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The Ectopic Expression of *Pax4* in the Mouse Pancreas Converts Progenitor Cells into α and Subsequently β Cells

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SUMMARY

We have previously reported that the loss of Arx and/or Pax4 gene activity leads to a shift in the fate of the different endocrine cell subtypes in the mouse pancreas, without affecting the total endocrine cell numbers. Here, we conditionally and ectopically express *Pax4* using different cell-specific promoters and demonstrate that *Pax4* forces endocrine precursor cells, as well as mature α cells, to adopt a β cell destiny. This results in a glucagon deficiency that provokes a compensatory and continuous glucagon$^+$ cell neogenesis requiring the re-expression of the pro-endocrine gene *Ngn3*. However, the newly formed α cells fail to correct the hypoglucagonemia since they subsequently acquire a β cell phenotype upon *Pax4* ectopic expression. Notably, this cycle of neogenesis and redifferentiation caused by ectopic expression of *Pax4* in α cells is capable of restoring a functional β cell mass and curing diabetes in animals that have been chemically depleted of β cells.

INTRODUCTION

The endocrine pancreas is organized in islets of Langerhans comprising five original cell subtypes, α, β, δ, ε, and PP cells, secreting glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide (PP), respectively (Collombat et al., 2006). The identification and characterization of the genetic determinants underlying endocrine pancreas morphogenesis and regeneration may potentially aid the design of cell replacement therapies to treat type 1 and 2 diabetes. In this context, a number of studies have demonstrated that, during development, the cooperation of several transcription factors successively specifies progenitor cells toward the pancreatic, endocrine, and ultimately islet cell fates. Hence, Pdx1 is required for pancreatic epithelium determination (Ahlgren et al., 1996, 1998; Grapin-Botton et al., 2001; Jonsson et al., 1994; Offield et al., 1996) and subsequently Neurogenin3 (Ngn3) for endocrine lineage specification (Gradwohl et al., 2000; Gu et al., 2002; Jensen et al., 2000; Johansson et al., 2007). Next to Ngn3 induction, a complex network of transcription factors, including Arx and Pax4, progressively and differentially promotes the particular endocrine fates (Collombat et al., 2003; Sosa-Pineda et al., 1997). In mice lacking *Arx*, the β and δ cell fates were found favored at the expense of α cell genesis, while the total endocrine cell content remained normal (Collombat et al., 2003). Conversely, in the absence of *Pax4*, the opposite phenotype was observed (Sosa-Pineda et al., 1997), indicating an inhibitory, cross-regulatory circuit between Arx and Pax4 (Collombat et al., 2005). Additional findings supported these conclusions and suggested that, first, Arx and Pax4 instruct endocrine precursor cells toward either an α cell or a β/δ cell fate, respectively. Next, through the analysis of double-mutant mice, a secondary function of Pax4 in specifying the β cell lineage in β/δ precursor cells was uncovered (Collombat et al., 2005). Recent evidence has demonstrated that the forced expression of *Arx* in early pancreatic cells drives endocrine progenitors toward either an α or, surprisingly, a PP cell fate (Collombat et al., 2007). It was therefore concluded that Arx is not only necessary, but also sufficient to instruct the α and PP cell lineages.

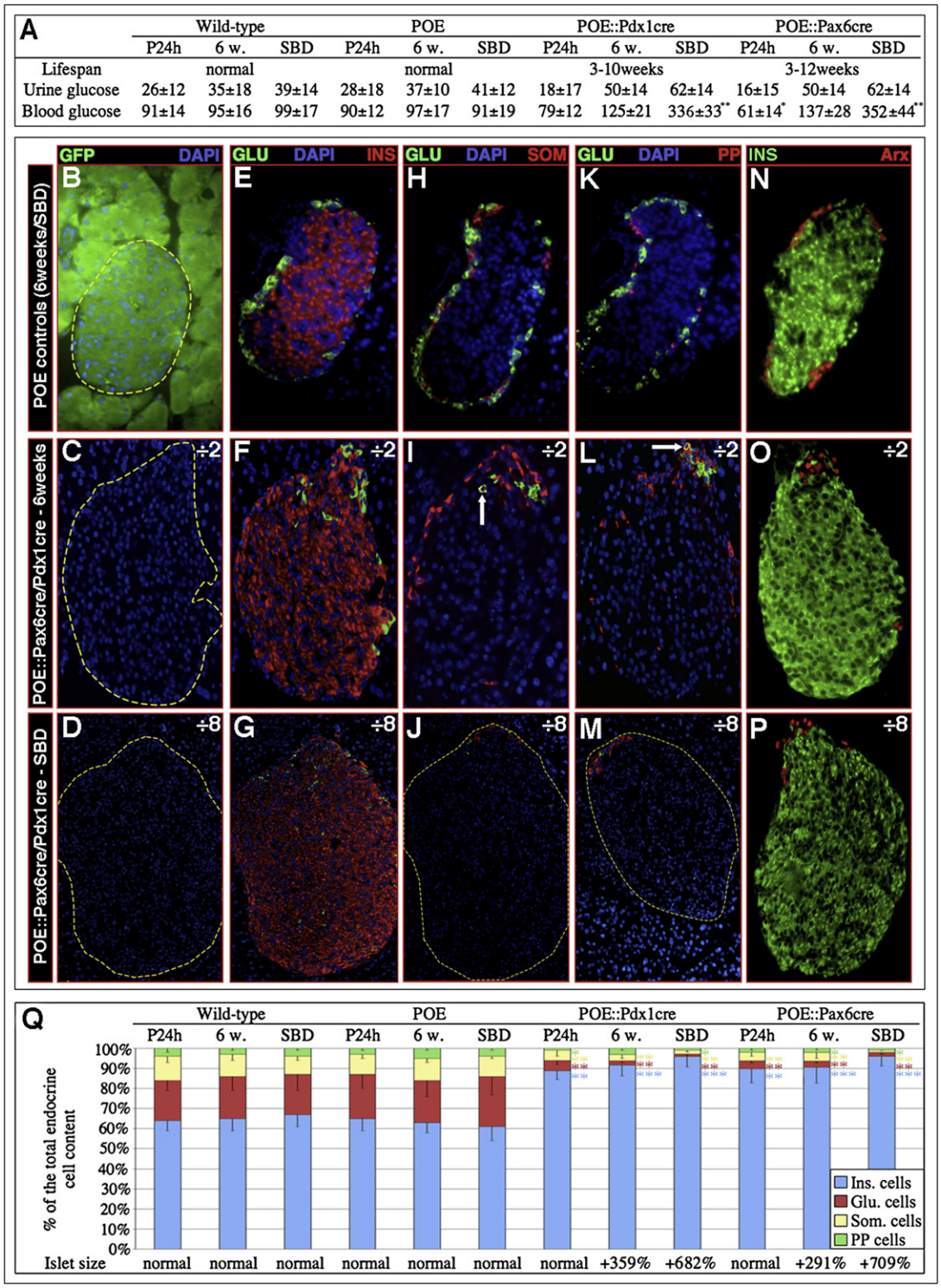


Figure 1. The Ectopic Expression of *Pax4* in *Pdx1* or *Pax6* Expression Domains Promotes the Genesis of Oversized Islets Mainly Containing Insulin-Expressing Cells

(A) Characterization of the life span and glycemia (in mg/dl) of POE::Pdx1cre and POE::Pax6cre mice 1 day and 6 weeks postpartum, as well as shortly before death (SBD). Note that initially, these animals are hypo- and subsequently hyperglycemic.

(B–P) Immunohistochemical analysis of the islets of double-transgenic mice. A dramatic increase in islet size and insulin-expressing cell mass is evident 6 weeks postpartum (C, F, I, L, and O) and is even more pronounced SBD (D, G, J, M, and P) as compared to representative control islets (B, E, H, K, and N). Concurrently,

Of particular interest was the finding that the forced expression of *Arx* in adult β cells induced their conversion into cells exhibiting α or PP cell phenotypes (Collombat et al., 2007). This discovery was of fundamental importance in the context of β cell-based therapy and implied that the opposite conversion might be achieved, that is, generation of β cells from other endocrine cells. To test this hypothesis, we generated mice conditionally expressing the *Pax4* gene. Our data indicate that the ectopic expression of *Pax4* in early pancreatic cells, and also in α cells, induces their respecification toward a β cell fate or identity. As a consequence of the ensuing glucagon deficiency, an ongoing neogenesis of α cells occurs. However, such α cells are continuously converted into β cells upon *Pax4* ectopic expression, resulting in the development of oversized islets of Langerhans. Importantly, a prominent expression of the proendocrine transcription factor Ngn3 in the pancreas of such animals is highlighted. Our results are consistent with the recently reported notion of facultative adult stem cells that reactivate *Ngn3* expression in injured pancreas (Xu et al., 2008). Finally, after streptozotocin (STZ)-induced depletion of β cells in young mice ectopically expressing *Pax4*, an α cell-mediated regeneration of the β cell mass, a progressive normalization of the glycemia, and an extended life span are observed.

RESULTS

Pax4 Ectopic Expression in the Developing Pancreas Promotes the Genesis of Oversized Islets Mainly Composed of β Cells

Taking advantage of the Cre-LoxP system, we generated transgenic mice able to conditionally express the *Pax4* gene (POE). Briefly, the construct used included the cytomegalovirus enhancer/chicken β-actin (CAG) promoter driving the expression of the *eGFP* gene followed by translation stop codons in all three frames and flanked by LoxP sites (Figure S1A available online). A *Pax4* cDNA-IRES-β-galactosidase sequence was subsequently cloned downstream of *eGFP*. Using pronuclear injection, we derived five independent transgenic mouse lines. The resulting mice constitutively expressed eGFP, but not β-galactosidase, at all ages examined (Figures S1B–S1E).

POE animals were bred with Pdx1cre or Pax6cre animals to target *Pax4* expression to the pancreatic epithelium or the endocrine tissue, respectively (Ashery-Padan et al., 2004; Gu et al., 2002). Double-transgenic POE::Pdx1cre and POE::Pax6cre mice were born and developed normally. However, animals of both genotypes eventually died at the age of 3 to 12 weeks, a majority after 9 weeks (Figure 1A and data not shown). To assess whether the ectopic expression of *Pax4* altered glucose homeostasis, we measured blood sugar levels. One day postpartum, a decrease in glycemia was evident in both POE::Pdx1cre and POE::Pax6cre genotypes (Figure 1A). Notably, at 6 weeks of age, blood glucose levels were found to be normal in these same animals compared to controls, whereas shortly before death (SBD), a dramatic hyperglycemia was uncovered. This indicates that POE::Pdx1cre and POE::Pax6cre mice initially present a hypoglycemic condition that progressively evolves toward a hyperglycemic one.

To assess the consequences of the forced expression of *Pax4* in *Pdx1* and *Pax6* expression domains, we performed immunohistochemical analyses of double-transgenic pancreata. As similar results were obtained in POE::Pax6cre and POE::Pdx1-cre mice, pictures representative of both genotypes are displayed for 6-week-old animals (Figures 1C, 1F, 1I, 1L, and 1O) and SBD (Figures 1D, 1G, 1J, 1M, and 1P). We did not observe any change in endocrine cell composition in POE animals as compared to their wild-type counterparts (Figures 1B, 1E, 1H, 1K, and 1N, and data not shown). The analysis of both POE::Pax6cre and POE::Pdx1cre pancreata showed a dramatic increase in islet size (note the 2- and 8-fold scale reduction in magnification in Figures 1C, 1F, 1I, 1L, and 1O and in Figures 1D, 1G, 1J, 1M, and 1P, respectively; Figures S2 and S3) containing mainly insulin-expressing cells (Figures 1F, 1G, 1O, 1P, S2, and S3) and only few δ- and Arx-labeled α and PP cells (Figures 1F, 1G, 1I, 1J, 1L, 1M, 1O, and 1P). Notably, the remaining α, δ, and PP cells were consistently located at one pole of the islet of Langerhans (Figures 1F, 1G, 1I, 1J, 1L, 1M, 1O, and 1P), a subset of these coexpressing the glucagon and somatostatin or PP hormones (arrows in Figures 1I and 1L, respectively). A quantification of these alterations (Figure 1Q, Table S1) ascertained (1) an age-dependent increase in islet size and β cell mass, concurrently with a reduction in non-β cell numbers (relative as well as absolute numbers), and (2) an abnormal location of the few remaining α, δ, and PP cells at one pole of the islet.

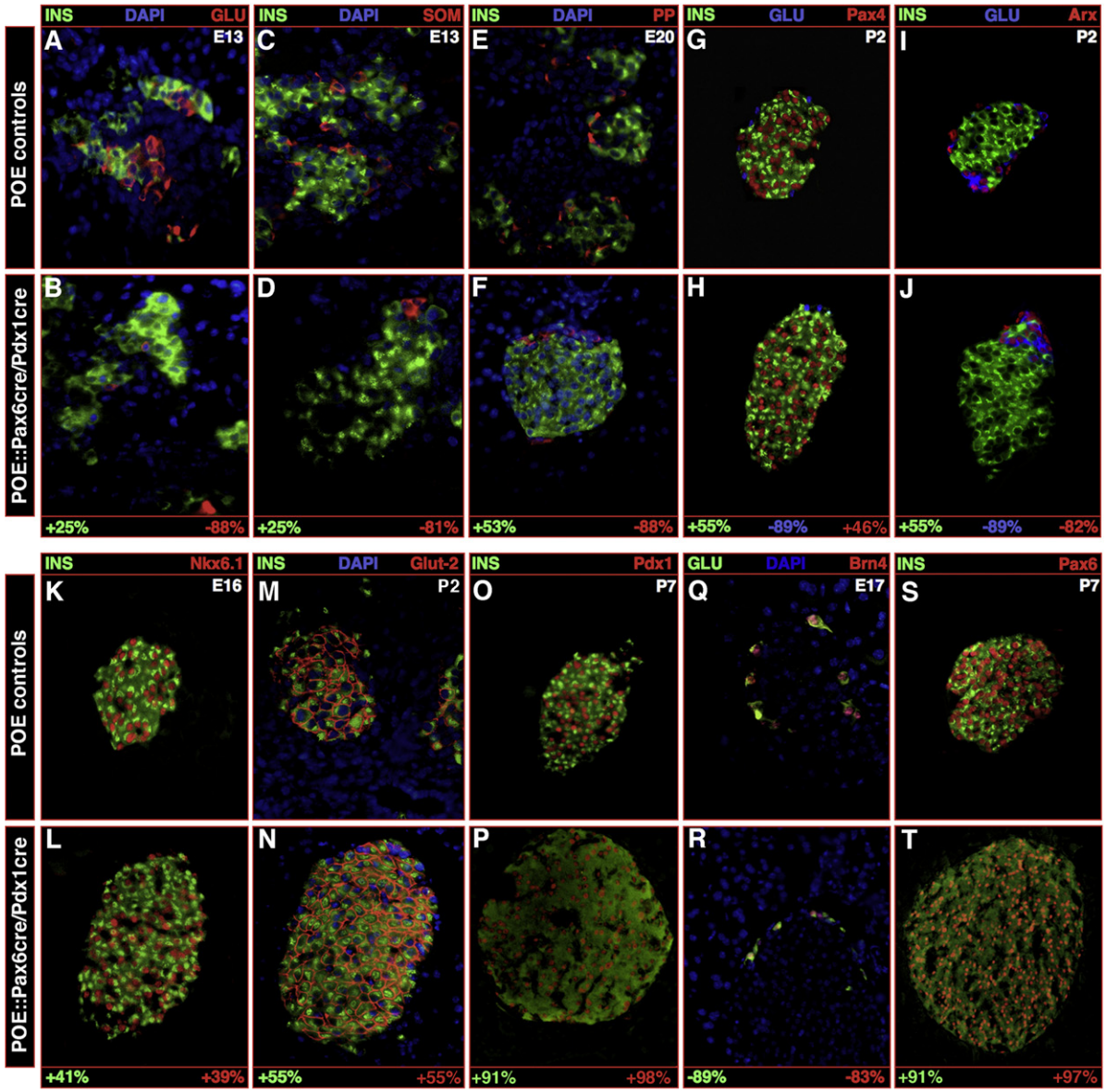
Pax4 expression was assayed with a newly developed antibody that detected Pax4 in wild-type embryonic pancreata (Wang et al., 2008) and specifically in adult β cells (Figures S4A, S4B, S5A, and S5D), as previously reported (Theis et al., 2004). Neither in *Pax4*-depleted nor in *Ngn3*-deficient islets did we find any expression of Pax4 (Figures S4C and S4D and data not shown). Importantly, in POE::Pax6cre mice, most insulin-producing cells were found positive for Pax4 (Figures S5E–S5H), whereas the vast majority of pancreatic cells were labeled in POE::Pdx1cre animals (Figures S5I–S5L). These results were confirmed by RT-PCR analysis (Figure S6) and assessment of β-galactosidase activity (Figures S1F and S1G).

Further examination of POE::Pax6cre and POE::Pdx1cre mice during the development of the endocrine pancreas indicated that α, δ, and PP cell lineages were disfavored to the benefit of an insulin-producing cell fate (Figures 2A–2H). Most insulin-labeled cells present in double-transgenic mice correctly expressed the β cell specific markers Nkx6.1, Pdx1, Glut2, and HB9 (Figures 2K–2P and data not shown). Accordingly, they lacked Arx (Figures 2I and 2J) and Brn4 (Figures 2Q and 2R), labeling α/PP and α cells, respectively, whereas Pax6 and Isl1 were

a loss of α- (E–M), δ- (H–J), PP- (K–M), and Arx-labeled (N–P) cells is evident, but, interestingly, few of these remain detectable at one pole of the islet, some coexpressing the glucagon and somatostatin (arrow in I) or PP (arrow in L) hormones.

(Q) A quantification of the endocrine cell alterations ascertains these observations (also see Table S2).

For the purpose of clarity, the magnification of double-transgenic islets is twice (C, F, I, L, and O) or eight times (D, G, J, M, and P) reduced compared to that of controls. ($n > 11$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; all values are expressed as means ± standard error of the mean).



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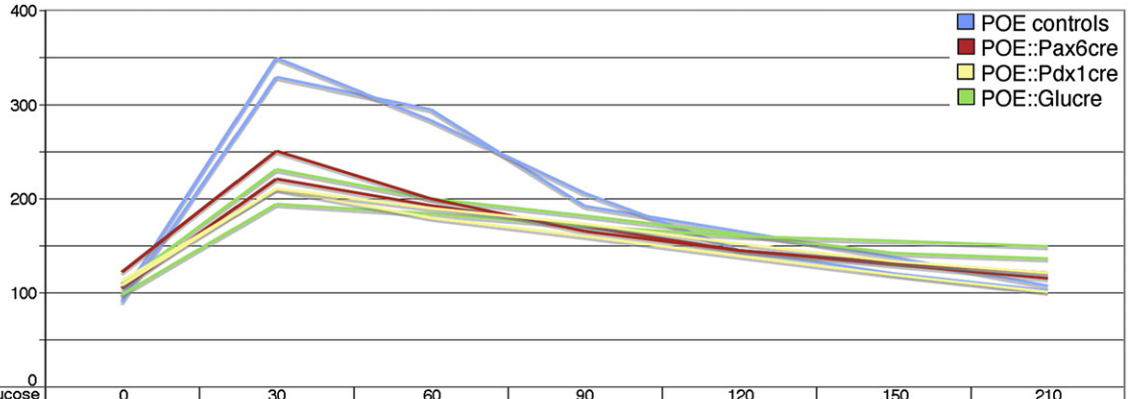


min post-glucose	0	30	60	90	120	150	210
Insulin (pmol/l)							
POE controls	44±5	391±17	319±22	212±31	165±47	97±14	51±9
POE::Pax6cre	62±9 (140%)*	843±121 (216%)**	710±89 (223%)**	404±68 (191%)**	285±54 (172%)	121±32 (125%)	74±23 (145%)
POE::Pdx1cre	76±9 (172%)*	1019±152 (261%)**	821±101 (258%)**	485±79 (228%)**	269±76 (163%)	152±53 (157%)	63±11 (124%)
POE::Glucre	69±8 (157%)**	969±109 (248%)**	701±80 (220%)**	382±61 (180%)**	198±78 (121%)	111±36 (115%)	45±14 (88%)

detected in all islet cell types, as expected (Figures 2S and 2T, and data not shown). To further assess the function of these insulin-producing cells postnatally, we performed glucose tolerance tests associated with systemic insulin measurements. Consistent with the increased β cell mass we previously noted, 3-week-old POE::Pdx1cre and POE::Pax6cre mice displayed an improved glucose clearance associated with a more than 2-fold increase in circulating insulin levels (Figure 2U). Altogether, our findings suggest that the ectopic expression of the *Pax4* gene drives endocrine precursor cells almost exclusively toward an insulin-expressing cell fate, and that these cells exhibit a functional β cell phenotype.

Conversion from an α to a β Cell Phenotype

As Pax4 specifically favors the β cell fate and identity throughout the morphogenesis of the endocrine tissue, we wondered whether this might also apply to mature endocrine cells. Therefore, animals conditionally expressing *Pax4* in glucagon-producing cells (Herrera, 2000) (Figure S8) were generated (POE::Glucre), and endocrine cell numbers were monitored. As early as 1 week postpartum, a 50% enlargement in islet size was outlined, these containing increased numbers of insulin$^+$/Pax4$^+$ cells compared to controls (Figure 3A). Notably, the content of glucagon-producing cells was found to be reduced by 77%, and most of the remaining glucagon$^+$ cells were located at one pole of the islet (Figures 3A–3C). As expected, δ and PP cell numbers were unchanged (Figures 3B and 3C). It is worth noticing that the expression of *Pax4* in glucagon-producing cells was accompanied by the production of the β-galactosidase protein (Figure S1A), allowing lineage-tracing experiments. One week after birth, this otherwise glucagon-cell-specific labeling was detected in the majority of insulin-producing cells (Figures 3D–3F) expressing a typical β cell complement of transcription factors (Table S2). Very few cells were found positive for both insulin and glucagon (Figure S9G). Besides, we observed an age-dependent increase in islet size and in the number of insulin-/β-galactosidase-producing cells, the latter exhibiting most β cell features (Tables S2 and S3, Figures 3G–3I and 2U). This suggests that, upon *Pax4* ectopic expression, adult glucagon-expressing cells are continuously converted into cells exhibiting a β cell phenotype.

Pax4 Ectopic Expression in β Cells Does Not Induce Their Proliferation

To further characterize the origin of the supernumerary β cells, we assayed proliferating cells combining bromodeoxyuridine (BrdU) incorporation and Ki67 staining. After a BrdU pulse in 5-week-old POE::Glucre mice, pancreata were quantitatively analyzed 1 week later, and a significant 2.35-fold increase in the number of BrdU-labeled cells was detected as compared to controls (Table S4). Interestingly, most BrdU$^+$ (Figure S9F), but also Ki67$^+$ (Figure S9D) cells were located outside the islets, within or adjacent to the neighboring duct epithelium.

The cell-autonomous effect of an augmentation of the Pax4 dose in β cells was analyzed by crossing of POE with Inscre mice expressing the cre recombinase under the control of the insulin promoter (Herrera, 2000). The quantification of the endocrine cell numbers in 3-week-old POE::Inscre pancreata did not reveal any increase in β cell numbers, or in islet size (Figure S10 and data not shown), despite the use of the POE mouse line inducing the highest *Pax4* expression (Table S5, line 5). Thus, although Pax4 may promote a modest proliferation of β cells in rat islet cultures (Brun et al., 2004), alternative mechanisms clearly act to boost the robust expansion of the β cell mass consistently observed in POE::Pdx1cre, POE::Pax6cre, and POE::Glucre mice (from hereon termed "double-transgenic animals"). One issue of concern to our approach was related to the overexpression of *Pax4*. However, through the use of different POE transgenic founder mice, we demonstrated that the ectopic expression of *Pax4* at a dose lower than found in normal β cells is sufficient to induce a loss of the α cell phenotype to the benefit of β cell features (Table S5).

Rescuing Glucagon Deficiency Prevents the Generation of Oversized Islets

As lineage-tracing experiments demonstrated that glucagon-expressing cells were converted into cells displaying a β cell phenotype, a depletion of α cells was expected. However, we consistently detected clusters of glucagon$^+$ cells in our double-transgenic mice (Figures 1–3), suggesting that α cell neogenesis occurred. We therefore hypothesized that the latter might be driven by the hypoglucagonemia resulting from the α to β cell conversion. To test this theory, we injected 3-week-old POE::Pdx1Cre, POE::Glucre, POE::Pax6cre, and control animals twice daily with glucagon during 3 weeks. A significant decrease in islet size was observed in glucagon-treated animals as compared to controls (Figure 4). This was mainly attributed to a reduction in the β cell mass (Figures 4E and 4G compared to Figure 4C, and Figure 4K compared to Figure 4I; Tables S3 and S4). Importantly, the number of α cells was also found to be significantly diminished (Figures 4F, 4H, and 4L and Tables S3 and S4). These results thus indicate that decreased glucagon levels are responsible for the continuous replenishment of α cells that acquire a β cell phenotype in double transgenic animals.

Figure 2. Insulin-Expressing Cells Detected in POE::Pdx1cre and POE::Pax6cre Pancreata Rxhibit a β Cell Identity

(A–T) Characterization of POE::Pdx1cre and POE::Pax6cre hormone-expressing cells. Sections of double-transgenic pancreata (of the indicated ages) were stained with the mentioned antibodies, and the number of labeled cells was counted and reported to the count obtained in POE control animals. The values are expressed in percentage of change in labeled cell number compared to controls. All values are statistically significant, with p values lower than or equal to 0.05. Note that the numbers of insulin-expressing cells are dramatically increased in double-transgenic animals at all examined stages (A–P and S–T) and this, as early as E13 (A and B), compared to controls. Concurrently, the mean contents of α- (A, B, G–J, Q, and R), δ- (C and D), PP- (E and F), and Arx- (I and J) marked cells appear drastically reduced. A thorough analysis of endocrine cell-specific markers demonstrates that all insulin-producing cells clearly express Pax4 (G and H), as well as the β cell markers Nkx6.1 (K and L), Glut-2 (M and N), and Pdx1 (O and P). These are negative for the α cell-specific factor Brn-4 (Q and R), whereas, all endocrine cells express Pax6 (S and T).

(U) An improved glucose tolerance and insulin release are highlighted in 3-week-old double-transgenic mice as compared to control POE mice ($n > 3$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$). Each picture is representative of at least four independent animals; note that, for the purpose of clarity, a POE::Pax6cre islet is displayed in (H).

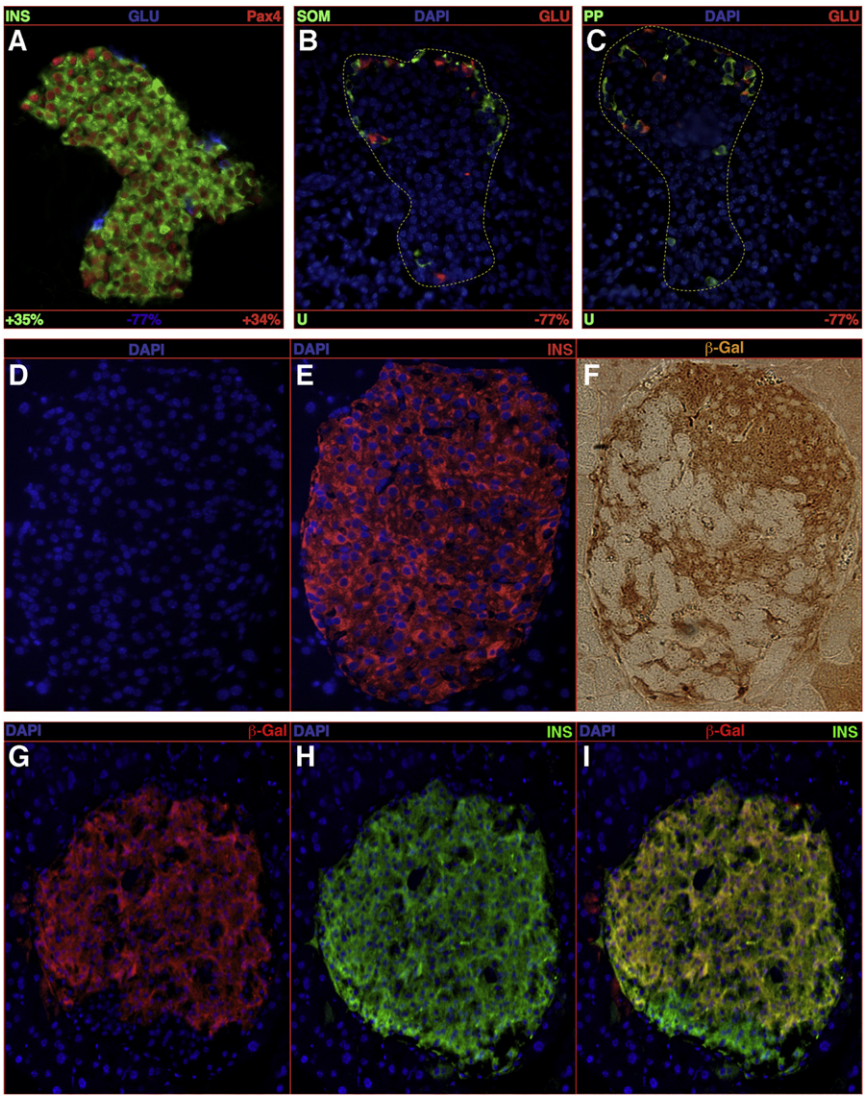


Figure 3. Conversion of Glucagon-Expressing Cells into Insulin-Producing Cells upon *Pax4* Ectopic Expression

(A–C) Quantification of the endocrine cell content alterations upon ectopic expression of the *Pax4* gene in glucagon-producing cells in 1-week-old animals. A clear increase in the number of insulin-/Pax4-labeled cells at the expense of glucagon-expressing cells is highlighted (A), whereas the δ and PP cell contents are found unchanged (B and C). Note the accumulation of the remaining glucagon-marked cells at one pole of the islet.

(D–F) The detection of insulin- and β-galactosidase-expressing cells on serial sections demonstrates that numerous insulin-labeled cells do express the *β-galactosidase* gene that normally marks glucagon-positive cells.

(G–I) The same observation is made in 6-week-old animals with coimmunofluorescence. All reported values are statistically significant ($p < 0.05$, $n = 5$). See Table S2 for a detailed analysis.

Requirement of *Ngn3* Re-expression for α Cell Neogenesis and Ensuing Acquisition of a β Cell Phenotype

The location of endocrine non-β cells at one pole of the islet of Langerhans of double-transgenic animals was remarkable. This observation was confirmed through Z stack analysis of POE::Pdx1Cre, POE::Glucre, and POE::Pax6cre whole islets stained with anti-glucagon antibodies (Movie S1). Most importantly, these glucagon-labeled cell clusters were consistently found adjacent to the ductal lining (Movie S2, in blue). The very detection of such glucagon$^+$ cells was intriguing as their conversion into β cells was expected upon *Pax4* expression. A likely explanation may be the continuous generation of glucagon-labeled cells, their detection indicating a transitional state prior to their conversion into insulin-expressing cells. This hypothesis was supported by the active cell proliferation observed near and within the duct epithelium (Figures 5B, S9D, and S9F), close to the pole of glucagon$^+$ cells. In addition, scattered glucagon$^+$ cells were found within the lining of the duct (arrowheads in Figures 5C and 5D), whereas the duct markers cytokeratin-19 (CK19) and SPP1 were detected not only within the duct epithelium, but also in few adjacent islet cells (arrowheads in Figures 5E and 5F). The existence of facultative progenitor cells in adult mice that, under specific injury conditions, reactivate *Ngn3* expression and develop into all four endocrine cell types has been recently reported (Xu et al., 2008). Interestingly, in pancreata ectopically expressing *Pax4*, we also noticed a reactivation of the islet cell progenitor marker Ngn3 (Figures 5G, 5H, S9A, S11, and S12, and Tables S3 and S4), while *Pax4* expression was never detected in duct cells (Figure S9B). However, because of the difficulties classically encountered to detect Ngn3 in adult tissues, the definitive location and counts of Ngn3-producing cells will await the generation of better antibodies/in situ probes. We therefore asked whether *Ngn3* reactivation was involved in the generation of supernumerary insulin$^+$ cells by tracing exocrine- and Ngn3-producing cells, or inhibiting *Ngn3* expression in the pancreas of double-transgenic mice. Because the expression of Cre, GFP, and β-galactosidase in our double-transgenic animals precluded the use of duct-Cre, Ngn3-Cre, Ngn3-GFP, or Ngn3-LacZ mouse lines for tracing purposes, a lentiviral approach was preferred. Specifically, recombinant lentiviruses constitutively expressing a c-Myc-tagged DsRED2 reporter were injected into the pancreatic duct of POE::Glucre mice, as previously described (Xu et al., 2008). Because of the high intensity of the GFP signal present in some animals, native DsRED2 fluorescence could not be unambiguously ascertained. We therefore employed immunohistochemistry to detect the exogenous c-Myc epitope-tag fused to DsRED2 (the endogenous c-Myc being unrecognized; Figure 5J). Two weeks after

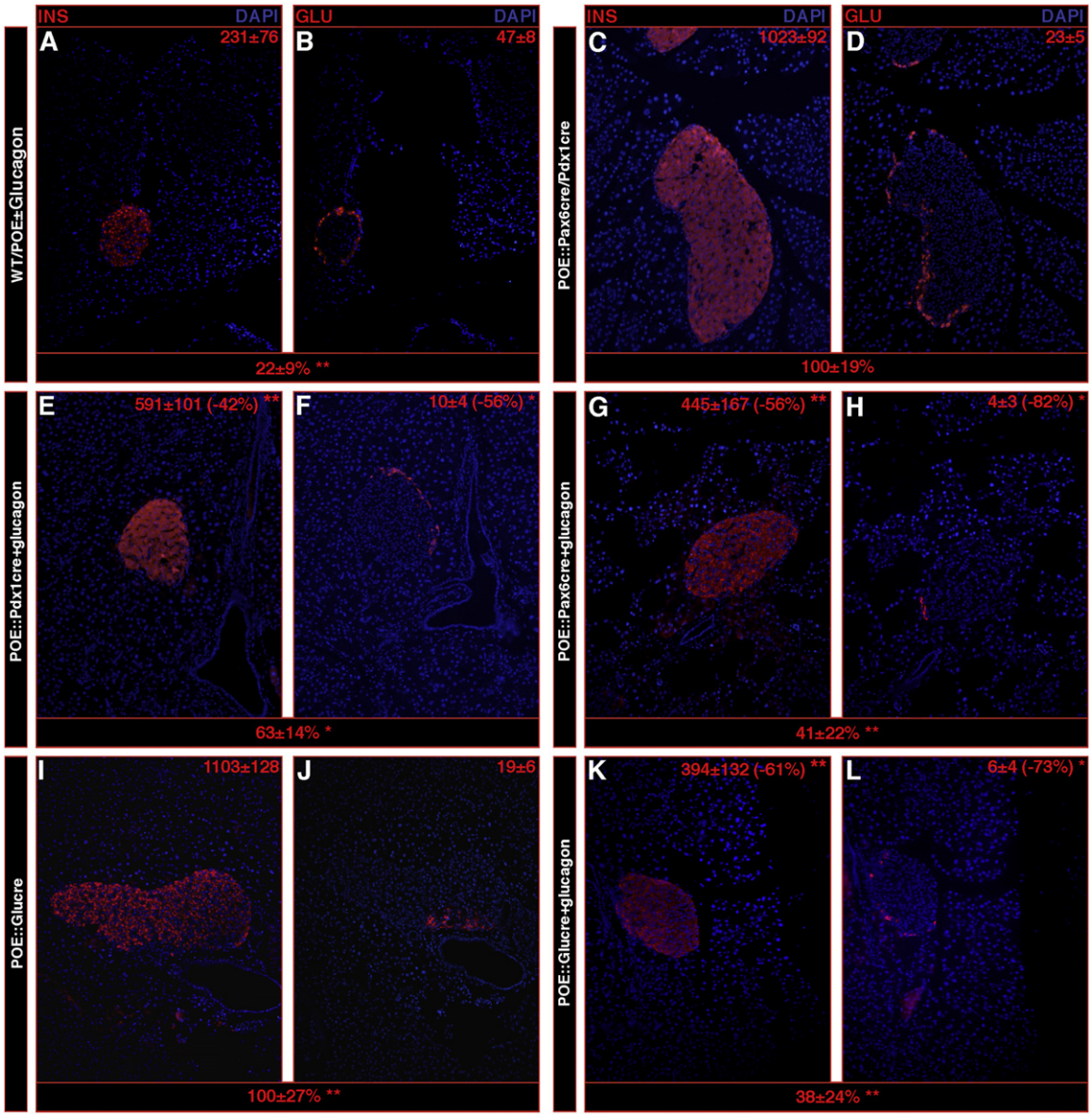


Figure 4. Exogenous Glucagon Supplementation Prevents Islet Overgrowth

Six-week-old pancreata of the indicated genotypes were sectioned and stained with anti-insulin (A, C, E, G, I, and K) or glucagon (B, D, F, H, J, and L) antibodies. The values at the top right corner of each photograph represent the number of hormone-producing cells per square centimeter of pancreas. The counts reported underneath each picture set represent variations in islet surface (estimated in silico), with POE::Pax6cre/Pdx1cre pancreata (C and D) used as a reference for photographs (A)–(H) and POE::Glucre pancreata (I and J) as reference for photographs (I)–(L). Note the overall diminution in islet size and glucagon$^+$ cell content, as well as the drastic decrease in insulin-expressing cell number (E–H compared to C–D and K–L compared to I–J) in animals supplemented with glucagon for 3 weeks compared to untreated ones (*$p < 0.05$, **$p < 0.01$, $n = 3$).

infection, Myc-tagged cells were noted in the vast majority of duct and acinar cells of control animals, whereas less than 5% of endocrine cells were labeled, as reported previously (Xu et al., 2008) (Figure 5K). In the pancreas of double-transgenic mice, however, 71% of endocrine cells were positive for the Myc tag (Figure 5L), most of them expressing insulin (Figure 5M) and β-galactosidase (Figures 5N and 5O), the latter labeling cells that previously expressed *glucagon*. This result suggests that exocrine cells were converted into endocrine cells in the double-transgenic mice. Double-transgenic mice were also infected with recombinant lentiviruses expressing either the c-Myc-tagged reporter under the control of the *Ngn3* promoter or a *Ngn3*-specific short hairpin (sh) interfering RNA controlled by the *CMV* promoter, as in Xu et al. (2008). Transduction with the first construct confirmed both the re-expression of *Ngn3* and the specificity of *Ngn3* promoter employed (Figures S13–S15). Interestingly, *Ngn3* knockdown caused a dramatic reduction in the number of Ngn3$^+$ (Figures 6A and 6E), insulin$^+$

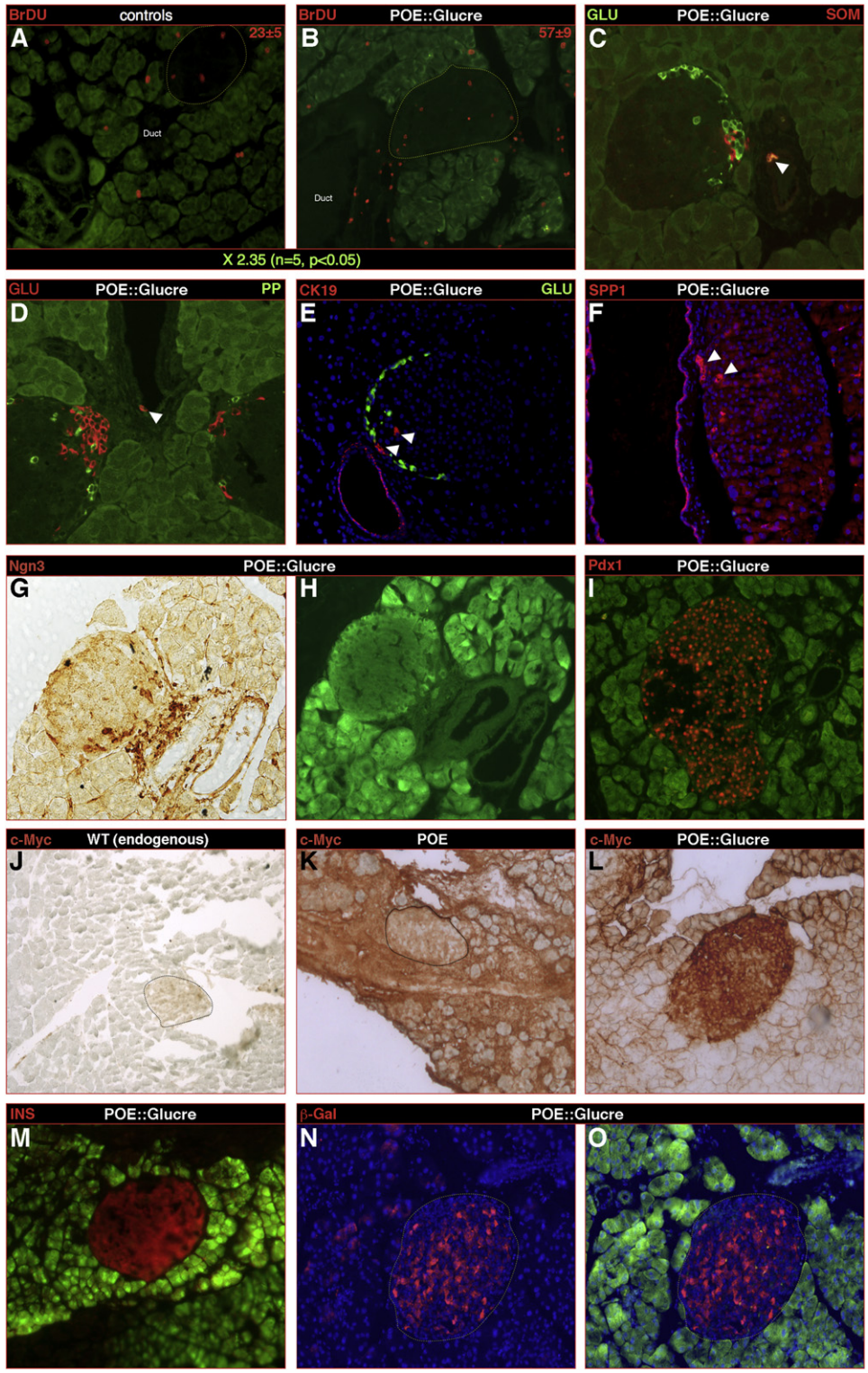


Figure 5. Progenitor Cells May Be Induced and Converted into Glucagon and Subsequently into Insulin-Expressing Cells

(A–I) After a pulse of BrdU at 3 weeks of age and examination a week later, a quantitative analysis established a 2.35-fold increase in the number of proliferating cells in POE::Glucre pancreas compared to POE controls (A and B). Importantly, most BrdU-labeled cells are not detected within the islets, but rather near or within the duct epithelium (B). It is worth noting that this location corresponds to the cluster of glucagon-producing cells consistently observed in this genotype. Within the duct epithelium, scattered endocrine cells could be detected, some coexpressing the glucagon and sometimes somatostatin hormones (arrowheads

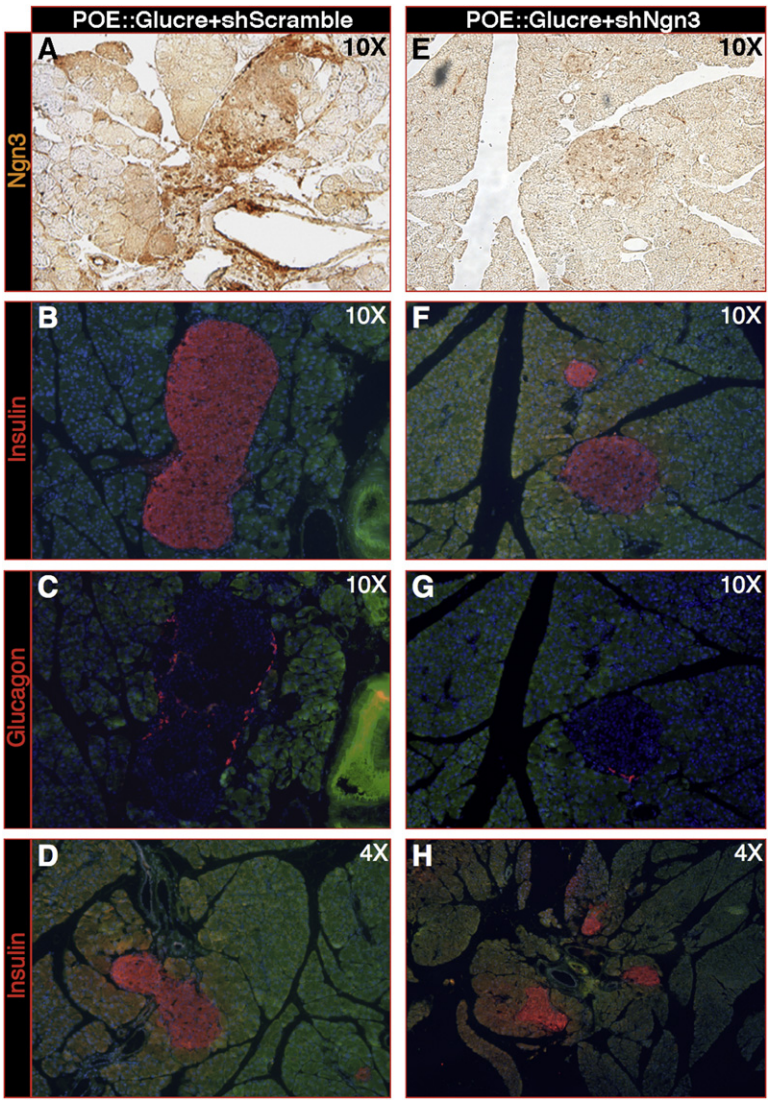


Figure 6. Knockdown of *Ngn3* Prevents the *Pax4*-Mediated β Cell Hyperplasia

Infection of POE::Glucre animals with lentiviruses producing either an shRNA targeting *Ngn3* transcripts (E–H) (Xu et al., 2008) or producing a scrambled shRNA (based on the former; A–D). Two weeks after infection, *Ngn3* knockdown pancreata (at the indicated magnifications) display an efficient 61% diminution in Ngn3 production (Xu et al., 2008) (A, E, and Table S6), but also a 69% decrease in insulin- (B, F, D, and H) or glucagon- (C and G) expressing cell numbers (Table S6) compared to scramble-infected counterparts.

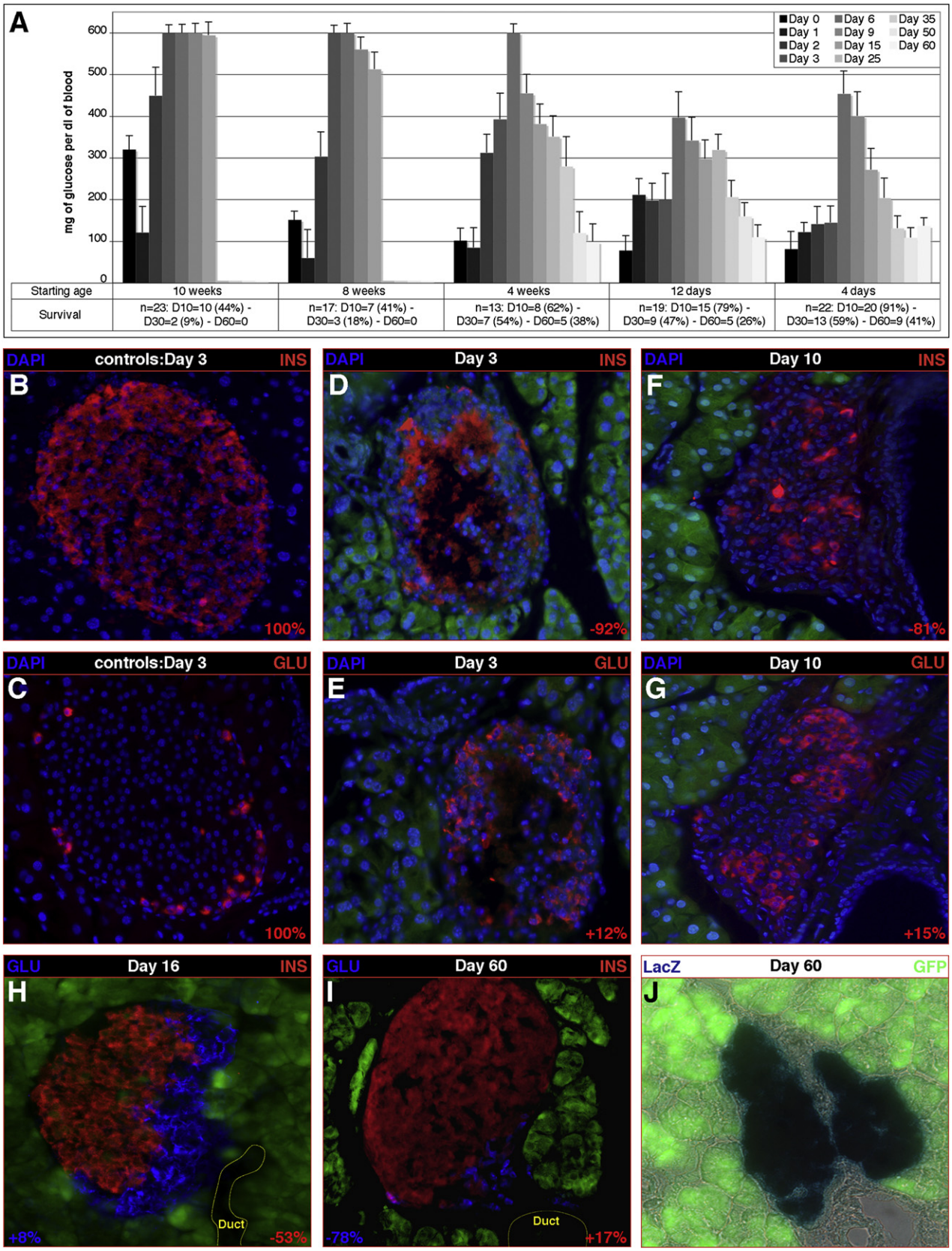
(Figure 6B, 6F, 6D, and 6H) and glucagon$^+$ (Figures 6C and 6G) cells, as well as in the level of transcripts encoding *Ngn3*, *insulin*, and *glucagon* (Table S6).

To test whether glucagon signaling deficiency may also induce the reactivation of *Ngn3*, in addition to POE::Glucre animals, we analyzed glucagon receptor-deficient mice (characterized by an α cell hyperplasia; Gelling et al. [2003]). When real-time RT-PCR and counts of immunostained sections were combined (Tables S3 and S4, Figure S16), an increase in *Ngn3*, but also in the β cell regeneration-associated *Reg3b* (or *INGAP*; Rosenberg [1998]) gene expression, was observed in both genotypes. Interestingly, *Ngn3* and *Reg3b* transcripts, as well as Ngn3$^+$ cell numbers, appeared to be dramatically reduced in POE::Glucre animals supplemented with glucagon for 3 weeks (Tables S3 and S4). Thus, our data support the notion that the glucagon deficiency mediated by the ectopic expression of *Pax4* results in *Ngn3* expression and in the successive conversion of progenitor cells into glucagon- and thereafter insulin-producing cells.

Pax4 Ectopic Expression Rescues Streptozotocin-Induced Diabetes

Thus far, our data suggested that the forced expression of *Pax4* in *Pax6*, *Pdx1*, or *glucagon* expression domains ultimately led to the development of oversized islets of Langerhans mostly composed of seemingly differentiated and functional β cells. We reasoned that such cells might be capable of replacing lost β cells in diabetic mice. Hence, POE::Glucre mice of different ages were injected with a single dose of the β cell toxin STZ, and both blood glucose levels as well as viability were monitored for 8 weeks (Figure 7A). Shortly after STZ injection, we often observed a decrease in glycemia, most likely caused by the discharge of insulin from killed β cells (Figure 7A). A higher mortality rate was found among animals older than 4 weeks, as compared to younger mice. It should be noted that these animals were already hyperglycemic and weakened prior to STZ injection because of a combined insulin insensitivity and β cell failure to optimally respond to a glucose challenge (Figure S17). In contrast, in 4-week-old and younger STZ-treated animals, we noted a normalization in blood glucose levels after a peak in glycemia and survival rates reaching 41% 2 months after injection (Figure 7A), while all controls died (data not shown). Immunohistochemical analyses demonstrated that after the rapid obliteration of the β cell mass (Figures 7B–7E), a massive neogenesis

in C and D). Along the same line, islet cells positive for the duct marker genes *CK19* and *SPP1* are observed adjacent to the duct epithelium (arrowheads in E and F). Notably, the duct lining also appears to contain numerous cells positive for the proendocrine marker gene *Ngn3* (G and H), but negative for *Pdx1* (I). (J–O) Infection of controls (K) and double-transgenic (L–O) pancreata with a construct containing a *CMV* promoter driving the constitutive expression of a c-Myc tag (the latter being not expressed in wild-type tissues, J). Because of the method used, 2 weeks after infection, most exocrine cells are labeled by the virus, whereas only very few islet cells are (islet underlined in K). Importantly, a majority of double-transgenic endocrine cells appear marked by the virus, suggesting their ductal or acinar origin (L). These cells express the insulin hormone (M) and are β-galactosidase positive (N and O), indicating that they once expressed the glucagon hormone. (H, I, M, and O: green fluorescence corresponding to residual GFP expression after bleaching of the sections.)


A
mg of glucose per dl of blood
600
500
400
300
200
100
0
Day 0
Day 1
Day 2
Day 3
Day 6
Day 9
Day 15
Day 25
Day 35
Day 50
Day 60
Starting age
10 weeks
8 weeks
4 weeks
12 days
4 days
Survival
n=23: D10=10 (44%) - D30=2 (9%) - D60=0
n=17: D10=7 (41%) - D30=3 (18%) - D60=0
n=13: D10=8 (62%) - D30=7 (54%) - D60=5 (38%)
n=19: D10=15 (79%) - D30=9 (47%) - D60=5 (26%)
n=22: D10=20 (91%) - D30=13 (59%) - D60=9 (41%)
DAPI controls:Day 3 INS
B
100%
DAPI Day 3 INS
D
-92%
DAPI Day 10 INS
F
-81%
DAPI controls:Day 3 GLU
C
100%
DAPI Day 3 GLU
E
+12%
DAPI Day 10 GLU
G
+15%
GLU Day 16 INS
H
Duct
+8%
-53%
GLU Day 60 INS
I
Duct
-78%
+17%
LacZ Day 60 GFP
J

of glucagon-expressing cells occurred, again near the duct lining (Figures 7F–7I). Ten days after injection, only few insulin-producing cells were detected (Figures 7F and 7G). However, a steady increase in glucagon- and insulin-expressing cell numbers was observed in the following days (Figure 7H). Approximately 2 months after injection, POE::Glucre animals exhibited almost normal-sized islets of Langerhans and normoglycemia (Figure 7I). Interestingly, at that age, the β-galactosidase lineage tracer was found to be uniformly distributed in islet cells (Figure 7J), demonstrating that the insulin-producing cells in these animals derived from cells that previously expressed *glucagon*. These insulin$^+$ cells displayed a β cell phenotype and expressed a β cell-specific complement of transcription factors (data not shown). Altogether, our analyses provide direct evidence that, in STZ-treated mice, the ectopic expression of *Pax4* in α cells continuously converts them into β cells and counters diabetes in animals younger than 4 weeks of age.

DISCUSSION

In this study, we report that the forced expression of the *Pax4* gene in the mouse endocrine pancreas results in oversized islets composed mainly of cells displaying a β cell phenotype. Our findings are consistent with the induction of progenitor cells that adopt an α cell identity as a consequence of decreased glucagon levels, and subsequently acquire β cell features upon *Pax4* ectopic expression, *Ngn3* reactivation being instrumental in this processes. The resulting β cells are functional at least at an early age, and can repopulate the islets of diabetic mice to normalize blood sugar levels.

Pax4 Promotes the β Cell Fate Specification during Embryogenesis and Induces an α Cell-Mediated β Cell Neogenesis Postpartum

The forced expression of *Pax4*, either in Pdx1$^+$ pancreatic progenitor cells (and ultimately in all pancreatic cells) or in Pax6$^+$ endocrine precursor cells (and ultimately in all islet cells), results in the nearly exclusive specification of cells exhibiting a β cell identity at the expense of the α, δ, and PP cell lineages. At birth, these animals display normal pancreas morphology, unaffected exocrine tissue, and well-sized islets of Langerhans, indicating that the main alterations observed are solely related to the allocation of the different endocrine cell lineages during embryonic development. This also suggests that Pax4 does not alter the pancreatic exocrine differentiation program, but rather acts on the specification of endocrine progenitor cells by promoting the acquisition of the β cell fate. Interestingly, after birth, an age-dependent increase in islet size is evident in pancreata ectopically expressing *Pax4* that was attributed to the continuous generation of cells displaying a β cell phenotype. In an effort to ascertain the origin of such cells, lineage-tracing experiments were performed with POE::Glucre mice. Our initial goal was to trigger the ectopic expression of *Pax4* in adult glucagon-producing cells, but an inducible glucagon-cre line is hitherto unavailable. Hence, the classical glucagon-cre mouse line (Herrera, 2000) was used to induce *Pax4* expression in glucagon-producing cells. It is important to note that during embryogenesis, the *glucagon* gene is initially expressed in early endocrine cells often coexpressing additional hormones, including insulin. However, by irreversibly tagging the progeny of cells with the Cre/LoxP system, Herrera (2000) demonstrated that mature glucagon- and insulin-producing cells do not derive from cells that previously expressed insulin or glucagon, respectively. Interestingly, lineage-tracing experiments performed in POE::Glucre mice revealed that the vast majority of newly formed β-galactosidase$^+$/insulin$^+$ cells originate from cells that previously expressed *glucagon* (note that endogenous β-galactosidase$^-$/insulin$^+$ cells remain detectable). Based on these results, but also on the age-dependent increase of β-galactosidase$^+$ β cell numbers and the concomitant decrease in α cell contents, the dramatic expansion of the β cell mass throughout the postnatal life span of double-transgenic mice was attributed to a continuous neoformation of β cells through α cell redifferentiation rather than to the slow self-renewal capacity of β cells (Dor et al., 2004). Although our findings are in agreement with a putative neogenesis/conversion of somatostatin- or PP-positive cells into β cells, testing of this hypothesis will have to await the generation of somatostatin-cre and PP-cre mice to allow lineage-tracing experiments.

Ngn3 Is Required for the Continuous Neogenesis of α Cells Ultimately Acquiring a β Cell Phenotype upon *Pax4* Ectopic Expression

We demonstrate that glucagon supplementation reduces the β cell hyperplasia in double-transgenic mice, most likely by compensating the deficiency in circulating glucagon resulting from the loss of α cells through Pax4-induced redifferentiation. Interestingly, compromised glucagon signaling has previously been associated with α cell neogenesis: both *Glucagon receptor* (*Gcgr*)- and *prohormone convertase 2* (*Pcsk2*)-deficient animals

Figure 7. *Pax4* Ectopic Expression Promotes the Reconstitution of the Insulin-Expressing Cell Mass upon β Cell Depletion

(A) After streptozotocin injection at the indicated age (starting age), the glycemia and survival of the treated animals was followed for 2 months. Note that animals older than 4 weeks of age become diabetic and die as a consequence of the obliteration of β cells, the same being true for all controls (data not shown). Importantly, in younger animals, a steady recovery leading to normoglycemia is observed next to a peak in glucose levels (all values are expressed as means ± standard error of the mean).

(B–J) Islet cell contents in 4-week-old POE::Glucre streptozotocin- (D–J) or sham- (B and C) treated animals were quantified (see at the bottom of the concerned pictures) 3 days (B–E), 10 days (F and G), 16 days (H), and 60 days (I and J) days after injection. Three days after injection, the β cell mass present in controls (B and C) is almost entirely lost in streptozotocin-treated mice (D and E), the only insulin labeling being observed in areas devoid of nuclei, suggesting a detection of hormone released from killed β cells. A 15% increase in glucagon-producing cells is highlighted 10 days after injection compared to controls, most of these cells neighboring duct structures (G). Importantly, few insulin-labeled cells are detected (F). This trend is ascertained at day 16 (H) with a major increase in the insulin-expressing cell number. A codetection with the glucagon hormone does not indicate any coexpression. Finally, at day 60, the β cell mass appears to be statistically normal as compared to control animals (I compared to B and C), most of the cells present in the islet expressing the β-galactosidase enzyme marking glucagon-producing cells (J).

display oversized islets, mainly composed of glucagon-producing cells (Furuta et al., 1997; Gelling et al., 2003). Similar to the present report, exogenous glucagon treatment significantly reduced the endocrine cell hyperplasia in *Pcsk2*-deficient animals (Blume et al., 1995; Petersson and Hellman, 1963; Webb et al., 2002). *Pcsk2* mutants were also found to contain glucagon$^+$ cells near the duct epithelium, and their contribution to the neogenesis of the supernumerary glucagon$^+$ cells was suggested. Accordingly, our findings indicate that, upon *Pax4* ectopic expression, a physiologically significant glucagon deficiency activates a continuous compensatory response resulting in α cell neogenesis, as seen in *Gcgr* and *Pcsk2* mutant mice. However, these cells are subsequently converted into β cells upon *Pax4* ectopic expression.

In animals ectopically expressing *Pax4*, but also in *Gcgr* mutants, a reactivation of *Ngn3*, a gene normally exclusively expressed during embryonic development of the pancreas and required for the endocrine differentiation program, was observed. However, because of the difficulties encountered to detect Ngn3 transcripts or protein, the determination of the origin of such Ngn3$^+$ cell will require more elaborated lineage-tracing experiments and/or more specific antibodies/in situ probes. Interestingly, lentivirus-mediated cell tracing and knockdown experiments showed that *Ngn3* re-expression is in fact crucial for the α cell-mediated β cell neogenesis. As important was a recent report demonstrating that, upon pancreatic duct ligation, facultative adult stem cells are activated along the lining of duct epithelium (Dor and Melton, 2008; Xu et al., 2008). It was also established that these cells reactivate *Ngn3*, differentiate into endocrine cells, and contribute to the formation of oversized islets. In mice ectopically expressing *Pax4*, our results suggest that duct-lining cells may represent the source of the neogenerated glucagon-expressing cells. Although we cannot exclude the contribution of acinar cells to this process, additional evidence favor a duct-to-islet cell conversion mechanism: (1) the continuous detection of neo-generated glucagon$^+$ islet cells adjacent to duct structures, (2) the active proliferation within the duct epithelium and near the islet pole where non-β endocrine cells accumulate, (3) the presence of glucagon$^+$ endocrine cells in the ductal lining, and (4) the detection of cells expressing duct markers within the islet. Our findings are supported by recent reports demonstrating the plasticity of pancreatic cells. For instance, Melton and coworkers proved that acinar cells could be reprogrammed into β cells upon the ectopic expression of selected genes in vivo (Zhou et al., 2008), whereas Inada et al. (2008) provided evidence that duct cells may give rise to endocrine and acinar cells in the adult pancreas. In agreement with these, our analysis reveals that hypoglucagonemia activates compensatory mechanisms provoking the conversion of progenitor cells into α cells that subsequently acquire a β cell phenotype upon *Pax4* ectopic expression, these Ngn3-dependent processes ultimately leading to the generation of oversized islets of Langerhans.

Pax4 Ectopic Expression Rescues from Streptozotocin-Induced Diabetes

We reasoned that the continuous β cell neogenesis observed in double-transgenic animals might be able to rescue experimentally induced diabetes. Therefore, the function of the newly formed β cells was assessed in diabetic mice with more than 95% β cell loss after STZ treatment. No insulin supplement was used to counter the sudden β cell loss, as the consequences of exogenous insulin treatment on islet cells formation are hitherto unclear. Accordingly, a high lethality was expected, especially in older hyperglycemic and weakened mice. However, while all control and double-transgenic mice older than 10 weeks died, 41% of the younger animals survived during 2 months after STZ injection. Closer examination revealed a progressive reconstitution of the islets and normalization of the glycemia and indicated that the new insulin$^+$ cells behaved as true β cells. Also under these conditions, clusters of glucagon$^+$ cells were detected at one pole of the islets, adjacent to duct structures, such cells subsequently adopting a β cell phenotype. Furthermore, the endocrine tissue in animals that died during the course of these experiments showed significant β cell neogenesis, but, most likely, not sufficient enough to allow survival.

The observation that only double-transgenic animals younger than 4 weeks of age could survive STZ treatment was intriguing. Interestingly, this age corresponds to the period when such animals, initially hypoglycemic, progressively develop a hyperglycemic condition. While the low blood glucose levels observed after birth can be easily explained by the β cell hyperplasia, the development of a diabetic condition was unexpected: the steady increase in insulin-producing cell content and the reduced number of glucagon-expressing cells are in contrast with the observed high glucose levels. In light of the data reported in this study, it appears that the newly formed insulin-expressing cells are functional for at least 4 weeks after birth and respond normally to a glucose challenge. During this time, they express all the β cell markers we tested and are negative for α, δ, and PP cell marker genes. However, glycemia, glucose tolerance, insulin secretion, and insulin sensitivity deteriorate in older animals despite a normal expression of a typical β cell complement of transcription factors, suggesting that older β cells fail to trigger an optimal response upon glucose challenge. Interestingly, these retain some capacity to secrete insulin when challenged with arginine or a long-acting GLP-1 analog. The reasons for the establishment of such a condition are unclear, but may result from progressive (1) β cell exhaustion, (2) β cell alterations, (3) desensitization of the insulin receptor/pathway as a consequence of increased insulin levels, and/or (4) unknown effects induced by the decreased glucagon/somatostatin/PP hormone contents. Defining the mechanisms involved would require further work in which the impact of β cell hyperplasia, but also of the decrease in α , δ, and PP cell contents, could be modulated and analyzed independently. It is interesting to note that younger double transgenics subjected to streptozotocin treatment display an extended life span in comparison to untreated counterparts (data not shown). This indicates that the development of the diabetic condition in these mice is not age-related, but may rather depend on the islet hyperplasia state. Clearly, using different activation times of exposure to Pax4 would allow us to determine its long-term effect on islet function. Together, our results suggest that the sole ectopic expression of *Pax4* in glucagon$^+$ cells can, in younger animals, reverse the consequences of streptozotocin-mediated diabetes through the

induction of α cell neogenesis and their ensuing conversion into functional β cells. Based on these findings, we suggest that drug-mediated modulation of Pax4 and/or its targets may open new avenues for treatment of diabetes and, in addition, may contribute to strategies aiming to differentiate β cells from stem, progenitor, or other cell types.

EXPERIMENTAL PROCEDURES

Mouse Manipulations

The strategy used to generate the POE mouse line is depicted is Figure S1. These mice were crossed with Pdx1-, Pax6-, Glucagon-, and Insulin-cre lines (Ashery-Padan et al., 2004; Gu et al., 2002; Herrera, 2000) and genotyped with a combination of fluorescence microscopy for GFP examination and genotyping PCR for *cre* and *β-galactosidase* genes.

So that the effects of glucagon on islet size could be assessed, mice were injected intraperitoneally twice daily (every 12 hr) with 5 μg glucagon and sacrificed after 3 weeks of treatment. For STZ-mediated diabetes induction, a freshly prepared 50 mg/ml solution in 0.1 mol/l sodium citrate (pH 4.5) was injected intraperitoneally (200 mg/kg). Lentivirus production and injection were performed as described previously (Xu et al., 2008). Lastly, BrdU was injected intraperitoneally (200 μl of 100 μg/ml) and was detected by immunohistochemistry (Invitrogen).

Immunohistochemistry

Tissues were fixed in 4% PFA overnight at 4°C and embedded in paraffin, and 8 μm sections were applied to slides. These sections were assayed as described previously (Collombat et al., 2003). For coimmunofluorescence, the GFP signal was bleached when necessary, as described (Collombat et al., 2007). The primary antibodies used were the following: mouse monoclonal anti-insulin, anti-glucagon (1/1000, Sigma), anti-Ngn3 (1/2000, 1F25A1B3, BCBC Antibody Core), anti-c-Myc (1/100, Abcam); guinea pig anti-insulin, anti-glucagon (1/1000, Sigma); rabbit anti-somatostatin (1/600, Dako), anti-PP (1/200, Dako), anti-Nkx6.1 (1/3000), anti-Nkx2.2 (1/1000, kindly provided by T. Jessell), anti-Pax6 (1/500, Chemicon), anti-Pax4 (1/500), and anti-Arx (1/1000); chicken anti-β-galactosidase (1/500, Biozol); and goat anti-Ngn3 (1/1000, kindly provided by M. Sander). The secondary antibodies (1/1000, Molecular Probes) used were the following: 594-alexa anti-mouse, 488-alexa anti-mouse, 594-alexa anti-rabbit, 488-alexa anti-rabbit, 594-alexa anti-guinea pig, and 488-alexa anti-guinea pig. Pictures were processed by confocal microscopy. For quantification purpose, stained cells were counted manually on every tenth section, and the count was reported to the pancreatic area estimated in silico.

Glucose Challenge and Circulating Glucose or Insulin Level Measurements

For glucose challenge tests, six mice per genotype were fasted for 24 hr and injected intraperitoneally with glucose (2 g/kg), and blood glucose levels were measured 0, 30, 60, 90, 120, 150, and 210 min afterward. At each time point, one animal per genotype was sacrificed immediately after glycemia assessment for serum insulin level determination with RIA (Linco). Glucose levels (mg/dl) were determined with the One Touch Glucose Monitoring Kit (Johnson & Johnson).

Data Analysis

All values are depicted as mean ± standard error of the mean from at least three independent experiments and considered significant if $p < 0.05$. All data were statistically analyzed by multivariate comparison (two-way ANOVA) with Bonferroni correction or one-way ANOVA with Newman-Keuls correction.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 17 figures, seven tables, and two movies and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00639-4.

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