, g), or conversely, 1–3% of the glucagon-expressing cell population (not shown). Interestingly, the glucagon$^+$/insulin$^+$ cells remained detectable at all times after ablation, with similar relative proportions 10 months

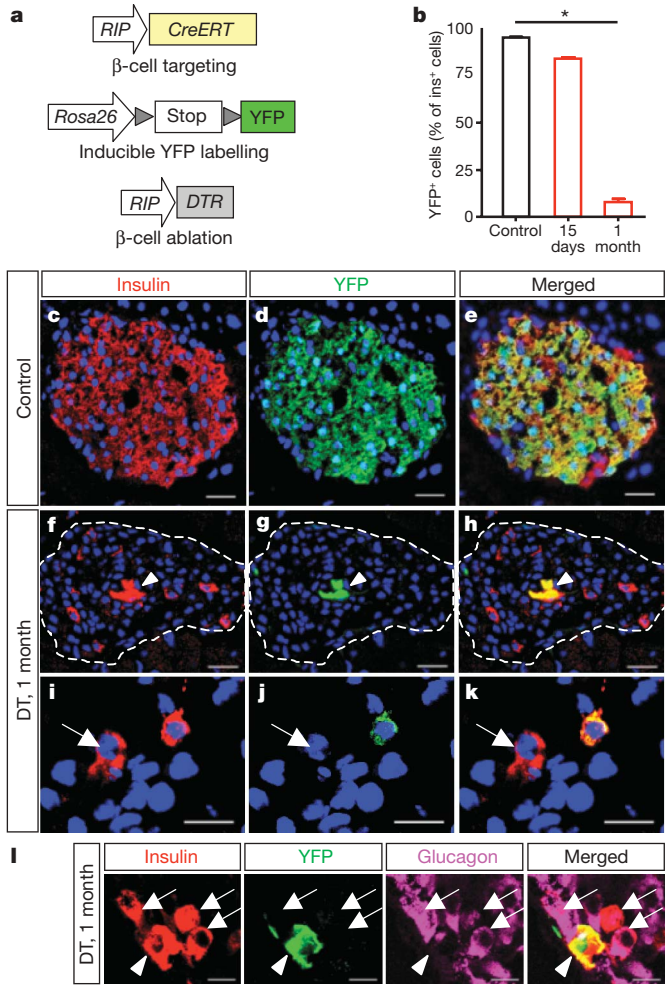

Figure 2 | Conditional β-cell lineage tracing. a, Transgenes. **b**, Proportion of YFP$^+$ β-cells, as a percentage of insulin$^+$ (ins$^+$) cells. Control: 95.4 ± 0.5% (mean ± s.e.m.) ($n = 4$; 159–499 β-cells per mouse; 5–12 islets per individual). Two weeks and 1 month after DT, 80.6 ± 2.9% and 7.6 ± 1.8% β-cells were labelled, respectively (15 days: $n = 3$ mice, 80–174 β-cells from 15–25 islets per mouse; 1 month: $n = 3$ mice, 54–73 β-cells from 15–24 islets per mouse). *$P < 0.01$. One-way ANOVA ($P = 0.0181$) and Dunn's multiple comparison test (*$P < 0.05$). Error bars denote s.e.m. **c–e**, Most β-cells express YFP in controls. **f–k**, Few β-cells are YFP$^+$ after 1 month (arrowhead) (**f–h**). **i–k**, Two β-cells are shown (arrow denotes YFP$^-$ β-cell). **l**, Glucagon$^+$/insulin$^+$ cells are YFP$^-$ (arrows); YFP$^+$/insulin$^+$ cells are glucagon$^-$ (arrowhead). Scale bars, 20 μm (**c–h**) and 10 μm (**i–l**).

after ablation (Supplementary Fig. 5f). In the *RIP-CreERT*-mediated lineage tracing described earlier, these bihormonal cells were never YFP-labelled, thus showing that they were not β-cells that escaped ablation and became glucagon-expressers (Fig. 2l). They should therefore be either pre-existing α-cells that start producing insulin, or undefined precursors that start producing and storing the two hormones, or both. Interestingly, other β-cell markers, such as the transcription factors Pdx1 and Nkx6.1 were also found in a fraction of glucagon-expressing cells very rapidly after DT treatment, and subsequently throughout the analysis period, up to 10 months (Supplementary Fig. 6).

Although marker colocalization per se is not proof of ontogenetic relationships between different cell types[12], we reasoned that cells co-expressing glucagon and β-cell-specific markers, in particular insulin, might represent emergent β-cells.

α-cells transdifferentiate to β-cells

We devised a conditional α-cell lineage analysis to determine whether pre-existing α-cells are at the origin of glucagon$^+$/insulin$^+$ co-expressing cells and new β-cells. We generated a transgenic strain in which only α-cells are selectively and irreversibly labelled before β-cell ablation (tetracycline-dependent Cre-*loxP* system). These mice were termed *glucagon-rtTA*. In addition the *TetO-Cre* responder transgene[30], they bore the reporter *R26-YFP*[26], and the *RIP-DTR* transgene (Fig. 3a and Supplementary Fig. 4b). *TetO* (tetracycline-responsive promoter/operator) drives the expression of Cre-recombinase after reverse tetracycline transactivator (rtTA) activation with the tetracycline analogue doxycycline (DOX, 'the pulse'). Almost no α-, β- or δ-cells were YFP-labelled without DOX in 2-month-old males (some 0.2%), whereas almost 90% of α-cells were irreversibly tagged when DOX was given during 15 days after weaning (Fig. 3b–f and Supplementary Fig. 7). The *glucagon-rtTA* inducible system is thus efficient. Two weeks after ending DOX administration, five 2-month-old males received DT and were euthanized after 1 more month, to assess for the possible direct contribution of adult α-cells to β-cell regeneration: any α-to-β-cell reprogramming would result in the presence of YFP-labelled β-cells (Supplementary Fig. 4b). Shortly after ablation, islets were composed almost exclusively of YFP$^+$ α-cells (Fig. 3g–j and Supplementary Fig. 10b). We found that nearly 90% glucagon$^+$/insulin$^+$ co-expressing cells were YFP-labelled (Fig. 3l–o), indicating that they were pre-existing α-cells that started expressing insulin.

On average, 65% of the cells expressing insulin one month after β-cell ablation were YFP$^+$ (Fig. 3k, p–s); almost 90% of them (YFP$^+$/insulin$^+$) still contained glucagon as well (Fig. 3l–s). This lineage tracing showed their direct origin from adult α-cells, which were irreversibly tagged before injury: they were reprogrammed α-cells.

This result was confirmed with two independent different experiments. In the first study, an important proportion of tagged insulin$^+$ cells appeared after the massive β-cell ablation using a constitutive lineage-tracing with the *glucagon-Cre* strain[21,31]; this further supports the concept of an adult α-cell origin for regenerated β-cells in *RIP-DTR* mice (Supplementary Fig. 8).

The second confirmatory experiment showed the absolute requirement of α-cells for the formation of glucagon$^+$/insulin$^+$ co-expressing cells after near-total β-cell loss: the bihormonal cells were absent when α-cells were co-ablated with β-cells in mice bearing a further transgene, termed *glucagon-DTR*, engineered to ablate α-cells (Supplementary Fig. 9).

We explored the proliferation rate of reprogrammed α-cells (that is, YFP$^+$/insulin$^+$ in *glucagon-rtTA* mice) and, again, we found no significantly increased insulin$^+$ cell replication one month after ablation (1.3%; 2 out of 149 β-cells scored were Ki67$^+$, one was YFP$^+$ and the other was YFP$^-$; 456 islets from 163 sections; five mice were analysed) as compared with healthy mice of the same age (0.9%; 19 β-cells Ki67$^+$ out of 2,192 from 68 islets of five mice. $P = 0.70$, non-significant; Fischer's test).

Gene expression quantification showed that several β-cell markers were severely downregulated after β-cell loss, as expected. Interestingly, expression of these very genes started to increase between one and two weeks after ablation, coincident with the beginning of regeneration (Supplementary Fig. 10a). This upregulation of β-cell-specific genes occurred selectively within islets, which are mainly composed of α-cells at this stage (Supplementary Fig. 10b–d). We confirmed the expression of some of these β-cell markers at the protein level in glucagon-expressing cells, in bihormonal cells (YFP$^+$/glucagon$^+$/insulin$^+$) and in the scarcer YFP$^+$/insulin$^+$ cells having lost glucagon expression: Nkx6.1 (Fig. 4a–c), Pdx1 (Supplementary Fig. 6) or the β-cell-specific glucose transporter type 2 (Glut2, also known as Slc2a2); Supplementary Fig. 11).

Together, these observations are compatible with a model in which α-cells become β-cells (Fig. 4d).

Discussion

We have observed that the adult pancreas has the ability of making β-cells from heterologous origins in a pathological situation whereby

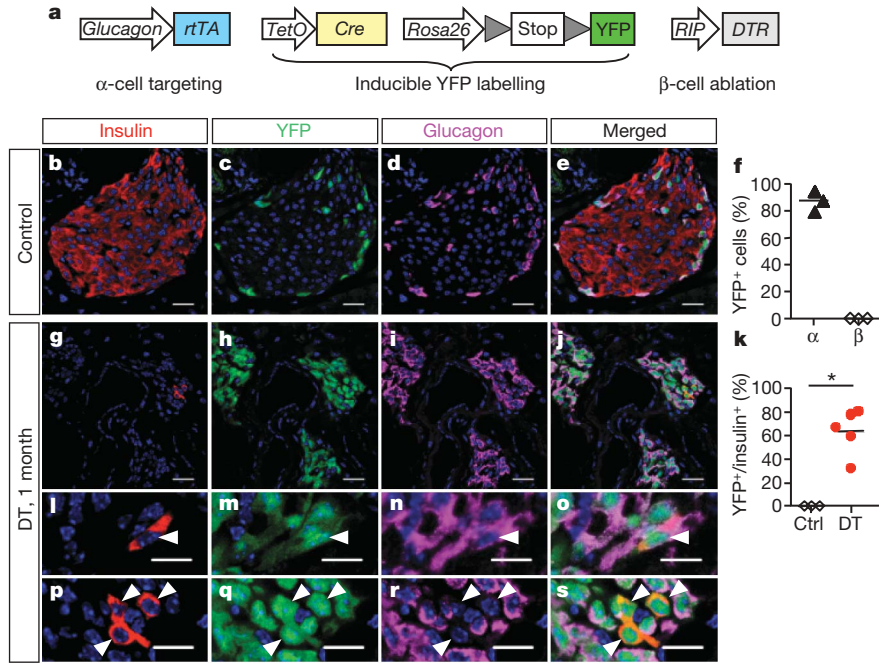

Figure 3 | α-to-β reprogramming. **a**, Transgenes used for the conditional α-cell lineage tracing. **b–e**, DOX-treated mice, without DT. **f**, Most α-cells are YFP$^+$ in controls (88.1 ± 4.42%; $n = 3$ mice; 2,258 α-cells scored, 108 islets). Horizontal bars (in **f** and **k**) denote mean values. **g–j**, One month after DT, islets are mostly composed of YFP$^+$ α-cells. **k**, Proportion of YFP$^+$/insulin$^+$ cells in DOX-treated mice. DT-treated group: 63.6 ± 8.6% (511 β-cells from 239 islets; five mice). *$P < 0.05$. **l–o**, YFP$^+$/insulin$^+$/glucagon$^+$ cell (arrowhead; 89.87 ± 3.04% of insulin$^+$ cells). **p–s**, YFP$^+$/insulin$^+$ cells, not expressing glucagon (arrowheads). Scale bars, 20 μm (**b–e**, **g–j**) and 10 μm (**l–o**, **p–s**).

β-cells have been completely lost, or almost. Regeneration in *RIP-DTR* mice is weaker than in other mouse models of less severe β-cell destruction[16,27,28] probably because there is almost no β-cells left after DT treatment; but why β-cell replication is not increased after the massive injury remains unclear, and is in contrast with observations reported after partial β-cell loss[16,27,28]. This fact alone shows the biological significance of studying regeneration and tissue responses under various contexts, such as the degree of injury or the age of disease onset.

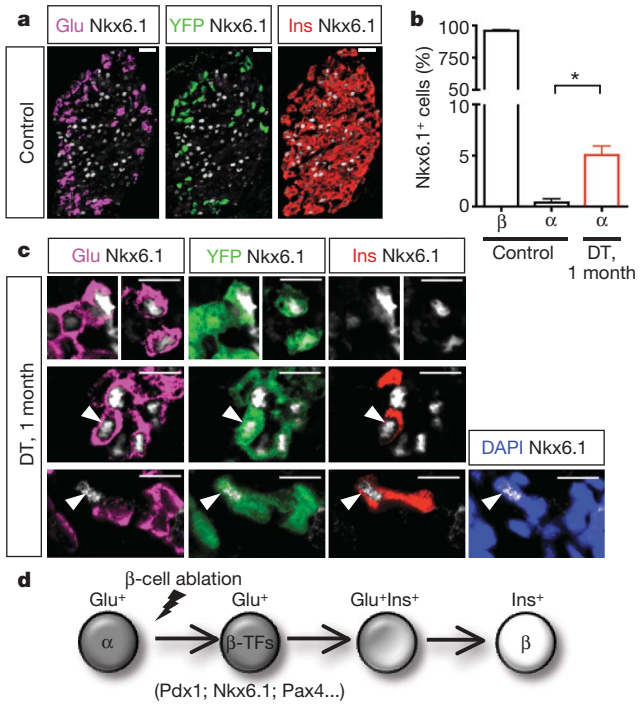

Figure 4 | β-cell marker expression. **a**, In control mice Nkx6.1 is expressed in β-cells. Scale bars, 20 μm. **b**, One month after ablation, 5.05 ± 0.8% of glucagon-expressing cells are Nkx6.1$^+$ (2,855 cells in 277 islets, five mice, versus 0.37 ± 0.3% in controls; 674 α-cells in 39 islets, three mice; $P = 0.035$). Error bars denote s.e.m. **c**, Nkx6.1 expression in glucagon$^+$ cells (top). Some cells also express insulin (middle). YFP$^+$/insulin$^+$/Nkx6.1$^+$/glucagon$^-$ cell (bottom). DAPI, 4′,6-diamidino-2-phenylindole. Scale bars, 10 μm. **d**, Proposed reprogramming sequence. TF, transcription factors.

Expression of Pdx1 may be part of the α-cell conversion mechanism: ectopic Pdx1 activity, alone or combined with other factors, drives hepatocytes or acinar cells into insulin production[3,32,33]. Pdx1 binds directly to insulin and glucagon promoters[34], thus inhibiting glucagon expression and inducing insulin transcription[35]. Other β-cell factors may determine the α-to-β reprogramming, such as Nkx6.1, which may also contribute to glucagon gene inhibition and activation of β-cell-specific genes[36], or Pax4, which regulates the balance between α- and β-cells by antagonizing Arx in endocrine progenitors[37]. In this respect, it was recently reported that expression of Pax4 in embryonic α-cells using the *glucagon-Cre* transgenics[21] induces their conversion into β-cells[38]. Because mature α- and β-cells share several transcription factors (such as Isl1 and Pax6)[39] and a common ancestor[21,39], the α-cell represents an appropriate candidate for reprogramming to β-cell phenotype. Moreover, α- and β-cells are functionally very close, with a similar machinery to metabolize glucose and secrete hormones[40]: both cell types express glucokinase and ATP-regulated K$^+$-channels, suggesting that they differ in glucose transport but not in glucose use[41,42]. Expression of Glut2 in insulin-producing reprogrammed α-cells, in addition to Nkx6.1 and Pdx1, should allow them to secrete insulin upon glucose stimulation, like functional β-cells.

Previous models of β-cell injury do not report heterologous regeneration of β-cells, yet this was not explored with lineage tracing analyses[16,27,28]. In these models, remaining β-cells were abundant (at least 20% of the initial β-cell mass), which suggests that β-cell loss must be near total for triggering heterologous β-cell formation. In this regard, milder β-cell ablation in *RIP-DTR* mice, by using hemizygous females (50% ablation) or in males treated with lower doses of DT (95–98% ablation), has a different outcome: in these situations, either there is no measurable regeneration (after 50% ablation;

Supplementary Fig. 12), or induction of α-cell reprogramming is decreased, with a more important contribution of spared β-cells (not shown). The amount of β-cell loss thus determines whether there is regeneration (Supplementary Fig. 12) and, together with the type of injury, it influences the degree of cell plasticity and regenerative resources of the adult pancreas (Supplementary Fig. 12).

These observations raise issues about cell plasticity and regenerative recovery from lesion: α-cells were never considered previously as a potential source of cells for β-cell therapy in diabetics. Our results argue that a deep lesion (total or near-total β-cell ablation, as in T1D) causes the release of some form of signal that allows prolonged and substantial β-cell regeneration. The presence of bihormonal cells (glucagon$^+$/insulin$^+$) a long time after lesion induction is compatible with α-cell reprogramming not being limited by temporal restrictions, and thus with regeneration in aged individuals. Alternatively or in addition, the persistence of glucagon staining in some reprogrammed α-cells may reflect impaired or inhibited glucagon granule exocytosis: in cells possessing several types of regulated secretory granules, exocytosis can be activated independently for each of them[43].

We found that the proportion of β-cells derived from reprogrammed α-cells is very variable among individuals having the same degree (>99%) of β-cell destruction: between 32% and 81% (Fig. 3k). This further stresses the adaptability of adult pancreas and reveals high versatility in response to injury. This plasticity is reminiscent of the various mechanisms of liver regeneration[44].

In long-term human T1D patients, occasional β-cells are found scattered in the pancreas, as well as circulating C-peptide, an indicator of proinsulin processing and insulin secretion[11,45,46]. Also, complete β-cell function recovery has been reported in young T1D patients[47,48]. Whether this is the consequence of a continuous regeneration of new β-cells, as we have seen in mice, or persistence of few β-cells, which would escape autoimmunity, is not known[45]. Nevertheless, our observations in mice should encourage attempts of treatment by inducing and enhancing regeneration after controlling the autoimmune aggression.

Finally, these findings indicate that the production of new models for selective and total cell ablation could lead to discoveries about regeneration induction and cell plasticity in other organs, including pathological conditions such as dysplasia or cancer.

METHODS SUMMARY

Generated mice. *RIP-DTR* transgene was prepared by sub-cloning the human HB-EGF complementary DNA[25] into a plasmid containing a 0.7-kb-long fragment of the rat insulin II promoter, and a 1.6-kb-long sequence containing an intron and the polyA signal of the rabbit β-globin gene, as described[21,23]. The transgene was introduced into a pDEST vector for homologous recombination at the HPRT locus in BPES cells (C57Bl/6-129 background, Speedy Mouse, Nucleis). Recombinant BPES cells were used to generate chimaeras.

The *glucagon-DTR* construct was arranged by replacing the Cre cDNA of *glucagon-Cre* plasmid[21] with the human HB-EGF cDNA. We generated seven independent F_0 founders by pronuclear injection[49], two of which had optimal expression of DTR.

The *glucagon-rtTA* construct was generated by replacing the Cre sequence of *glucagon-Cre* plasmid[21] by the rtTA-Advanced cDNA sequence of pTet-On Advanced vector (Clontech). Thirteen F_0 founders were obtained by pronuclear injection. Two of the thirteen strains showed equivalent efficiency of glucagon cell labelling after DOX treatment.

Diphtheria toxin, tamoxifen, doxycycline and insulin treatments. DT (Sigma) was given to 2-month-old mice in three intraperitoneal (i.p.) injections (126 ng of DT per injection, on days 0, 3 and 4). Tamoxifen was freshly prepared (50 mg ml^{-1}; TAM; Sigma) and administered with a gastric catheter (five doses of 10 mg, every 2 days). TAM (10 mg) was diluted in 10 μl 100% ethanol, completed to 200 μl with 0.9% NaCl and sonicated for 60 s at minimum intensity. DOX (1 mg ml^{-1}) (Sigma) was added to drinking water for 2 weeks. After DOX removal, mice were kept during 15 further days without treatment before DT administration; this period is sufficient for DOX clearance[50]. Mice received subcutaneous implants of insulin (Linbit) when hyperglycaemic (>20 mM) in the long-term regeneration experiments.

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