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Pancreatic β-Cell Neogenesis by Direct Conversion from Mature α-Cells

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ABSTRACT

Because type 1 and type 2 diabetes are characterized by loss of β-cells, β-cell regeneration has garnered great interest as an approach to diabetes therapy. Here, we developed a new model of β-cell regeneration, combining pancreatic duct ligation (PDL) with elimination of pre-existing β-cells with alloxan. In this model, in which virtually all β-cells observed are neogenic, large numbers of β-cells were generated within 2 weeks. Strikingly, the neogenic β-cells arose primarily from α-cells. α-cell proliferation was prominent following PDL plus alloxan, providing a large pool of precursors, but we found that β-cells could form from α-cells by direct conversion with or without intervening cell division. Thus, classical asymmetric division was not a required feature of the process of α- to β-cell conversion. Intermediate cells coexpressing α-cell- and β-cell-specific markers appeared within the first week following PDL plus alloxan, declining gradually in number by 2 weeks as β-cells with a mature phenotype, as defined by lack of glucagon and expression of MafA, became predominant. In summary, these data revealed a novel function of α-cells as β-cell progenitors. The high efficiency and rapidity of this process make it attractive for performing the studies required to gain the mechanistic understanding of the process of α- to β-cell conversion that will be required for eventual clinical translation as a therapy for diabetes. STEM CELLS *2010;28:1630–1638*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

β-cell regeneration to replace the cells lost in both type 1 and type 2 diabetes is an attractive approach to therapy. To study β-cell regeneration, a number of models of pancreatic damage have been developed, including chemical and genetic β-cell ablation, partial pancreatectomy, and pancreatic duct ligation (PDL), in which the pancreatic duct is ligated between the head and tail of the organ. Each of these models has resulted in studies that claim to show β-cell regeneration, either by replication of pre-existing β-cells or by neogenesis from endogenous progenitors [1]. Genetic lineage tracing studies have found evidence for β-cell replication as the predominant mechanism [2], while other studies have found evidence for neogenesis from ducts [3, 4]. Previously, we used a lineage tracing methodology with lentiviral vectors to demonstrate that epithelial cells from the human exocrine pancreas could be induced to undergo endocrine differentiation under the influence of inductive factors from the human fetal pancreas [5].

In some cases, differences in the mechanism by which β-cell regeneration occurs could be attributed to differences in the model, for example, partial pancreatectomy versus PDL. In others, discrepancies between genetic lineage trace studies using the same model may be attributed to differences in the transgenic mice being used, for example, different promoters are used to drive cre recombinase expression. A striking example of this comes from the results of genetic lineage tracing studies of the fate of ductular epithelial cells following PDL. One study using a fragment of the carbonic anhydrase II promoter to drive the expression of tamoxifen-inducible cre found substantial β-cell neogenesis from CAII-positive precursors [3], while another study using the hepatocyte nuclear factor 1 beta (HNF1b) promoter to drive inducible cre expression found no evidence for β-cell neogenesis [6]. Thus, the issue of whether β-cell neogenesis occurs in adults at all remains unresolved.

A significant problem with most previous studies is the persistence of pre-existing β-cells which can confound the assessment of the origin and extent of β-cell neogenesis. Most recently, a model using complete β-cell ablation with diphtheria toxin found evidence for β-cell neogenesis from α-cells [7]. However, in this damage model, several months were required before significant β-cell neogenesis could be achieved from α-cells. The low efficiency not only makes it difficult to study α-cell to β-cell conversion but also raises concerns about the potential for clinical translation. To study β-cell neogenesis better, we developed a new model that

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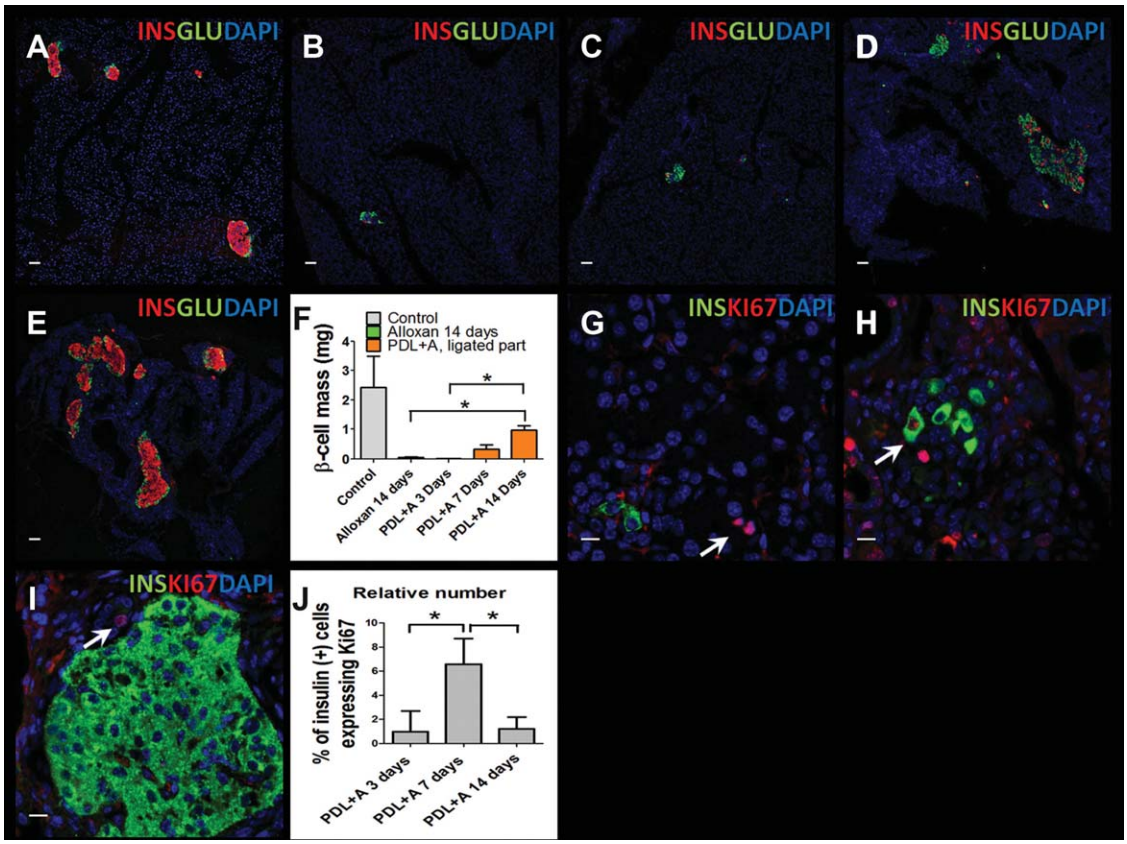

Figure 1. Alloxan plus PDL induces rapid and robust β-cell neogenesis in the adult mouse pancreas. Representative sections from the pancreas of a normal control (**A**), a mouse 14 days after alloxan injection (**C**), and from the distal pancreas 3 (**B**), 7 (**D**), and 14 (**E**) days following alloxan and PDL. Sections were immunostained with antibodies against insulin (red) and glucagon (green). Nuclei were visualized with DAPI (blue). Scale bar = 50 μm. Alloxan injection eliminated 99% of pre-existing β-cells by 3 days after injection, while α-cells remained intact (compare [**A**] with [**B**], quantified in [**F**]). There was no change in the β-cell mass between 3 and 14 days after alloxan injection ([**B, C**], quantification in [**F**]). α-Cell hyperplasia and numerous insulin (+) cells appear at day 7 post PDL+A (**D**). Large islets mainly composed of β-cells are seen 14 days post PDL+A (**E**). Quantification of β-cell mass (**F**). β-cell replication does not contribute significantly to β-cell regeneration in the PDL+A model (**G–J**). The replication of insulin (+) cells (green) at 3 days (**G**), 7 days (**H**), and 14 days (**I**) after PDL+A was analyzed by immunohistochemistry for KI67 (red). β-cell replication was low at all time points (quantification in [**J**]). Arrows indicate representative cells positive for KI67. Scale bars = 10 μm. Data are presented as mean $\pm$ SD, $n = 3$ animals; *, $p < .05$. Abbreviations: DAPI, DAPI (4′,6-diamidino-2-phenylindole); PDL, pancreatic duct ligation.

combines PDL with quantitative elimination of pre-existing β-cells using alloxan, which enters cells through the glut2 transporter and generates reactive oxygen species that induce cell death [8]. By combining PDL and alloxan (PDL+A) treatment, we created a model in which virtually all β-cells that appear in the pancreas must arise by neogenesis rather than replication, allowing for a quantitative assessment of the degree of β-cell regeneration by a neogenic mechanism.

Unlike PDL or alloxan individually, in which there was little evidence of β-cell neogenesis, PDL plus alloxan resulted in rapid and robust β-cell neogenesis, with the formation of islets composed almost entirely of neogenic β-cells within 2 weeks. These new β-cells did not arise from the ductular epithelium, which was considered to be the source of β-cell progenitors on the basis of most previous studies. Rather, PDL plus alloxan resulted in the recruitment of a β-cell precursor pool residing in the α-cell population, which responded quickly to environmental cues by replication and differentiation. Induction of insulin in intermediate cells coexpressing glucagon and other β- and α-cell markers, as well as substantial replication of α-cells, occurred within a week after PDL plus alloxan. Two weeks after treatment, the wave of β-cell neogenesis from α-cells had substantially abated, with the formation of islets primarily composed of neogenic β-cells. The rapidity of the process made it possible to follow β-cell formation over time, allowing us to recognize and characterize the process by which α-cells converted into β-cells.

In summary, this study presents a new model of β-cell regeneration in which there is efficient and rapid in vivo α-cell to β-cell conversion.

Materials and Methods

Animal and Animal Procedures

All animal experiments were approved by the "Institutional Animal Care and Use Committee" of the Sanford-Burnham Medical Research Institute and in accordance with national regulations. Six- to ten-week-old C57/B6 or ICR (Institute of Cancer Research) mice (Harlan Sprague Dawley, Inc.; Placentia, California, USA) were injected intravenously with Alloxan (Sigma Aldrich, St. Louis, Missouri, USA) at 90 mg/kg. PDL was conducted essentially as described [4, 9] with the following minor modifications. Approximately 50% of the pancreas (the head) remained proximal to the ligation. For the

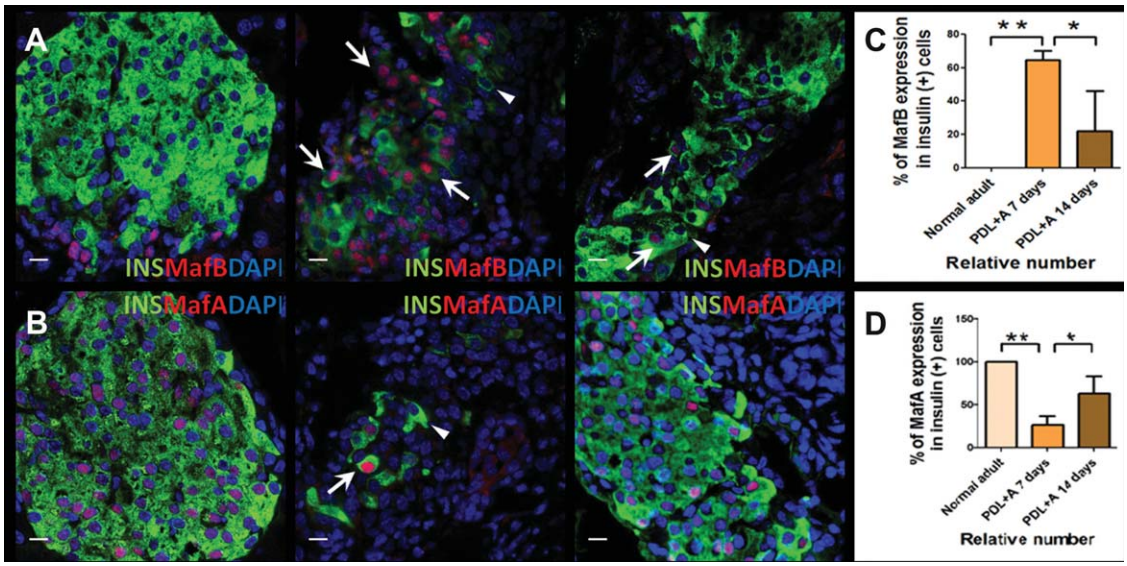

Figure 2. Neogenic insulin (+) cells are initially immature but mature over time. Mouse pancreas from normal adult ([**A**] and [**B**], left panel), 7 days after PDL+A ([**A**] and [**B**], middle panel), and 14 days after PDL+A ([**A**] and [**B**], right panel) were examined. Costaining with insulin (green), MafB (red), and DAPI (blue) in (**A**) and insulin (green), MafA (red), and DAPI (blue) in (**B**) are shown. As expected, mature normal adult β-cells expressed MafA, not MafB (left panel in [**A**] and [**B**]). At day 7 after PDL+A, numerous insulin (+) cells expressed MafB (middle panel in [**A**]) and only a small number of insulin (+) cells expressed MafA (middle panel in [**B**]). However, at day 14 post PDL+A, numerous insulin (+) cells expressed MafA (right panel in [**B**]) and only a small number of insulin (+) cells expressed MafB (right panel in [**A**]), consistent with β-cell maturation over time. Scale bar = 10 μm (**A, B**). Quantification of MafB (**C**) and MafA (**D**) expression in insulin (+) cells. Data are presented as mean ± SD, $n = 3$; *, $p < .05$. **, $p < .01$. Abbreviations: DAPI, DAPI (4′,6-diamidino-2-phenylindole); PDL, pancreatic duct ligation.

mice receiving PDL+A, PDL was performed 30 minutes after alloxan injection. Only mice with a blood sugar greater than 500mg/dl (checked 48 hours after alloxan injection) were selected for experiments. Those mice were injected subcutaneously once daily with insulin glargine (Sanofi-Aventis, Paris, France). The initial insulin dose was 1 unit per mouse (around 25 mg in weight). If hyperglycemia was not controlled at that dose, it was raised gradually until diabetes was controlled. Even with careful management of diabetes, the mortality following PDL plus alloxan was high, ∼50%.

Immunohistochemical Staining

Tissue was fixed in 4% formaldehyde for 6 hours at 4°C, washed in PBS (Phosphate buffered saline), followed by overnight in 30% sucrose at 4°C, then embedded in OCT (Optimal Cutting Temperature) compound and frozen at −80°C. Cryosections of 5 μm thickness were incubated with antisera specific for insulin (1/200, guinea pig, USBIO, Swampscott, Massachusetts, USA), insulin (1/200, rabbit, Santa Cruz Biotechnology, Santa Cruz, California, USA), glucagon (1/2,000, mouse, Sigma-Aldrich, St. Louis, Missouri, USA), glucagon (1/50, rabbit, Abcam, Cambridge, Massachusetts, USA), cytokeratin (Wide Spectrum Screening; 1/500, rabbit, DakoCytomation, Glostrup, Denmark), somatostatin (1/200, goat, Santa Cruz Biotechnology, Santa Cruz, California, USA), KI67 (1/50, mouse, BD Pharmingen, San Diego, California, USA), MafA (1/100, rabbit, Bethyl Laboratories, Montgomery, Texas, USA), MafB (1/100, rabbit, Bethyl Laboratories, Montgomery, Texas, USA), PDX1 (1/2,000, goat, Abcam, Cambridge, Massachusetts, USA), Nkx6.1 (1/1,000, mouse, Beta Cell Biology Consortium, Nashville, Tennessee, USA), 5-bromodeoxyuridine (BrdU; mouse, GE healthcare, Piscataway, New Jersey, USA), and C-peptide (1/100, rabbit, Cell Signaling, Danvers, Massachusetts, USA). Secondary antibodies for detection of guinea pig, rabbit, goat, or mouse antibodies were labeled with: Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA), Rhodamine Red (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA), Cy5 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Nuclei were visualized with DAPI (4′,6-diamidino-2-phenylindole) (Sigma Aldrich, St. Louis, Missouri, USA).

Quantitation of β-Cell Mass

β-cell mass was calculated as the relative β-cell area multiplied by pancreatic weight. For the quantitative analysis of insulin (+) cell area, we studied sections spaced 100 μm apart from each other from the tail of the pancreas per mouse. These sections were incubated with antisera to insulin (1:200, rabbit, Santa Cruz Biotechnology, Santa Cruz, California, USA) and the bound antibody was visualized by DAB (3,3′-Diaminobenzidine) (Vector Laboratories, Burlingame, California, USA). The nuclei were stained by Hematoxylin (Surgipath, Richmond, Illinois, USA). All slides were scanned and analyzed by using the Aperio ScanScope XT system (version 10, Aperio Technologies, Vista, California).

Image and Statistical Analysis

All images were acquired by confocal scanning microscopy (Radiance 2100/AGR-3Q BioRad Multi-photon Laser Point Scanning Confocal Microscope). Images were processed with Image J software. The colocalization patterns in Figure 4, Supporting Information Figures 3 and 4 were determined by the colocalization threshold algorithm in WCIF image J. Confocal z stacks were captured at 0.5 μm increments. Three-dimensional reconstructions and measurements were performed by using Volocity software (version 5.3). Statistical significance of cell-specific changes in controls versus experimental groups was calculated by an unpaired Student's *t*-test. We considered *p*-values below .05 as statistically significant. For all statistical analysis, Graphpad Prism 5 was used. All results are expressed as mean ± SD.

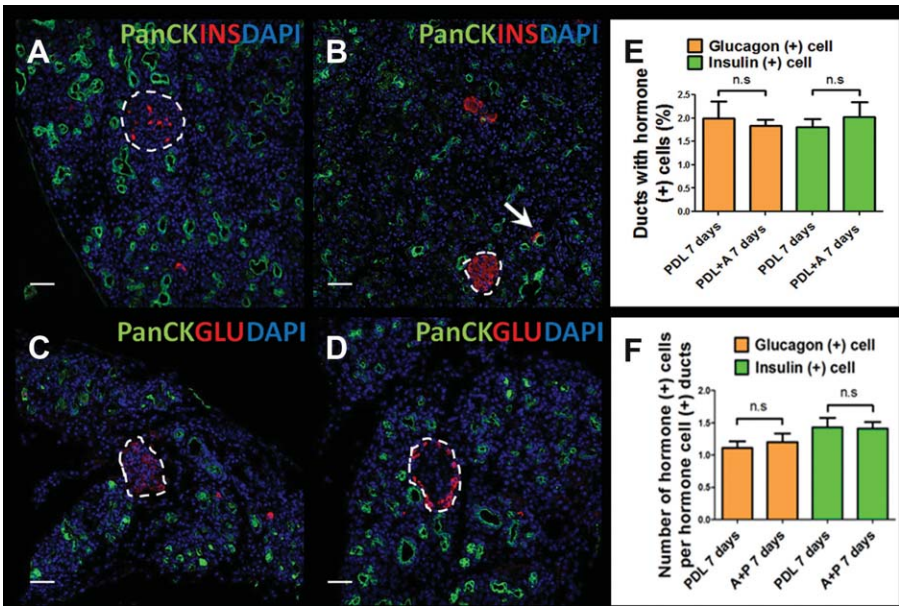

Figure 3. Endocrine neogenesis from ducts does not change with PDL+A compared with PDL alone. Representative pancreatic sections 7 days after PDL+A (**A, C**) and 7 days after PDL alone (**B, D**). Sections were immunostained with antibodies against insulin (red in [**A**] and [**B**]), glucagon (red in [**C**] and [**D**]), and pancytokeratin (PanCK, green). DAPI (blue) was used to visualize nuclei. Scale bar = 50 μm. Islets are outlined with white dashed lines. Insulin (+) and glucagon (+) cells in the ducts are rare in both PDL+A and PDL alone models (**A–D**). The arrow in (**B**) indicated an insulin (+) cell in the hyperplastic duct. Quantification of the frequency of ducts with insulin (+) or glucagon (+) cells (**E**). Quantification of the number of endocrine cells in each duct containing endocrine cells (**F**). In (**E**) and (**F**), over 5,000 ducts were counted in the insulin group. Over 4,000 ducts were counted in the glucagon group. Data are presented as mean ± SD, $n = 3$ animals. Student's t-test results are shown. Abbreviations: DAPI, (4′,6-diamidino-2-phenylindole); n.s., not significant; PanCK, pancytokeratin; PDL, pancreatic duct ligation.

Results

β-Cell Neogenesis Following PDL Plus Alloxan Is Rapid and Robust

To determine the extent to which β-cell mass in adults can be regenerated by neogenesis, as opposed to replication, we combined PDL with elimination of pre-existing β-cells using the β-cell toxin alloxan. Following PDL plus alloxan, greater than 99% of β-cells were eliminated (compare Fig. 1A with Fig. 1B, quantitated in Fig. 1F), leaving residual clusters of α-cells marking the location of pre-existing islets (Fig. 1B). Two weeks later, there was no evidence of β-cell regeneration with alloxan treatment alone (Fig. 1C, Supporting Information Fig. 1D, quantitated in Fig. 1F). PDL alone resulted in the appearance of insulin- and glucagon-positive cells within the hyperplastic ducts that are present in that model, but such cells were uncommon (Fig. 3B, 3D) [4, 10].

PDL plus alloxan resulted in a dramatically different regenerative response than alloxan alone. Two weeks following PDL plus alloxan, large islets were found in the ligated but not unligated part of the pancreas (Fig. 1E, Supporting Information Fig. S1A–S1C, quantitated in Fig. 1F). Some islets present 2 weeks after PDL plus alloxan were substantially larger than control islets (compare Figs. 1A, 4B with Fig. 1E). Thus, the combination of alloxan and PDL resulted in the induction of rapid and robust β-cell regeneration. However, while the α-cells existed in a small cluster 3 days following alloxan treatment, by 14 days, the islets had the same relative arrangement of α- and β-cells as islets from control animals, with the α-cells surrounding the β-cells. This suggests that the sorting mechanism responsible for separating α- and β-cells during embryonic development was preserved in the adult pancreas.

β-Cells in the PDL Plus Alloxan Model Are Newly Formed and Do Not Arise by Replication of Pre-Existing β-Cells

The β-cells that appeared at 2 weeks following PDL plus alloxan must have arisen either by replication of pre-existing β-cells that were not eliminated by alloxan or by neogenesis from a precursor. Given that almost all pre-existing β-cells were eliminated by alloxan, residual β-cells (less than 1% of pre-existing β-cells, Fig. 1) could account for regeneration only if there was highly efficient and rapid replication of the rare remaining β-cells. To address this possibility, we determined the frequency of β-cell replication at different times following PDL plus alloxan. By Ki67 immunohistochemistry, the frequency of replicating insulin-positive cells 3 days following alloxan plus PDL was very low (0.98%; Fig. 1G, quantified in Fig. 1J). It increased transiently to 6% by day 7 (Fig. 1H), but had decreased to basal levels by day 14 (Fig. 1I, quantified in Fig. 1J). Given the low level of β-cell replication, the small number of residual β-cells cannot account for the insulin-positive cells at days 7 and 14.

To further examine the nature of the β-cells that were found at 7 days following PDL plus alloxan, we examined the expression of the transcription factors MafA and MafB. MafB is expressed in α-cells and in developing β-cells, and it is then replaced by MafA as β-cells mature [11, 12]. In the adult, all β-cells express MafA (Fig. 2B, 2D), with no MafB expression being detectable (Fig. 2A, 2C) [12]. When mature β-cells replicate, MafA continues to be expressed, with no dividing β-cells expressing MafB (Supporting Information Fig. S2) [12]. Thus, if the insulin-positive cells found following PDL plus alloxan were derived from pre-existing β-cells that survived the alloxan treatment, they should be uniformly positive for MafA, while if they arose from a neogenic process, they could express either MafA or MafB, depending on the extent of maturation.

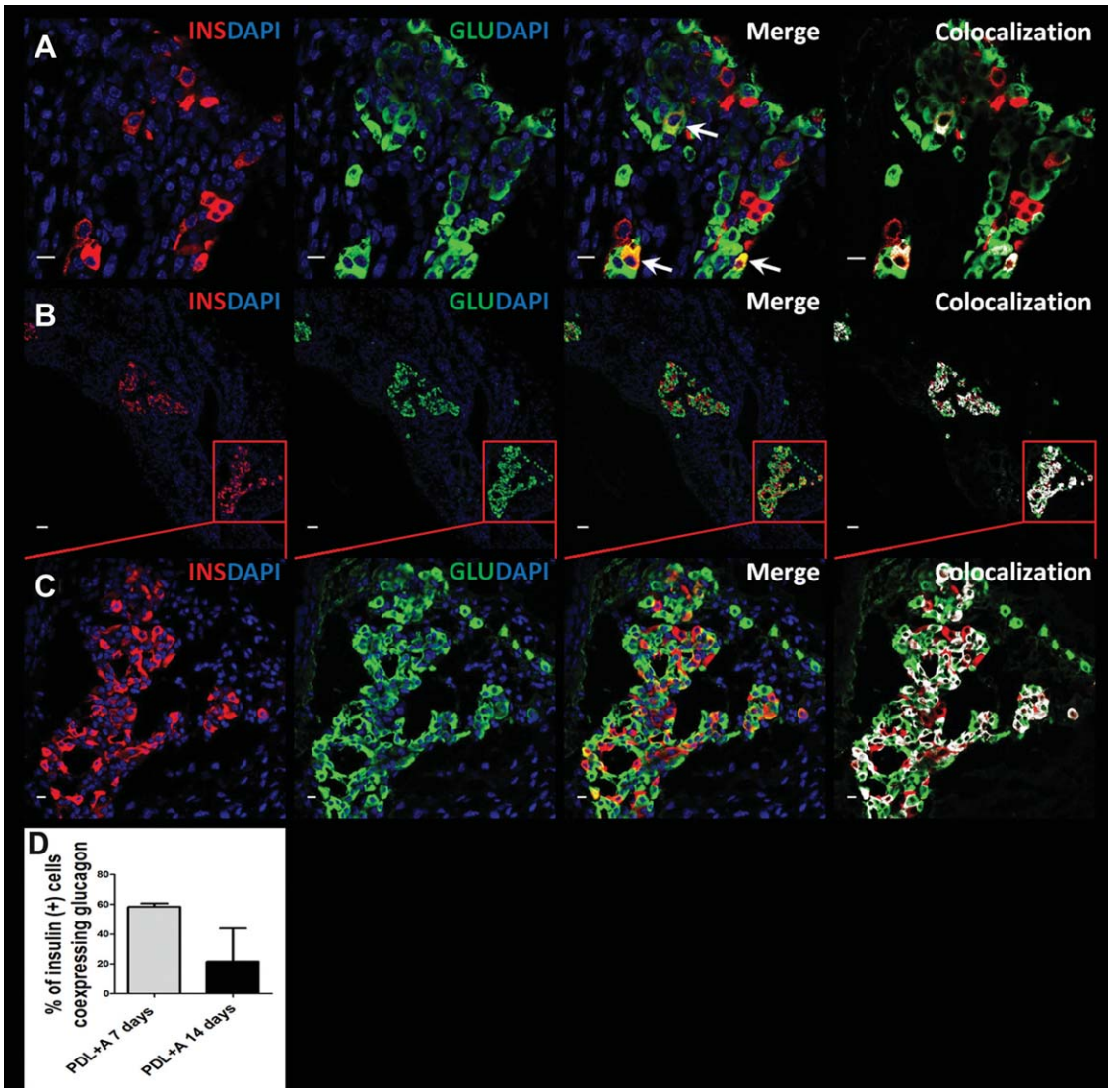

Figure 4. Neogenic β-cells induced by PDL plus alloxan arise from pre-existing α-cells. Photomicrographs of adult mouse pancreas seven (**A**) and 14 (**B, C**) days after PDL+A. The boxed regions in (**B**) are shown at high power in (**C**). Insulin (red) and glucagon (green) staining show numerous cells coexpressing both markers. Colocalization of insulin and glucagon (white) was determined by the colocalization threshold algorithm in Image J (right panel). Scale bar = 10 μm (**A, C**); 50 μm (**B**). Quantification of insulin (+) cells coexpressing glucagon (**D**). Data are presented as mean ± SD, $n = 3$ animals. Abbreviations: DAPI, (4′,6-diamidino-2-phenylindole); PDL, pancreatic duct ligation.

Seven days following PDL plus alloxan, 64% of insulin-positive cells expressed MafB (Fig. 2A, 2C), while 26% expressed MafA (Fig. 2B, 2D). This indicates that the great majority of insulin-positive cells must be newly formed and have arisen from a source other than replication of pre-existing β-cells, and further indicates that the neogenic process involves an intermediate that has at least some characteristics of an immature β-cell. By 14 days following alloxan plus PDL, the ratio of MafB/MafA expressing β-cells had reversed, with 63% expressing MafA (Fig. 2B, 2D), and only 22% expressing MafB (Fig. 2A, 2C), as would be expected with β-cell maturation.

Neogenic β-Cells Do Not Arise Predominantly from Progenitors in the Duct

Having ruled out replication of pre-existing β-cells as a source of the regenerating β-cells following PDL plus alloxan, we concluded that the vast majority of insulin-positive cells must have arisen by neogenesis from a progenitor. Although substantial controversy exists [6, 13], a number of studies have reported that duct epithelial cells can function as endocrine progenitors in the PDL model [3, 4]. Thus, it was important to examine the possibility that duct cells were the source of the neogenic β-cells following PDL plus alloxan.

Given the tremendous increase in the number of β-cells that occurred following PDL plus alloxan compared with PDL alone, where none of the pre-existing β-cells were eliminated, the frequency of insulin-positive cells within ducts would have to be much higher in PDL plus alloxan compared with PDL alone if duct cells were the source of the neogenic β-cells. Thus, we examined the occurrence of insulin-positive cells within ducts in the PDL and PDL plus alloxan models, finding that there was no difference in the number of ducts that contained insulin-positive cells (Fig. 3A, 3B, 3E) or in the number of insulin-positive cells per duct (Fig. 3F). As it was recently shown that ectopic expression of Pax4 under the

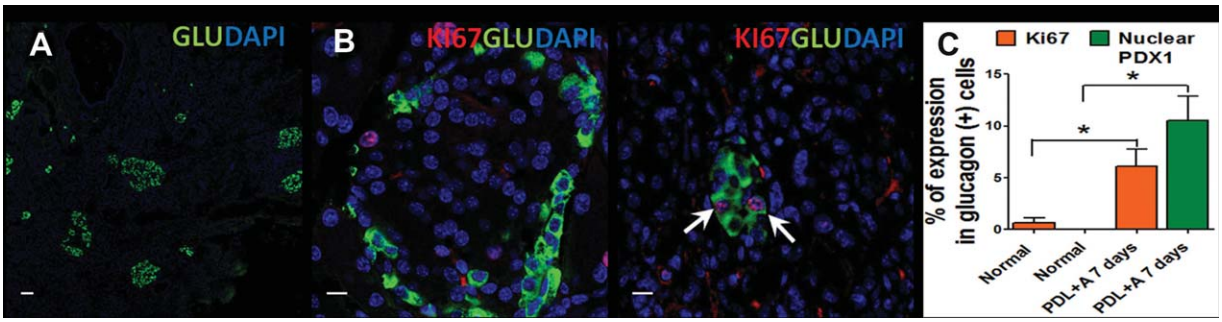

Figure 5. PDL plus alloxan induces α-cell proliferation and PDX1 expression. PDL+A induced α-cell proliferation. Seven days after PDL+A, the endocrine compartment of the ligated part of the pancreas was mainly composed of hyperplastic α-cells (green) (**A**). Most α-cells in the normal adult mouse pancreas did not express Ki67 ([**B**], left panel). However, PDL+A induced a large increase in Ki67 expression in α-cells (arrows in [**B**], right panel). Quantification of Ki67 and PDX1 expression in α-cells (**C**). Data are presented as mean + SD, $n = 3$ animals. *, $p < .05$. Scale bar = 50 μm (**A**); 10 μm (**B**). Abbreviations: DAPI, (4′,6-diamidino-2-phenylindole); PDL, pancreatic duct ligation.

control of the glucagon promoter could shift the lineage of newly formed glucagon-positive cells in ducts to a β-cell lineage [14], we examined the occurrence of glucagon-positive cells within ducts. Similar to the insulin-positive cells, there was no difference in the number of ducts that contained glucagon-positive cells (Fig. 3C, 3D, 3E) or in the number of glucagon-positive cells per duct (Fig. 3F). Thus, we concluded that duct-associated insulin or glucagon-positive cells were highly unlikely to be the major source of neogenic β-cells.

Additional evidence that the neogenic β-cells were not arising primarily from ducts came from the fact that the insulin-positive cells appeared in large numbers in association within pre-existing islets primarily composed of the α-cells that persisted following alloxan treatment (Figs. 1D, 3A, 7A) rather than outside the pre-existing islets. This led us to conclude that the neogenic β-cells were likely to be arising from a progenitor located within the pre-existing islets.

α-Cells Replicate and Differentiate Efficiently into β-Cells in the PDL and Alloxan Model

Apart from β-cells, the two most common cell types in the islet are α- and δ-cells. Because δ-cells have been reported to transdifferentiate into β-cells following low-dose streptozotocin treatment [15], we performed double-labeling by immunohistochemistry to look for transitional cells coexpressing somatostatin and insulin. A very low frequency of coexpressing cells was found (Supporting Information Fig. S3), making it unlikely that this pathway was a significant contributor to the large number of neogenic β-cells in the islet.

By 7 days following PDL plus alloxan, α-cell hyperplasia and numerous β-cells were present (Figs. 1D, 5A). This was not found with alloxan (Fig. 1C) or PDL (Fig. 3D) alone. The replication rate of glucagon-positive cells increased from almost undetectable levels to 5% (Fig. 5B, 5C).

Strikingly, at 1 week following PDL plus alloxan, 58% of insulin-positive cells coexpressed glucagon (Fig. 4A), suggesting that α-cells could be the source of the neogenic β-cells. By 14 days following PDL plus alloxan, colocalization had decreased. Overall 21% of insulin-positive cells coexpressed glucagon (Fig. 4D). However, in some cases, substantial colocalization of insulin and glucagon was still present at 14 days (Fig. 4B, 4C, Supporting Information Fig. S4, Supporting Information Movie S1). Insulin-positive cells at day 14 also expressed C-peptide (Supporting Information Fig. S7).

If α-cells were the source of neogenic β-cells, the transcription factors that contribute to cell type-specific hormone expression should play a role in α-cell to β-cell conversion, and intermediates expressing both β-cell and α-cell markers should be present. Thus, we determined the expression of the transcription factors MafA, PDX-1, and Nkx6.1, which in the adult pancreas are restricted to β-cells and MafB, which is restricted to mature α-cells. (Figs. 2, 6A, 6C, 6E, 6G). In the PDL plus alloxan model, 11% of α-cells began to express PDX1 within 1 week (Figs. 5C, 6B). Some of the α-cells expressing PDX-1 started to coexpress insulin (Fig. 6I). α-cells expressing Nkx6.1 (Fig. 6D), another β-cell marker, as well as insulin, and Nkx6.1 (Fig. 6J) were also found, suggesting that the induction of Nkx6.1 in α-cells was also an early event in α-cell to β-cell conversion. Intermediates coexpressing glucagon, PDX-1, MafB (Fig. 6F) and insulin, glucagon, MafB (Fig. 6H) were present as well. Compared with normal adult pancreas, the frequency of MafB expression in insulin-positive cells increased at day 7 and then decreased at day 14, suggesting that the expression of MafB is still preserved in the early stage of α-cell to β-cell conversion, but is gradually turned off and replaced by MafA as neogenic β-cells become mature (Fig. 2). The model of α- to β-cell conversion is summarized in Supporting Information Figure S5.

α-Cell Replication and α- to β-Cell Conversion Are Independent Processes

The occurrence of both α-cell hyperplasia and α- to β-cell conversion raised the possibility that the two phenomena might be linked, that is, that asymmetric division of replicating α-cells might be required for conversion to β-cells. The alternative was direct α- to β-cell conversion through a nonreplicative intermediate. To distinguish between those possibilities, we performed continuous labeling with BrdU. If replication was required for β-cell neogenesis, then almost all β-cells present at 14 days following PDL plus alloxan should be positive for BrdU, while if replication and β-cell neogenesis from α-cells were unlinked, then neogenic β-cells could be either BrdU-positive or negative. Consistent with the latter model, we found insulin-positive cells that were BrdU-negative and BrdU-positive (Fig. 7A). Interestingly, we found numerous cells that were negative for BrdU but coexpressed insulin and glucagon, consistent with direct conversion without a replicative intermediate (Fig. 7A). This was further supported by examination of Ki67, which was absent from many cells coexpressing insulin and glucagon (Fig. 7B). Some α-cells remained negative for BrdU at 14 days following PDL plus alloxan (Fig. 7A), consistent with an origin of these α-cells from pre-existing islets rather than neogenic α-cells from a replicative precursor, for example, a duct cell. Overall, it shows that α-cells could directly convert into β-cells with or

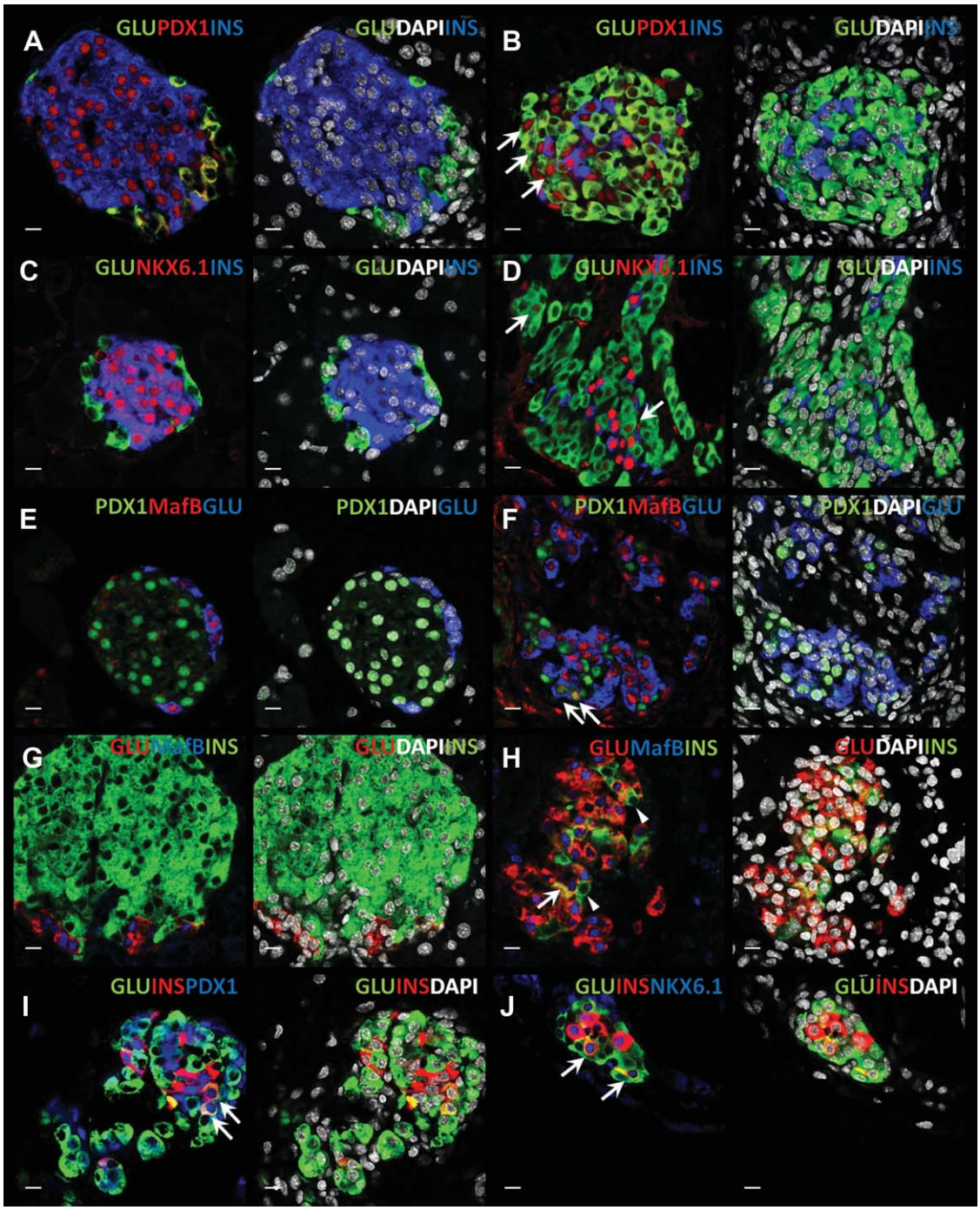

Figure 6. Characterization of endocrine cells following PDL+A. (**A, C, E, G**): Endocrine cells from normal adult pancreas. (**B, D, F, H, I, J**): Endocrine cells are from 7 days post PDL+A. As expected, glucagon (+) cells in the normal adult pancreas did not express PDX1 (**A**). PDL+A induced PDX1 expression in glucagon (+) cells ([**B**], representative cells indicated by arrows). As expected, glucagon (+) cells in adult pancreas did not express Nkx6.1 (**C**). PDL+A induced Nkx6.1 expression in glucagon(+) cells ([**D**], representative cells indicated by arrows). As expected, glucagon (+) cells in the normal adult mouse pancreas expressed MafB, but not PDX1 (**E**). PDL+A induced MafB (red) and PDX1 (green) coexpression in glucagon (+) cells ([**F**], yellow nuclei indicated by arrows). As expected, mature β-cells in the normal adult pancreas did not express MafB (**G**). PDL+A induced cells coexpressing insulin (green), glucagon (red), and MafB (blue; [**H**], a representative cell indicated by the arrow). Cells (+) only for insulin did not express MafB (arrowheads). Cells coexpressing insulin (red) and glucagon (green; yellow cells indicated by arrows) also expressed nuclear PDX1 (**I**) and Nkx6.1 (**J**). Scale bar = 10 μm (**A–J**). Abbreviation: DAPI, (4′,6-diamidino-2-phenylindole).

without intervening replication and asymmetric division of α-cells is not required to generate new β-cells. The processes of α-cell replication and α- to β-cell conversion are unlinked in the PDL plus alloxan model.

Effect of Age on β-Cell Neogenesis in the PDL Plus Alloxan Model

β-cell replication declines precipitously with age [16], but little is known about the effect of age on β-cell neogenesis, as

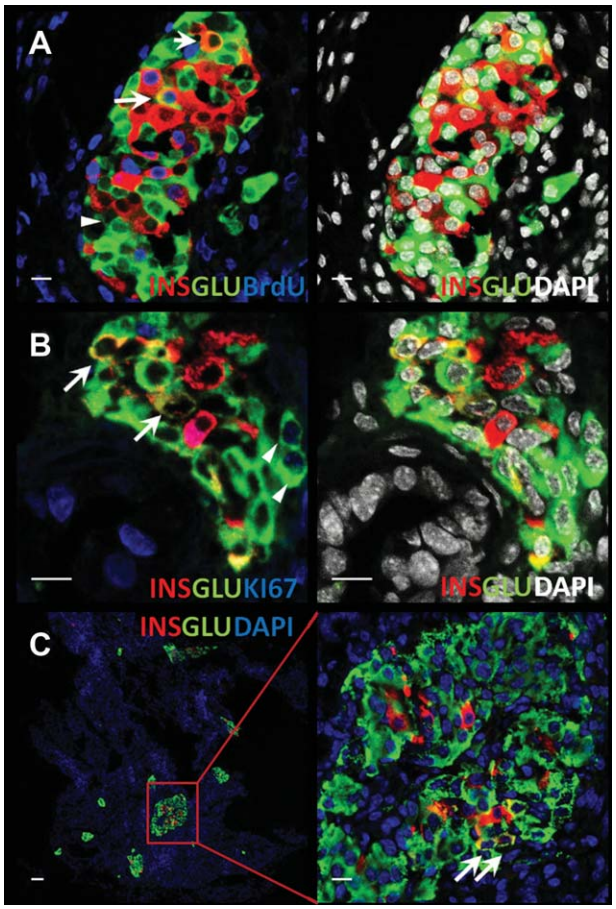

Figure 7. Replication is not required for α- to β-cell conversion (**A, B**). Continuous labeling with BrdU for 12 days (day 3–14 after PDL+A) (**A**). Cells (yellow) coexpressing insulin (red) and glucagon (green) can develop with (long arrow) or without (short arrow) having replicated as indicated by BrdU incorporation. The lack of BrdU incorporation in some α-cells (denoted by an arrowhead) in this islet is expected for α-cells that were part of a pre-existing islet. Immunohistochemistry, insulin (red), glucagon (green), and KI67 (blue), 7 days after PDL+A reveals cells coexpressing insulin and glucagon (yellow cells) that are negative for Ki67 (**B**). Arrowheads denotes KI67 (+) and glucagon (+) cells. β-cell neogenesis in mature mice (**C**). Pancreatic sections from 7-month-old mice 14 days after PDL+A. The boxed area is shown at high power on the right. Transitional cells coexpressing insulin and glucagon (arrows) still formed in mature mice. Scale bar = 10 μm ([**A, B**] and right panel of [**C**]); 50 μm (left panel of [**C**]). Abbreviations: BrdU, 5-bromodeoxyuridine; DAPI, (4′,6-diamidino-2-phenylindole).

most studies of β-cell regeneration have been performed in juvenile animals. Thus, we examined mature mice (7 months of age) for β-cell neogenesis in the PDL plus alloxan model. Although there was a decline in β-cell neogenesis with age, substantial numbers of neogenic β-cells still formed in 7-month-old mice (Fig. 7C, Supporting Information Fig. S6). This indicates that induction of β-cell neogenesis may be a viable approach for the treatment of diabetes even in mature adults.

Discussion

The studies presented here demonstrate for the first time that β-cell neogenesis from α-cells can be a rapid and robust process, resulting within 2 weeks in the formation of islets in which essentially all of the β-cells are neogenic (Fig. 4). Most previous studies using different damage models such as partial pancreatectomy concluded that β-cell neogenesis did not occur, at least to a significant extent [2]. Even studies in which β-cell neogenesis was proven to occur, including our previous study with human pancreatic exocrine cells [5], did not demonstrate a high efficiency of β-cell differentiation from progenitors [3], leading to skepticism about the extent to which β-cell neogenesis could occur and consequent doubts about the possibility of it being a viable route to enhance β-cell mass in patients with diabetes [17]. A recent study in which essentially complete elimination of β-cells was accomplished using a transgenic approach to express the diphtheria toxin receptor in β-cells also found evidence for α- to β-cell conversion, although it took several months to generate significant number of β-cells [7]. Also, the efficiency of α- to β-cell conversion in that model was much lower than with PDL plus alloxan. The rapidity and efficiency of in vivo α- to β-cell conversion in our model makes it much easier to study the process and also facilitates mechanistic studies required for future clinical translation.

The fact that the great majority of insulin-positive cells at day 7 expressed MafB, together with the almost complete β-cell ablation and low β-cell replication rate, excluded the possibility that pre-existing β-cells were the origin of new β-cells. We scrutinized carefully the conversion process to define the nature of intermediate cells. The intermediate cells coexpressing multiple α-cell and β-cell markers, providing strong evidence for α-cell to β-cell conversion. The rapidity of the process in our model compared with β-cell ablation using diphtheria toxin [7] allowed us to carefully examine the intermediates over time. Furthermore, the continuous BrdU labeling experiment and Ki67 expression showed that many transitional cells coexpressing insulin and glucagon during α-cell to β-cell conversion did not incorporate BrdU. This provided evidence that those insulin-positive cells were formed by direct conversion from adult α-cells without replication rather than from other potential progenitors, such as ductal cells or rare putative stem cells, which would need to proliferate extensively to generate the large number of β-cells present following PDL plus alloxan. Because of the large literature supporting a ductal origin for neogenic β-cells, we examined that possibility in great detail in our model, but there was no change in the frequency of endocrine neogenesis from ducts following PDL plus alloxan compared with PDL alone.

Some have put forth the possibility that β-cells might arise from sources, such as mesenchymal cells and neural cells within islets or dedifferentiated β-cells [18]. However, such cells cannot account for the time course of hormone expression in our model, in which large numbers of cells expressing glucagon alone begin to coexpress insulin and then progress to mature β-cells.

Conventionally, differentiation of a stem/progenitor cell involves replication of the progenitor with asymmetric division to provide for self-renewal of the stem/progenitor population as well as the formation of progeny with a different differentiated state. However, there has been increasing interest in direct conversion of one cell type into another. For example, direct conversion of fibroblasts into neurons and acinar into endocrine cells have been reported [19, 20]. But, in those cases, conversion required the introduction of multiple genes into the precursor cell. The direct conversion of α- to β-cells described here occurred under the influence of endogenous factors, raising the possibility that this phenomenon occurs normally as part of tissue homeostasis or in response to damage stimuli.

The signals that control α-cell proliferation and conversion into β-cells remain unknown. α-cell proliferation occurs in a number of settings, including type 2 diabetes and selective deletion of the insulin receptor in α-cells [21]. However, in

most cases, α-cell proliferation does not appear to be accompanied by substantial β-cell neogenesis. In PDL plus alloxan model, we found α-cells could rapidly replicate and differentiate to β-cells. However, the processes of α-cells replication and conversion into β-cells are unlinked. Thus, conversion may require an additional signal that is present in the environment of the duct-ligated pancreas. Ectopic expression of Pax4 in duct cells that expressed glucagon following PDL promoted insulin expression in those cells that could be inhibited by injection of glucagon [14]. However, in that case, the genetic alteration occurred during development and Pax4 expression in glucagon-positive cells in more mature animals did not result in β-cell conversion, suggesting that factors in addition to Pax4 are important in α-cell to β-cell conversion. Our result which greatly accelerated α-cell to β-cell conversion in vivo is important, as (in contrast to β-cells) α-cells are retained in type 1 diabetes, are increased in number in type 2 diabetes, and are cotransplanted with β-cells in islet transplantation. Thus, β-cell progenitors are present in essentially all of the clinical settings in which β-cell regeneration is desirable. However, clinical translation would require a method that does not require irreversible surgical intervention as with PDL. Although PDL plus alloxan promoted efficient β-cell neogenesis from α-cells, the mice did not achieve normoglycemia, most likely due to the profound and continuing inflammation and disruption of normal organ homeostasis that occur following PDL.

We found that even older animals were able to generate a considerable number of β-cells by α-cell conversion, albeit not as efficiently as in juvenile animals. Overall, the studies presented here provide strong evidence that β-cell neogenesis from α-cells can be a robust and efficient process and with further mechanistic understanding might be harnessed as a therapeutic for patients with diabetes.

Conclusion

In this study, we present a new model for β-cell neogenesis in which the β-cells arise primarily from mature α-cells. In this model, combining PDL and alloxan, adult α-cells rapidly replicate and convert efficiently into β-cells. However, replication with asymmetric division is not required for α-cell to β-cell conversion. The rapid and efficient β-cell neogenesis following PDL plus alloxan provides an attractive model to study the mechanism of α-cell to β-cell conversion which is important for eventual clinical translation.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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