

In vivo reprogramming of adult pancreatic exocrine cells to β -cells

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One goal of regenerative medicine is to instructively convert adult cells into other cell types for tissue repair and regeneration. Although isolated examples of adult cell reprogramming are known, there is no general understanding of how to turn one cell type into another in a controlled manner. Here, using a strategy of re-expressing key developmental regulators *in vivo*, we identify a specific combination of three transcription factors (*Ngn3* (also known as *Neurog3*) *Pdx1* and *Mafa*) that reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β -cells. The induced β -cells are indistinguishable from endogenous islet β -cells in size, shape and ultrastructure. They express genes essential for β -cell function and can ameliorate hyperglycaemia by remodelling local vasculature and secreting insulin. This study provides an example of cellular reprogramming using defined factors in an adult organ and suggests a general paradigm for directing cell reprogramming without reversion to a pluripotent stem cell state.

Cells of adult organisms arise from sequential differentiation steps that are generally thought to be irreversible¹. Biologists often describe this process of development as proceeding from an undifferentiated (embryonic) cell to a terminally differentiated cell that forms part of an adult tissue or organ. There are rare examples, however, in which cells of one type can be converted to another type in a process called cellular reprogramming or lineage reprogramming^{2,3}. Various forms of cellular reprogramming are referred to in the literature as transdifferentiation, dedifferentiation or transdetermination⁴. For example, cellular reprogramming occurs in amphibian limb regeneration and fly imaginal disc identity switches^{5,6}, and it may be central to certain types of pathological metaplasia⁴. There is long-standing interest and fascination in reprogramming studies, in part because of the promise of harnessing this phenomenon for regenerative medicine whereby abundant adult cells that can be easily collected would be converted to other medically important cell types to repair diseased or damaged tissues.

Somatic cell nuclear transfer (SCNT), developed in the 1960s, demonstrated that nuclei from differentiated adult cells could be reprogrammed to a totipotent state after injection into enucleated eggs^{2,7}. More recently, it was shown that a small number of transcription factors can reprogram cultured adult skin cells to induced pluripotent stem (iPS) cells^{8–13}. These studies point to the possibility of regenerating mammalian tissues by first reverting skin or other adult cells to pluripotent stem cells and then redifferentiating these into various cell types. Alternatively, it should be possible to convert one cell type into another directly, without the need to first revert the cell to an undifferentiated pluripotent state. Indeed, there are examples in the literature that suggest that this approach is feasible. For example, studies with embryonic cells have shown that dermal fibroblasts and retinal epithelial cells can be converted into muscle-like cells¹⁴, and pancreatic tissue to liver¹⁵. In adult animals, mature B lymphocytes have been reprogrammed into macrophages¹⁶ or pro-B cells¹⁷. Today, well documented examples of cellular reprogramming, especially in adult animals, remain rare and have generally been restricted to cases in which a single inducing factor is involved. The recent work on iPS formation suggests that a specific combination of multiple factors, instead of a single one, might be the most effective way to reprogram adult cells^{8–13}.

We developed a strategy to identify adult cell reprogramming factors by re-expressing multiple embryonic genes in living adult animals. Our focus on embryonic genes is based in part on regeneration studies in newts, frogs and fish, wherein it has been shown that dedifferentiation of adult cells to progenitors, a form of cellular reprogramming, is accompanied by reactivation of embryonic regulators^{5,18,19}. These studies suggest that re-expression of appropriate embryonic genes may reprogram differentiated cells.

To search for factors that could reprogram adult cells into β -cells, we focused on transcription factors, a class of genes enriched for factors that regulate cell fates during embryogenesis. An *in situ* hybridization screen of more than 1,100 transcription factors identified groups of transcription factors with cell-type-specific expressions in the embryonic pancreas²⁰. There are at least 20 transcription factors expressed in mature β -cells and their immediate precursors, the endocrine progenitors (Supplementary Table 1). Of these, 9 genes exhibited β -cell developmental phenotypes when mutated^{21,22}, and these were selected for initial reprogramming experiments.

We chose mature exocrine cells of the adult pancreas as target cells for reprogramming. Exocrine cells derive from pancreatic endoderm, as do β -cells²³, and exocrine cells can turn on endocrine programs when dissociated and cultured *in vitro*^{24,25}. We carried out our experiments *in vivo* so that any induced β -cells would reside in their native environment, which might promote their survival and/or maturation. In addition, this approach allows for a direct comparison of endogenous and induced β -cells. The transcription factors were delivered into the pancreas in adenoviral vectors. It has been shown that adenovirus preferentially infects pancreatic exocrine cells, but not islet cells²⁶, and, because most endogenous β -cells reside within islets (Fig. 1b), any newly formed (induced) β -cells could be easily detected as extra-islet insulin⁺ cells.

Induction of insulin⁺ cells in adult mice

Adenovirus that co-expresses each transcription factor together with nuclear GFP (nGFP) was purified. All nine viruses were pooled and injected as a mixture (referred to as M9, for mixture of nine) into the pancreata of 2-month-old adult mice (Fig. 1a). The immune-deficient

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Rag1^{-/-} strain was used to avoid complications associated with viral-elicited immune response²⁷. One month after viral delivery, immunohistochemistry revealed a modest increase of extra-islet insulin⁺ cells among viral infected cells (nGFP⁺) in two out of three animals (Fig. 1d). To determine which of the nine factors are required, individual factors were removed from the pool one at a time. Pools lacking Nkx2.2, Nkx6.1 or Pax4 continued to produce increased extra-islet insulin⁺ cells (data not shown), suggesting that these genes are dispensable. Results for the other six genes were inconclusive. We conducted another round of factor withdrawal with mixtures of the remaining six genes (M6); three of them, *Ngn3*, *Pdx1* and *Mafa*, proved to be absolutely required (Fig. 1d). The combination of these three factors (referred to as M3) converted >20% of infected cells to insulin⁺ cells (red cells with green nuclei, Fig. 1c, e). Notably, single factors or combinations of any two factors did not elicit this effect (Fig. 1e). Antibody labelling confirmed that these three inducing factors are co-expressed in the induced insulin⁺ cells (Supplementary Fig. 1). NeuroD (also known as Neuro1) can functionally replace *Ngn3* in M3, but the resulting cocktail has reduced induction efficiency (Fig. 1e).

We noticed that the percentage of insulin⁺ cells among infected cells increases with progressive removal of factors from the pool such that M3 induces more insulin⁺ cells than M6, whereas M6 is better than M9 (Fig. 1d, e). This is probably due to the fact that a constant volume of virus was injected into each animal, regardless of the viral

combinations. The effective concentration of *Ngn3*, *Pdx1* and *Mafa* viruses in a cocktail, therefore, increases when fewer factors are included. New insulin⁺ cells were detected 3 days after injection, but the expression level was low. The intensity of insulin staining increased gradually so that, by day 10, the level was comparable to that of endogenous β -cells (Supplementary Fig. 2). These new insulin⁺ cells were still present after 3 months, the longest time point that we analysed, and remained as scattered individual cells or small clusters and did not form islets (Fig. 1c). The reprogramming effect of the three factors appeared to be rather specific for pancreatic exocrine cells: infection of skeletal muscle *in vivo* or fibroblasts *in vitro* with M3 did not induce insulin expression, despite extensive co-expression of the three factors in the target cells (Supplementary Fig. 1).

New insulin⁺ cells come from exocrine cells

Lineage analysis was performed to determine the origin of the new insulin⁺ cells. The five major cell types in the adult pancreas can be detected with lineage-specific molecular markers: exocrine (amylase), duct (Ck19), endocrine (insulin, glucagon, somatostatin and pancreatic polypeptide), vascular (PECAM) and mesenchymal (nestin and vimentin) cells. On injection with a control nGFP virus, most infected cells (>95%) were found to be mature amylase⁺ exocrine cells (Fig. 2a, b), consistent with previous reports²⁶. Non-exocrine cells together accounted for approximately 5% of the infected population. Because more than 20% of M3-infected cells become insulin⁺ 10 days after viral delivery, it suggests that non-exocrine cells can contribute, at most, to a minor fraction of these new insulin⁺ cells. As there is little cell death and no enhanced proliferation during this reprogramming (Supplementary Fig. 3), most insulin⁺ cells would thus appear to originate from mature exocrine cells. To confirm the exocrine origin of the new insulin⁺ cells, we genetically labelled mature exocrine cells with a mouse line (*Cpa1CreER*^{T2}) that expresses an inducible form of Cre recombinase (*CreER*^{T2}) specifically in adult exocrine cells²⁰ (Fig. 2c). When crossed with the *R26R* reporter line, tamoxifen induction in double heterozygous *Cpa1CreER*^{T2}; *R26R* adults indelibly labelled 5–10% of mature

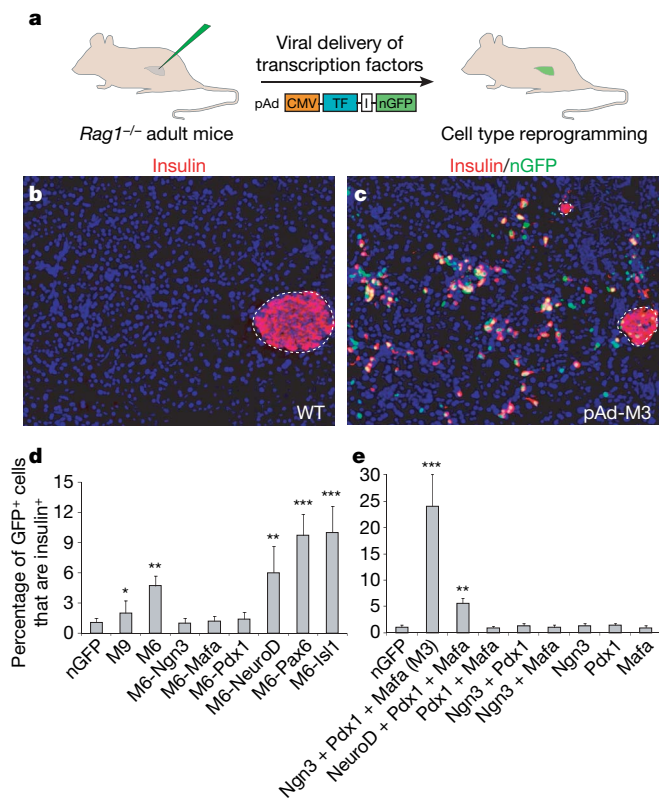


Figure 1 | A combination of three transcription factors induces insulin⁺ cells in adult mouse pancreas *in vivo*. **a**, Schematic diagram of the experimental strategy. Adenoviruses encoding bicistronic transcription factor (TF) and nGFP linked by an IRES element (I) were injected into the pancreas of an adult mouse (*Rag1*^{-/-}). CMV, cytomegaloviral promoter. **b**, Wild type (WT) pancreas is predominantly exocrine tissue with insulin⁺ β -cells in the islet (outlined). Nuclei were stained blue with DAPI. **c**, One month after infection with a combination of *Ngn3*, *Pdx1* and *Mafa* viruses (pAd-M3), numerous insulin⁺ cells appear outside of islets. **d, e**, Quantification of induction one month after infection. M9, M6: mixture of 9 and 6 different viruses, respectively. Data are presented as mean + s.d.; $n = 3$ animals. ~1,000 nGFP⁺ cells were counted per animal. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$.

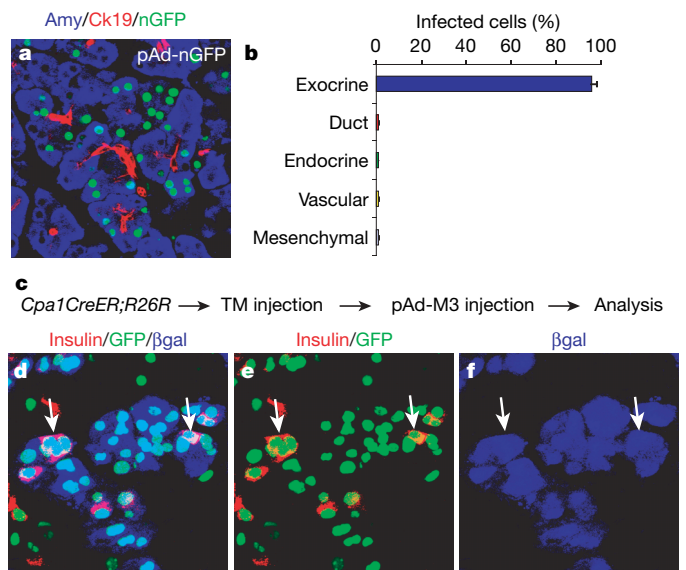


Figure 2 | Induced new β -cells originate from differentiated exocrine cells. **a**, Ten days after nGFP viral infection, most infected cells are amylase⁺ (*Amy*⁺) mature exocrine cells, not duct cells (*Ck19*⁺). **b**, Quantification of nGFP-infected cell types. Data are presented as mean + s.d., $n = 3$ animals. ~1,000 nGFP⁺ cells were counted. **c**, Double heterozygous *Cpa1CreER*^{T2}; *R26R* adult mice are injected with tamoxifen (TM), which labels the mature exocrine cells with β -galactosidase (β gal). Reprogramming is subsequently induced by infection with pAd-M3. **d-f**, Ten days after infection, many β gal⁺ insulin⁺ cells (arrows) are present. **e** and **f** are insulin (red)/GFP (green) and β gal (blue) channels of **d**, respectively.

exocrine cells with β -galactosidase (Fig. 2d, f); no label was found in other cell types. After pAd-M3 injection, many β -galactosidase⁺ cells become insulin⁺ (Fig. 2d–f, pink cells), providing direct evidence that mature exocrine cells give rise to new insulin⁺ cells.

Induced β -cells closely resemble islet β -cells

We next examined the new insulin⁺ cells to determine the extent to which they have been reprogrammed. Morphologically, exocrine cells are large with a cobble stone appearance (Fig. 3a, b) whereas islet β -cells are much smaller and spindle shaped (Fig. 3a). When dissociated into single cells, the diameter of amylase⁺ exocrine cells range from 25 μ m to 17 μ m whereas insulin⁺ β -cells range from 9 μ m to 15 μ m. The induced cells are indistinguishable from islet β -cells in size and shape (Fig. 3b, c).

At the ultrastructural level, the reprogrammed cells have all the hallmarks of islet β -cells (Fig. 3d, e). They possess the small dense secretory granules characteristic of insulin granules, and lack the large zymogen granules and dense assemblies of endoplasmic reticulum that are characteristic of exocrine cells (Fig. 3d, e). Immunoelectron microscopy further showed that the induced β -cells express both GFP in the nucleus and abundant insulin in the granules (Supplementary Fig. 4). Interestingly, the induced β -cells often appeared on the electron micrograph as intercalated within exocrine acinar rosettes (Fig. 3e). In wild-type pancreatic samples, rare single or small clusters of β -cells reside outside islets, but they often associate with duct but not exocrine cells. The unique position of induced cells probably reflects their exocrine origin.

Molecular marker analysis reveals that most of the insulin⁺ cells co-express genes essential for β -cell endocrine function including glucose transporter 2 (Glut2, also known as Slc2a2, expressed in

92.8% of the new insulin⁺ cells), glucokinase (GCK, 96.7%), prohormone convertase (PC1/3, also known as Pcsk1, 86.7%; Fig. 4a–c and Supplementary Fig. 5), and the key β -cell transcription factors NeuroD (88.9%), Nkx2.2 (85.3%) and Nkx6.1 (85.9%; Fig. 4d–f and Supplementary Fig. 5). The induced insulin⁺ cells express C-peptide (part of proinsulin; Fig. 4h). Expression profile analysis of the reprogrammed cells further indicates a strong overlap of endocrine-enriched genes between reprogrammed cells and islet cells, suggesting a high degree of similarity between their endocrine programs (Supplementary Fig. 6).

The new β -cells do not express exocrine genes such as amylase or Ptf1a, the duct marker Ck19 (also known as Krt19), mesenchymal markers nestin and vimentin, nor the neuronal marker Tuji (β -tubulin III, also known as Tubb3) (Fig. 4g, Supplementary Fig. 5 and data not shown). Nor do the new β -cells express any other pancreatic hormones such as glucagon, somatostatin or pancreatic polypeptide (Fig. 4h, i and Supplementary Fig. 5). Thus, the new β -cells do not exhibit a hybrid or mixed phenotype, indicating silencing of non- β -cell programs.

The primary function of β -cells is to synthesize and release insulin. To facilitate the release of insulin into the circulation, β -cells, unique among pancreatic cell types, synthesize vascular endothelial growth factor (VEGF), which promotes local angiogenic remodelling²⁸. Notably, induced β -cells similarly synthesize VEGF and induce angiogenesis so that blood vessels form next to these new cells (Fig. 5a, b). Quantification indicates that, in nGFP controls, 32% of infected cells lie adjacent to blood vessels whereas 61% and 83% of induced β -cells are directly juxtaposed to blood vessels 10 days and 30 days after induction, respectively.

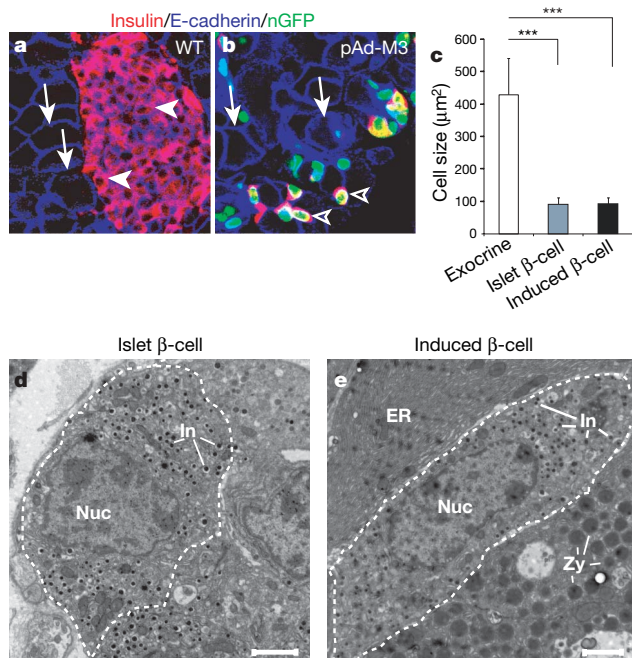


Figure 3 | Endogenous and induced β -cells are indistinguishable in morphology and ultrastructure. **a, b**, Islet β -cells (**a**, arrowheads) and induced β -cells (**b**, arrowheads) are similar in size and shape but distinctly different from exocrine cells (**a, b**, arrows). E-cadherin staining was used to visualize cell boundaries. **c**, Size comparison of exocrine cells, islet β -cells and induced β -cells. Data are presented as mean \pm s.d., $n = 3$ animals. > 100 cells per animal were used. Three asterisks, $P < 0.001$. **d**, Electron micrograph of a β -cell (outlined) in an islet. **e**, Example of an induced β -cell situated between two exocrine cells. Endogenous and induced β -cells contain small insulin granules (In) and lack zymogen granules (Zy) of exocrine cells and extensive endoplasmic reticulum (ER). Nuc, nucleus. Scale bars, 2 μ m.

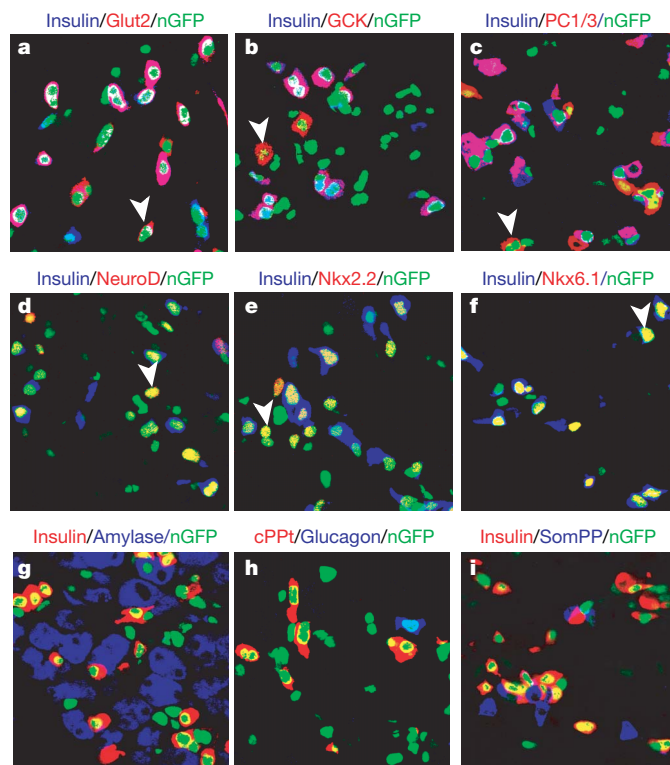


Figure 4 | Molecular marker characterization of induced β -cells. **a–f**, One month after infection with pAd-M3, most insulin⁺ induced β -cells co-express endocrine genes: glucose transporter 2 (Glut2, **a**), glucokinase (GCK, **b**), prohormone convertase 1/3 (PC1/3, **c**) and β -cell transcription factors NeuroD (**d**), Nkx2.2 (**e**) and Nkx6.1 (**f**). Arrowheads indicate examples of cells that express marker genes but not insulin. **g–i**, Induced β -cells do not express amylase (**g**), glucagon (**h**) or somatostatin/pancreatic polypeptide (**i**, SomPP). cPPT, c-peptide.

To test whether induced β -cells release insulin, mice were rendered diabetic by streptozotocin (STZ) injection, which specifically ablates islet β -cells. When subsequently injected with pAd-M3, fasting blood glucose levels of hyperglycaemic animals showed a significant and long-lasting improvement compared to animals injected with control (nGFP) virus (Fig. 5d). In addition, the pAd-M3 animals showed increased glucose tolerance (Supplementary Fig. 7), had increased insulin levels in the serum (non-fasting, $P < 0.01$, Fig. 5e) and possessed large numbers of induced β -cells (Fig. 5f). Polymerase chain reaction with reverse transcription (RT-PCR) analysis and direct observation revealed that virus injected into the pancreas does not spread to other internal organs such as liver and intestine that, theoretically, could modulate insulin secretion and/or response (Supplementary Fig. 8). In addition, we found no evidence that STZ-treated animals show spontaneous conversion of exocrine cells to β -cells (Supplementary Fig. 8). As the data in Fig. 5 show, the total number of induced β -cells is rather small compared to the number of β -cells in normal animals and this may account for the limitation to the effectiveness in restoring glucose homeostasis. Alternatively, because the new β -cells are not reorganized into islet structures, this may limit their effectiveness. Together, these data show that induced β -cells can produce and secrete insulin *in vivo*.

Inducing factors are required only transiently

Our results thus far support the contention that a combination of three transcription factors fully reprograms exocrine cells to β -cells *in vivo*. To determine whether continued presence of these factors is required to maintain the phenotype of reprogrammed cells, we used RT-PCR and primers specific to viral transgenes to detect their presence. Transgene expression from all three viruses was substantially diminished after 1 month and was undetectable after 2 months

(Supplementary Fig. 9). Ngn3 protein was undetectable by antibody staining 1 month after infection (Supplementary Fig. 9). Pdx1 and Mafa protein expression in the induced β -cells, however, remains consistently strong even after 2 months, indicating the activation of endogenous genes (Supplementary Fig. 9). These results are consistent with the fact that endogenous islet β -cells do not express Ngn3, but do express Pdx1 and Mafa^{21,22}. Thus, a transient expression of the inducing factors is sufficient to convert exocrine cells to a stable new β -cell state.

β -cell reprogramming does not involve dedifferentiation

In principle, the conversion of exocrine cells to β -cells could be direct or involve dedifferentiation to common progenitors that then redifferentiate into β -cells. Indeed, exocrine and β -cells share a common progenitor during embryogenesis that is characterized by rapid division and expression of genes including *Sox9* and *Hnf6* (also known as *Onecut1*; ref. 20). Continuous 5-bromodeoxyuridine (BrdU) labelling over the first 10 days of reprogramming, however, shows that few induced β -cells (3.2%) have divided (Supplementary Fig. 3). In comparison, 12.9% of endogenous islet β -cells in the same animals incorporated BrdU (Supplementary Fig. 3). In addition, we detected no induction of *Sox9* or *Hnf6* (data not shown). These results suggest that *in vivo* reprogramming of exocrine to β -cells is a direct conversion of cell types and does not involve dedifferentiation. We can not formally exclude the possibility that a very transient or partial dedifferentiation may occur, but our results indicate that extensive replication and reversion to a dedifferentiated cell for an appreciable time does not occur.

Discussion

Our results provide evidence that fully differentiated exocrine cells can be directly reprogrammed into cells that closely resemble β -cells

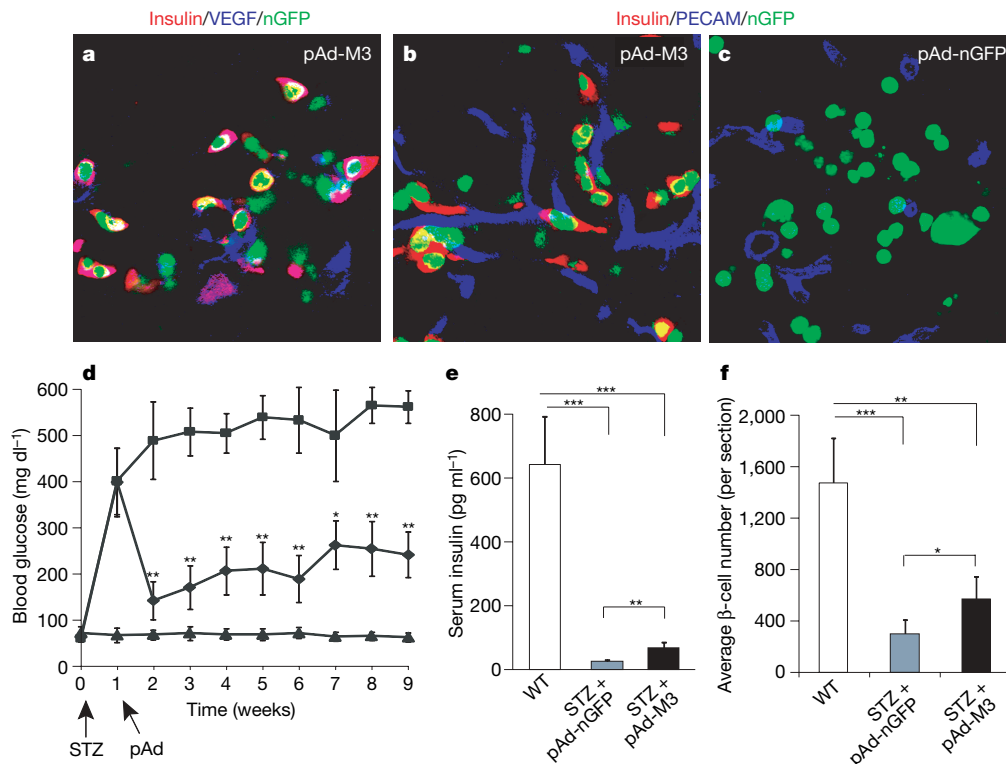


Figure 5 | Induced new β -cells remodel vasculature and ameliorate hyperglycaemia. **a–c**, New β -cells synthesize VEGF (**a**) and induce local angiogenic remodelling (**b**). Note the proximity of blood vessels (PECAM⁺) to the induced β -cells (**b**) versus control infected cells (**c**). **d**, Improvement of fasting blood glucose level in diabetic mice after injection with pAd-M3 (diamond) compared to controls with nGFP virus (square). Triangle,

non-diabetic controls. STZ, streptozotocin. Arrows indicate timing of injection. $n = 6–8$ animals. **e**, Non-fasting serum insulin levels 6 weeks after injection. $n = 6–8$ animals. **f**, Average insulin⁺ β -cell number per section 8 weeks after injection. $n = 3$ animals. Both islet and induced β -cells were counted for the pAd-M3 samples. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. Data are presented as mean + s.d.

in adult animals by a combination of just three transcription factors. The three reprogramming factors, *Ngn3*, *Pdx1* and *Mafa*, are known to be important in the embryonic development of pancreas and β -cells^{21,22}. In contrast, many additional factors are also required for β -cell development^{21,22}. Further studies will be necessary to understand why this particular combination is sufficient for adult β -cell reprogramming.

The induced β -cells do not organize into islet structures and remain as single cells or small clusters. Signalling between β -cells inhibits basal insulin secretion and enhances glucose-stimulated insulin secretion²⁹. The lack of organization of induced β -cells undoubtedly impairs their function. Strategies that promote aggregation of the induced β -cells in adult should help to restore full glucose responsiveness.

There have been previous attempts to convert adult liver cells to β -cells *in vivo* by expressing pancreatic transcription factors^{27,30–32}. These factors were able to induce expression of some pancreatic genes, but not phenotypic or morphological conversion into functional β -cells^{27,30–32}. Mature exocrine cells can turn on endocrine programs when dissociated and cultured *in vitro*^{24,25,33,34}. Interestingly, dissociation itself is apparently sufficient to initiate endocrine programs whereas the addition of growth factors is necessary for cell survival^{24,25,33,34}. However, the molecular mechanisms of this process remain largely unknown. Other studies have shown that pancreatic duct cells and liver cells could be induced to express certain β -cell gene products in culture^{35–37}. Most of these studies, however, did not address whether these cells possess a hybrid phenotype. In addition, RT-PCR on populations of cells, instead of single-cell resolution immunohistochemistry, was routinely used to evaluate the expression of β -cells markers. It is unclear how many cells actually expressed these markers or at what level. Finally, β -cells exhibit highly unstable phenotypes when cultured and appear to transform into fibroblast-like cells^{38,39}. *In vitro* generation of β -cells will probably require suitable culture conditions that have yet to be discovered.

It is surprising that the reprogramming of exocrine cells to β -cells does not involve multiple rounds of cell proliferation. It is generally thought that epigenetic changes that underlie reprogramming events are most easily made during cell division². It may be the case that many reprogramming events do indeed involve obligatory proliferation steps⁴. In contrast, reprogramming of B lymphocytes to macrophages seems to be cell-cycle-independent¹⁶. Early SCNT experiments also provided evidence for reprogramming without DNA replication⁴⁰.

Reprogramming of exocrine cells to β -cells occurs at a relatively fast speed, with the first insulin⁺ cells appearing at day 3, and with efficiency of up to 20%. This is in contrast with recent reports of reprogramming fibroblasts to embryonic stem cells^{8–13}, where it takes a considerably longer time (7–30 days) and the efficiency is much reduced (typically less than 0.1%). This may be due to the fact that pancreatic exocrine and β -cells are closely related cell types and share much of their epigenomes whereas the epigenomes of fibroblasts and embryonic stem cells are largely dissimilar. Conversion between exocrine and β -cells may therefore require fewer epigenetic changes.

Recent advances in mammalian cellular reprogramming with defined genes collectively point to the possibility that a limited number of factors could reprogram any given adult cell to a different type of cell such as a stem cell, a committed progenitor or another mature cell type. All these studies relied on knowledge of the normal development of these cell types, which enabled the manipulation of key developmental regulators in adult cells. This approach may prove to be a general strategy for directing adult cell reprogramming. The recent reprogramming of human skin cells to iPS cells raises the possibility of generating patient-specific human embryonic stem lines for therapies^{9,10,13}. This would be the first step in a process that will then require directed differentiation of the iPS cells to produce therapeutically important cell types such as neurons, cardiomyocytes or pancreatic β -cells. In principle, patient-specific cell therapies

could be achieved more directly by reprogramming abundant and easily accessible patient-specific human cells such as fibroblasts, blood cells or adipocytes.

METHODS SUMMARY

Adenovirus construction and purification. Genes of interest were first cloned into a shuttle vector containing an internal ribosome entry site linked to nuclear GFP (*IRES-nGFP*), and then into the pAd/CMV/V5-DEST adenoviral vector (Invitrogen). High titre virus ($>1 \times 10^{10}$ plaque-forming units (p.f.u.) per ml) was obtained by purification with the AdEasy Kit (Stratagene).

Animals, surgery and physiological studies. *Rag1*^{-/-} and *Rag1*^{-/-}; *NOD* animals were obtained from Jackson Laboratories. Adult animals (>2 months old) were injected with 100 μ l ($>1 \times 10^9$ p.f.u.) of purified adenovirus directly into the splenic lobe of the dorsal pancreas. Blood glucose was measured with Ascensia Elite blood glucose meter. Insulin levels were determined with an Ultrasensitive insulin ELISA kit (Alpco).

Immunohistochemistry, BrdU labelling and TUNEL analysis. This was performed as previously described⁴¹. BrdU (1 mg ml⁻¹) was provided in drinking water for BrdU labelling after surgery. Apoptotic cells were recognized by TUNEL (terminal dUTP nick-end labelling) with a TMR red cell death kit (Roche).

Electron microscopy. Dissected pancreas was fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for 2 h at room temperature (24 °C). For conventional transmission electron microscopy, samples were further fixed by osmium tetroxide, embedded in Epon resin and sectioned at 60–80 nm. For immunogold labelling, ultrathin sections were cut at -120 °C and stained with gold-conjugated antibodies. Images were obtained with a Tecnai G² Spirit BioTWIN transmission electron microscope.

FACS analysis and gene profiling. Pancreas was digested with liberase and elastase (Roche) to single cells. GFP⁺ cells were isolated by FACS with FACSaria (BD Bioscience). Biotin-labelled complementary RNA probes were synthesized with the Illumina TotalPrep RNA Amplification kit (Ambion). Gene profiling was performed with Sentrix BeadChip Array MouseRef-8 v1.1 (Illumina). Data were analysed with the BeadStudio software.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The microarray data were deposited in the Gene Expression Omnibus (GEO) under accession number GSE12025. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to D.A.M. (dmelton@harvard.edu).

METHODS

Viral injection and tissue collection. For adult pancreas, ~100 μ l purified virus was injected directly into 2–3 foci of the dorsal splenic lobe with a 3/10 cc Insulin Syringe (Becton Dickinson). For skeletal muscle, ~20 μ l virus was injected into the upper thigh. At the time of tissue collection, the infected portion of the tissue was visualized by GFP fluorescence and dissected out. For adult pancreas, typically ~50% of the dorsal pancreas was taken.

Immunohistochemistry. Adult mouse pancreata were fixed by immersion in 4% paraformaldehyde for 2 h at 4 °C. Samples were subsequently incubated in 30% sucrose solution overnight (6–12 h) and embedded with optimal cutting temperature compound (Tissue-Tek).

The following primary antibodies were used: rat anti-E-cadherin (Zymed), rat anti-Pecam1 (PharMingen), goat anti-Ngn3 (Santa Cruz), guinea-pig anti-insulin (Dako), guinea-pig anti-glucagon (Linco), guinea-pig anti-Pancreatic polypeptide (Linco), rabbit anti-somatostatin (Dako), rabbit anti-pancreatic polypeptide (Dako), goat anti-somatostatin (Santa Cruz), goat anti-Pdx1 (Santa Cruz), guinea-pig anti-Pdx1 (gift from C. Wright), goat anti- β -galactosidase (Biogenesis), goat anti-amylase (Santa Cruz), mouse anti-BrdU (Amersham), rabbit anti-mafA (Bethyl), chick anti-*nestin* (Ames), chick anti-*vimentin* (Chemicon), goat anti-Glut2 (Santa Cruz), goat anti-VEGF (R&D), rabbit anti-PC1/3 (Chemicon), goat anti-glucokinase (Santa Cruz), rabbit anti-Ck19 (Melton laboratory stock), rabbit anti-chromogranin A/B (RDI), rabbit anti-Ptf1a (gift from H. Edlund), goat anti-*NeuroD* (Santa Cruz), rabbit anti-Nkx6.1 (BCBC), rabbit anti-*Sox9* (Santa Cruz), goat anti-Nkx2.2 (Santa Cruz) and rabbit anti-c-peptide (Linco).

Rodamin-Red-X-, FITC-, Cy5- and Alexa-dye-conjugated donkey secondary antibodies were obtained from the Jackson Immunoresearch Laboratories and Molecular Probes Inc. Tyramide amplification system (PerkinElmer) was used for PC1/3 and glucokinase staining. Immunofluorescence pictures were taken with a Zeiss LSM 510 META confocal microscope.

***Cpa1CreER^{T2}* labelling of mature exocrine cells.** *Cpa1CreER^{T2};R26R* double heterozygous animals were generated by mating homozygous *Cpa1CreER^{T2}* males with *R26R* homozygous females (Jackson laboratory). Two-month-old *Cpa1CreER^{T2};R26R* adults were injected with tamoxifen at 6 mg per animal every third day four times to label mature exocrine cells.

Physiological studies. Diabetic animals were produced with intraperitoneal injection of streptozotocin (120 μ g per g body weight) after overnight fasting with 2-month-old adult animals of the Rag1 strain (Jackson laboratory). Hyperglycaemic animals that displayed >250 mg dl⁻¹ fasting blood glucose levels for at least two consecutive days were used for experiments.

Fasting blood glucose was measured on tail-vein blood with an Ascensia Elite glucometer (Bayer) after 6–8 h fasting. The non-fasting insulin level was determined from tail-vein blood collected around 9 to 10 am with an Ultrasensitive Insulin ELISA kit (Alpco).

The average β -cell number per section was determined by sectioning through the entire pancreas at 15 μ m and collecting every third section. Twenty randomly selected sections were immunostained for insulin and 4,6-diamidino-2-phenylindole (DAPI) to visualize individual β -cells. The total number of β -cells was counted and averaged from three animals.

The glucose tolerance test was performed by fasting animals overnight (12 h), followed by intraperitoneal injection of glucose (3 g per kg body weight).

Electron microscopy. Small pieces of pancreatic samples (1–2 mm) were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 2 h at room temperature.

For conventional electron microscopy, samples were further refixed with a mixture of 1% osmium tetroxide (OsO₄) plus 1.5% potassium ferrocyanide (K₃Fe(CN)₆) for 2 h, were washed in water and stained in 1% aqueous uranyl acetate for 1 h followed by dehydration in grades of alcohol (50%, 70%, 95%, 2 \times 100%) and propyleneoxide (1 h), and then infiltrated in propyleneoxide:Epon 1:1 overnight and embedded in TAAB Epon (Marivac

Canada Inc.). Ultrathin sections (about 60–80 nm) were cut on a Reichert Ultracut-S microtome, picked up on to copper grids, stained with 0.2% lead citrate and examined in a Tecnai G² Spirit BioTWIN transmission electron microscope. Images were taken with an AMT CCD camera.

For immunoelectron microscopy, fixed samples were infiltrated with 2.3 M sucrose in PBS for 30 min then frozen in liquid nitrogen. Frozen samples were sectioned at –120 °C, the sections transferred to formvar–carbon-coated copper grids and floated on PBS until the immunogold labelling was carried out.

The gold labelling was carried out at room temperature on a piece of parafilm. All antibodies and protein-A gold were diluted in 1% BSA. The diluted antibody solution was centrifuged for 1 min at >10,000g before labelling to avoid possible aggregates. Grids were floated on drops of 1% BSA for 10 min to block unspecific labelling, transferred to 5- μ l drops of primary antibody and incubated for 30 min. The grids were then washed in four drops of PBS for a total of 15 min, transferred to 5- μ l drops of protein-A gold (G. Posthuma) for 20 min, and washed in four drops of PBS for 15 min and six drops of double-distilled water.

For double labelling, after the first protein-A gold incubation, grids were washed in four drops of PBS for a total of 15 min and then transferred to a drop of 0.2% glutaraldehyde in PBS for 5 min, and washed in four drops of PBS/0.15 M glycine (to quench free aldehyde groups). Following this, the second primary antibody was applied, followed by PBS wash and different size protein-A gold as described previously. The antibodies used were rabbit anti-GFP (Invitrogen) and guinea-pig anti-insulin (Dako).

Contrasting/embedding of the labelled grids was carried out on ice in 0.3% uranyl acetate (Electron Microscopy Sciences) in 2% methyl cellulose (Sigma) for 10 min. Grids were picked up with metal loops (diameter slightly larger than the grid) and the excess liquid was removed by streaking on a filter paper (Whatman, number 1), leaving a thin coat of methyl cellulose (bluish interference colour when dry).

The grids were examined in a Tecnai G² Spirit BioTWIN transmission electron microscope and images were recorded with a 2k AMT CCD camera.

FACS analysis, islet isolation and gene profiling. For FACS sorting of GFP⁺ cells, pancreata infected by the M3 inducing factors for one month were perfused through the common bile duct, digested with liberase and elastase (Roche), and further dissociated into single cells with EDTA incubation. GFP⁺ cells were isolated by FACS with FACSaria (BD Bioscience). Staining of sorted cells indicates that ~70% of total sorted cells are GFP⁺ and ~22% are insulin⁺.

Islets were isolated by liberase digestion of the pancreas of Pdx1–GFP animals. Islets were picked manually under a fluorescent dissecting scope. Pancreatic cells devoid of GFP⁺ islets were collected as the non-islet sample.

RNA was extracted with Trizol reagent (Invitrogen). Biotin-labelled cRNA probes were synthesized with the Illumina TotalPrep RNA amplification kit (Ambion). Gene profiling was performed with Sentrix BeadChip Array MouseRef-8 v1.1 (Illumina) that contains probes for ~19,000 genes. Data were analysed with BeadStudio software. For identifying differentially enriched genes, the following parameters suggested by Illumina were used: *P* value < 0.05, Diff score > 30, average signal > 100.

RT–PCR. Pancreatic tissues were harvested and immediately frozen in liquid nitrogen. Total RNA was extracted with the RNeasy kit (Qiagen). First-strand cDNA was synthesized with Superscript III kit (Invitrogen). Thirty cycles of semiquantitative RT–PCR were performed using the standard protocol. The following primer pairs were used: *Ngn3* viral transgene: ~350 bp, *Ngn3*:F: CAGACGCTGCGCATAGCGGACCAC, IRES2.R: GCGGCTTCGGCCAGTAA CGTTAG. *Pdx1* viral transgene: ~1.2 kb, *Pdx1*:F: GGAGCAAGATT GTGCGGTGACCTC, IRES2.R: GCGGCTTCGGCCAGTAAACGTTAG. *Mafa* viral transgene: ~300 bp, *Mafa*:F: ACATTCTGGAGAGCGAGAAGTGCC, IRES2.R: GCGGCTTCGGCCAGTAAACGTTAG. *GADPH*: ~400 bp, F: ACCA CAGTCCATGCCATCAC, R: TCCACCACCTGTTGCTGTA.