

Pancreatic Duct Ligation After Almost Complete β -Cell Loss: Exocrine Regeneration but No Evidence of β -Cell Regeneration

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There has been great interest in the extent of β -cell regeneration after pancreatic duct ligation (PDL) and whether α - to β -cell conversion might account for β -cell regeneration after near-complete β -cell loss. To assess these questions, we established a PDL-model in adult male rats after almost complete beta-cell depletion achieved by giving a single high dose of streptozocin (STZ) in the fasted state. Because of the resultant severe diabetes, rats were given islet cell transplants to allow long-term follow-up. Although animals were followed up to 10 months, there was no meaningful β -cell regeneration, be it through replication, neogenesis, or α - to β -cell conversion. In contrast, the acinar cell compartment underwent massive changes with first severe acinar degeneration upon PDL injury followed by the appearance of pancreatic adipocytes, and finally near-complete reappearance of acini. We conclude that β -cells and acinar cells, although originating from the same precursors during development, have very distinct regenerative potentials in our PDL model in adult rats. (*Endocrinology* 154: 4493–4502, 2013)

Because reduced β -cell mass and function are fundamental to the pathogenesis of types 1 and 2 diabetes (1), there has been considerable interest in the regenerative potential of the endocrine pancreas (2). Less studied is regeneration of the exocrine pancreas. β -Cell regeneration could, in theory, result from replication of existing β -cells, neogenesis, the formation of new β -cells from pancreatic nonendocrine-cells, or conversion of α -cells to β -cells. Replication of β -cells is quantitatively the most important determinant of expansion in postnatal mice (3, 4) and after β -cell loss (5). β -Cell neogenesis, thought to originate from multipotent duct cells, appears to contribute to β -cell expansion during the neonatal period (6, 7). Neogenesis also occurs after partial pancreatectomy in rodents (8, 9) and has been thought to take place after pancreatic duct ligation (PDL) in adult mice (6, 10–12). However, there is controversy as to whether postnatal β -cell neogenesis from duct cells occurs. Some studies employing lineage

tracing and other techniques provide support (6, 11, 13), whereas others using similar approaches have challenged the hypothesis (14–17). In addition, the validity of PDL as a model of endocrine regeneration has recently been called into question (16, 18–20).

The possibility of α - to β -cell conversion has also attracted considerable interest. Genetically induced diphtheria toxin β -cell ablation in mice resulted in α - to β -cell conversion that took place after many months (21). Another study used alloxan combined with PDL and reported rapid α - to β -cell conversion within weeks (22). We have found no reports of α - to β -cell conversion after streptozocin (STZ).

The aim of our study was 1) to assess whether α - to β -cell conversion occurs in another model of extreme β -cell loss as in the diphtheria-toxin model (21), and 2) to address the question of whether any type of β -cell regeneration can be induced by PDL after severe β -cell deple-

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Abbreviations: EQ, equivalent; IE, islet equivalent; L, ligated; PDL, pancreatic duct ligation; PP, pancreatic polypeptide; STZ, streptozocin; TX, transplantation; UL, unligated.

Table 1. Average and Proportion of Insulin and Glucagon/PP-Positive Cells in STZ-Only Controls, Short-Term Therapeutically and Subtherapeutically Transplanted STZ+/TX+/PDL+ and Long-Term STZ+/TX+/PDL+ Rats

Variable	STZ Only (n = 9)	PDL+0 (n = 3)	PDL+3 (n = 5)		PDL+7 (n = 8)		PDL+10 (n = 8)	
			L	UL	L	UL	L	UL
Insulin+ cells								
Average per animal \pm SEM	17 \pm 3	27 \pm 8	23 \pm 7	19 \pm 2	32 \pm 7	16 \pm 3	26 \pm 8	11 \pm 2
Proportion (%) \pm SEM	1.4 \pm 0.2	1.1 \pm 0.1	1.0 \pm 0.2	1.9 \pm 0.3	1.3 \pm 0.3	1.7 \pm 0.4	1.2 \pm 0.3	1.2 \pm 0.2
Glucagon/PP+ cells								
Average per animal \pm SEM	1257 \pm 187	2378 \pm 405	2417 \pm 547	1058 \pm 97	2497 \pm 351	970 \pm 188	2428 \pm 312	1051 \pm 231
Proportion (%) \pm SEM	98.6 \pm 0.2	98.9 \pm 0.1	99.0 \pm 0.2	98.1 \pm 0.3	98.7 \pm 0.3	98.3 \pm 0.4	98.8 \pm 0.3	98.8 \pm 0.2
Average no cells per animal \pm SEM	1274 \pm 188	2405 \pm 413	2441 \pm 553	1077 \pm 96	2529 \pm 355	986 \pm 190	2454 \pm 315	1063 \pm 232
Total cells counted	11 469	7215	12 203	5386	20 231	7886	19 630	7438
Total islets counted	219	115	222	113	326	139	344	139

Continuous variables are presented as mean \pm SEM.

tion. The rationale for combining severe β -cell depletion by STZ and PDL was to eliminate potential misinterpretation of β -cell regeneration due to shrinkage of exocrine tissue after PDL. Because of severe diabetes, the rats were given islet cell transplants to allow long-term observation. We found no evidence of β -cell regeneration for up to 10 months after PDL. However, the acinar cell compartment first underwent severe degeneration followed by extensive appearance of adipocytes and then slow, but near-complete, recovery of the acinar compartment.

Materials and Methods

Animals

Male 7- to 10-week-old Lewis rats (Harlan Laboratories) were kept under conventional conditions with free access to water and food. All procedures were approved by the Joslin Institutional Animal Care and Use Committee.

Administration of STZ to rats with varying glucose levels

A single dose of STZ (ip, 95 mg/kg, Sigma) freshly dissolved in citrate buffer (pH 4.5) was injected into 4 groups of Lewis rats with varying blood glucose levels. These groups were: 1) fed rats given a glucose bolus (Fed+Glc; 1 g/kg of 20% glucose ip [Mediatech] 15 minutes before STZ), 2) rats in the fed state (Fed), 3) fasted overnight (Fast), and 4) fasted overnight plus an insulin bolus (Fast+Ins; 1 U insulin lispro ip [Eli Lilly] 20 minutes before STZ). Untreated rats were controls. Glucose levels were measured on blood from snipped tails with a glucometer (Accu-Check, Boehringer-Mannheim Biochemicals). Animals were killed after 1 week, and β -cell loss was assessed by pancreatic insulin content (rat insulin ELISA by Alpco Immunoassays) and immunostaining on paraffin sections for β -cells (insulin) and non- β -islet cells (glucagon/pancreatic polypeptide [PP]/somatostatin) as described below.

Islet isolation and transplantation

Islets were isolated from 8- to 10-week-old male Lewis rats by collagenase digestion (23) with rodent Liberase RI (Roche), purified by gradient separation using Histopaque-1077 (Sigma), and cultured overnight in RPMI 1640 containing 11.8 mM glu-

cose (Mediatech) with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Mediatech). On the next day, 7-week-old animals, 2 days after receiving STZ (after fasting), were anesthetized and transplanted under the left kidney capsule with either 2000 or 1300 islet equivalents (IEs), as previously described (24).

Pancreatic duct ligation (PDL)

At 4–5 days after transplantation, animals were anesthetized and the main pancreatic duct was ligated in the direct line below the pylorus (10). The ligated (L) tail portion accounted for approximately 50% of the pancreas; the unligated (UL) portion was an internal control. Animals receiving STZ and islet transplants but no PDL (STZ+/transplantation[TX]/PDL–) served as controls to the STZ+/TX+/PDL+ condition. Additional control experiments were performed with nondiabetic animals with (Ctrl+14 PDL only) or without PDL (Ctrl+14 Sham).

Nephrectomy

In a subset of rats, the kidney containing the islet graft was removed at 5 or 10 months after PDL. After nephrectomy, animals were killed when they developed severe diabetes. The 10-month time point was chosen because in another model of extreme β -cell loss β -cell regeneration has been reported within this period (21).

Immunostaining

With overdose anesthesia, pancreases were excised, weighed, and fixed with 4% (para)formaldehyde followed by paraffin embedding. Sections were stained with hematoxylin and eosin or immunostained. We observed strong autofluorescence by immune cells infiltrating after PDL, which could have led to overestimation of β -cells. Pretreatment with 0.025% Pontamine Blue Sky (Sigma) in 1% dimethylsulfoxide (Sigma) in PBS for 30 minutes at room temperature before the primary antibody (25) raised background levels to block out the autofluorescence whereas insulin-positive cells could be easily distinguished. The following primary antibodies were used: rabbit antiglucagon (1:3000; Millipore Corp.), rabbit antipancreatic polypeptide (1:200; Millipore Corp.), rabbit antisomatostatin (1:200; Millipore); guinea pig antiinsulin (1:200; Sigma); rabbit anti-Ki67 (1:200; Abcam) followed by biotinylated antirabbit IgG (1:200; Jackson ImmunoResearch Laboratories); mouse anti-E-cadherin (1:200; BD Biosciences). The following were secondary antibodies: Alexa fluor 488 antirabbit IgG (1:500; Invitrogen);

Table 1. Continued

PDL+14 (n = 6)		PDL+7 (hyper) (n = 6)		PDL+5 mos (n = 10)		PDL+10 mos (n = 6)	
L	UL	L	UL	L	UL	L	UL
18 ± 4 1.2 ± 0.4	19 ± 4 1.2 ± 0.2	57 ± 9 2.0 ± 0.2	25 ± 4 2.0 ± 0.3	42 ± 7 1.5 ± 0.2	39 ± 6 2.1 ± 0.4	24 ± 5 1.1 ± 0.2	21 ± 5 1.7 ± 0.4
1677 ± 301 98.8 ± 0.4	1611 ± 353 98.8 ± 0.2	2862 ± 418 98.0 ± 0.2	1280 ± 175 98.0 ± 0.3	2928 ± 405 98.5 ± 0.2	1941 ± 227 98.0 ± 0.4	2139 ± 80 98.9 ± 0.2	1266 ± 236 98.3 ± 0.4
1694 ± 301 9779	1630 ± 356 10 166	2919 ± 425 17 514	1305 ± 177 7830	2970 ± 411 29 700	1980 ± 230 19 801	2162 ± 82 12 973	1287 ± 240 7721
207	196	309	126	515	348	151	238

Dylight 594 antiguinea pig IgG (1:200; Jackson ImmunoResearch Laboratories); Streptavidin Alexa fluor 488 IgG (1:200; Invitrogen); and Cy3 antimouse IgG (1:200; Jackson ImmunoResearch Laboratories). DAPI was used for nuclear staining. Confocal images were taken on a Zeiss LSM 710.

Endocrine cell quantification

We used the proportion of β /islet cells for β -cell quantification because determining mass with point counting was not prac-

tical due to the paucity of β -cells. Because the UL pancreatic portion contains the ventral, PP-rich part of the pancreas, we combined glucagon and PP-staining for determination of non- β -cells, which allowed comparison of β /islet cell proportions not only of L portions of different animals but also L and UL portions of the same animals. δ -Cells, which account for approximately 2%–4% of the endocrine pancreas (26), were not stained. For quantification of β /islet cells in STZ+TX+/PDL+ or STZ+TX+/PDL– experiments, 10 000–20 000 cells in the L portion and 5000–10 000 in the UL were counted per group (Table 1). Endocrine cell aggregates were classified as islets (>8 endocrine cells), clusters (2–8 cells), and single cells.

Ki67-quantification in duct and acinar cells

For quantification of Ki67-positive acinar and duct cells, at least 4 fields (one field 0.12 mm²) in both L and UL portions (~1500 cells each) were counted for each animal. Sections were double-stained for Ki67 and E-cadherin: duct cells could be discriminated from acinar cells by their morphology and stronger, more even, E-cadherin staining.

Insulin content

Pancreases were excised at 2 weeks and 5 months and dissected into L and UL portions under a dissecting microscope. Control pancreases were similarly dissected to obtain ligated equivalent (L EQ) and unligated equivalent (UL EQ) portions. Portions were weighed, placed into 70% acid ethanol, sonicated, kept overnight at 4°C, and stored at –20°C. Insulin was measured with ELISA (Alpco Immunoassays).

Data analysis

Data are expressed as mean ± SEM. The unpaired Student's *t* or Mann-Whitney tests were used. A *P* value <0.05 was considered statistically significant. Correlations were calculated with the Pearson's *r* test.

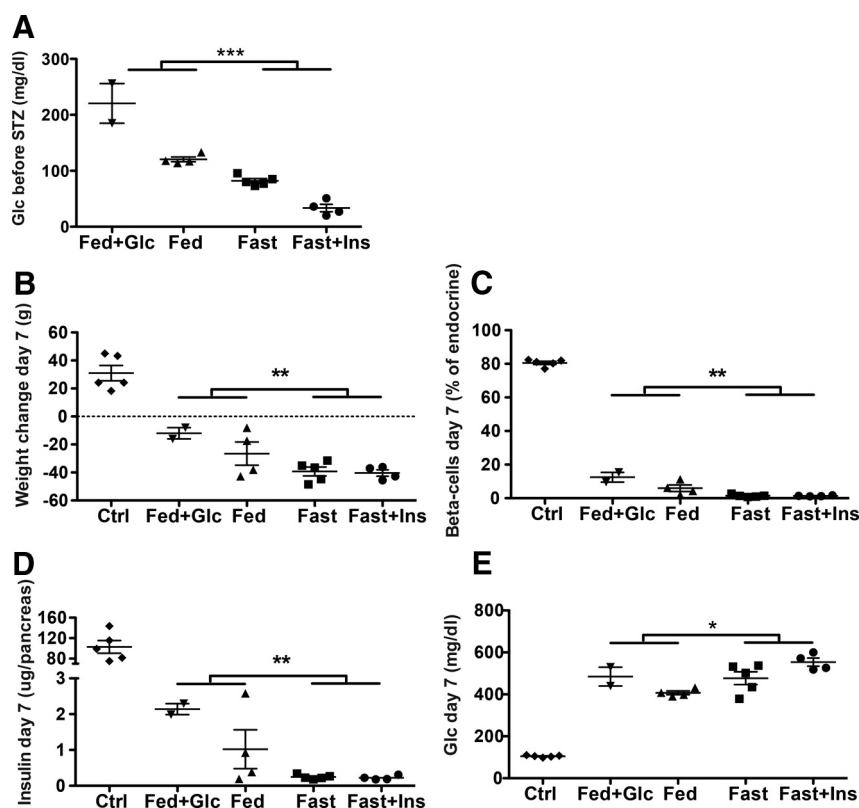


Figure 1. β -Cell Loss by STZ Is More Severe With Lower Glucose Levels at Time of STZ Injection. A, Lewis rats of groups Fed+Glc (fed plus 1 g/kg 20% glucose ip 15 minutes before STZ; n = 2), Fed (n = 4), Fast (overnight fast; n = 5), and Fast+Ins (overnight fast plus 1 U insulin lispro ip 20 minutes before STZ; n = 4) had varying glucose levels before receiving a single STZ-injection (95 mg/kg, ip). B, Weight change 7 days after STZ. C, Percentage of β -cells as a proportion of all endocrine cells (β -, α -, PP, and δ -cells). D, Pancreatic insulin content was lowered in a glucose-dependent fashion. E, Glucose levels 7 days after STZ. Untreated animals served as controls (Ctrl; n = 5). Changes were statistically significant when comparing the two fed conditions (Fed/Fed+Glc) with the two fasting conditions (Fast/Fast+Ins). *, *P* < .05; **, *P* < .01; ***, *P* < .001. Ctrl, control; Glc, glucose; Ins, insulin.

Results

Extreme β -cell loss achieved by lowering blood glucose at time of STZ injection

Glucose levels at the time of STZ injection were 221 ± 36 mg/dL for fed plus glucose bolus (Fed+Glc, $n = 2$), 121 ± 4 mg/dL for fed alone (Fed, $n = 4$), 82 ± 4 mg/dL for fasting overnight alone (Fast, $n = 5$), and 34 ± 7 mg/dL for fasting overnight plus insulin bolus (Fast+Ins groups, $n = 4$) (Figure 1A). At day 7 after STZ injection, body weight, percentage of remaining β -cells as proportion of all endocrine cells (β , α , PP, and δ), and insulin content were lowered in a glucose-dependent fashion (Figure 1, B–D), whereas glucose levels were higher in fasting compared with fed conditions (Figure 1E). These surrogate measures of β -cell mass differed statistically when fed conditions (Fed and Fed+Glc) were compared with fasting conditions (Fast+Ins and Fast). β -Cells as a percentage of all endocrine cells (β -, α -, PP, and δ -cells) after STZ accounted for $12.5 \pm 2.8\%$ in Fed+Glc, $6.0 \pm 2.0\%$ in Fed, $1.4 \pm 0.4\%$ in Fast, and $1.4 \pm 0.1\%$ in Fast+Ins groups (Figure 1C). In untreated controls, $80.6 \pm 0.9\%$ of all endocrine cells were β -cells. When the dorsal, L portion of the pancreas was stained for PP and glucagon, PP-positive cells accounted for $2.6 \pm 0.4\%$ and glucagon-positive cells

accounted for $97.5 \pm 0.4\%$. Insulin content (μg) decreased from 103 ± 12.4 μg in untreated controls to only 0.22 ± 0.03 μg in Fast+Ins and 0.25 ± 0.03 μg in Fast groups (Figure 1D). There was a strong correlation between all different surrogate measures of β -cell mass (body weight and insulin content, $r = 0.90$; insulin content and percentage of β -cells, $r = 0.96$; and body weight and percentage of β -cells, $r = 0.84$). These data indicate a degree of extreme β -cell depletion similar to that induced by diphtheria toxin in a transgenic mouse model (21) or by alloxan (22). Extreme hypoglycemia produced by insulin injection after overnight fasting did not lead to even greater β -cell loss; therefore, fasting alone before the STZ injection was chosen for extreme β -cell depletion.

Lack of β -cell regeneration after extreme β -cell loss combined with PDL in the short term

PDL had no effect on glucose levels in rats transplanted with islets (Figure 2A). Histologically, the atrophic islets looked unaffected by PDL. The proportion of β /islet cells (β -, α - and PP, but not δ -cells) was very low at all time points in both L (days 3, 7, 10, and 14: 1.0 ± 0.2 , 1.3 ± 0.3 , 1.2 ± 0.3 , and $1.2 \pm 0.4\%$, respectively) and UL portions (days 3, 7, 10, and 14: 1.9 ± 0.3 , 1.7 ± 0.4 , 1.2 ± 0.2 , and $1.2 \pm 0.2\%$, respectively) (Figure 2B and Table 1) in contrast to the $80.6 \pm 0.9\%$ β -cells in normal controls, suggesting that no α - to β -cell conversion took place.

Two weeks after PDL, L portions of the pancreas were atrophied, weighing only $40.3 \pm 6.4\%$ of equivalent portions of controls (L 0.19 ± 0.03 g vs. L EQ 0.47 ± 0.01 g; $P < .001$; Figure 2C). Insulin concentrations ($\mu\text{g/g}$) were significantly higher in L portions (L 2.03 ± 0.3 $\mu\text{g/g}$; $n = 12$) compared with equivalent portions of control rats receiving STZ and islet transplants but no PDL (L EQ 1.09 ± 0.2 $\mu\text{g/g}$, $n = 7$; $P = .048$) (Supplemental Figure 1A published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). However, this increased insulin concentration is misleading due to marked acinar atrophy. Total insulin content (μg) correlates well with β -cell mass as long as glucose levels remain normal and therefore can be considered a useful surrogate. Thus, although in-

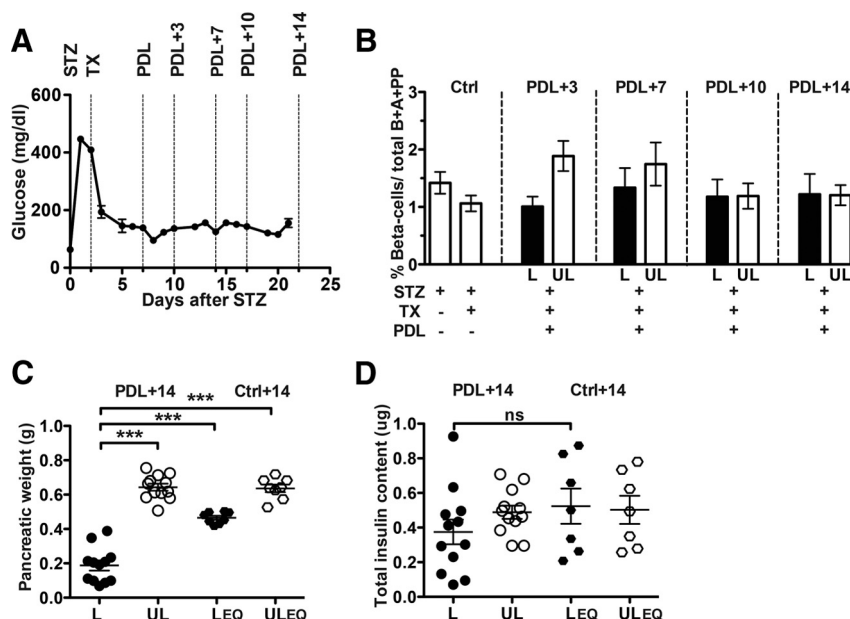


Figure 2. Short-Term Changes (2 Weeks) After Extreme β -Cell Loss Combined With PDL: Blood Glucose, Histology, and Insulin Content. A, PDL had no effect on glucose levels. B, Animals were killed at 5 time points after PDL: PDL+0 ($n = 3$), PDL+3 ($n = 5$), PDL+7 ($n = 8$), PDL+10 ($n = 8$), and PDL+14 days ($n = 6$). The proportion of β /islet cells (comprised of β -, α -, and PP, but not δ -cells) remained very low at $1.2 \pm 0.2\%$ in the L and $1.5 \pm 0.2\%$ in the UL portions, respectively. STZ+ ($n = 9$) and STZ+TX+ animals ($n = 3$) served as controls without PDL. C, Weight loss of the L pancreatic portion reflected the degeneration of the acinar tissue. The total insulin contents (μg) of L portions (panel D) ($n = 12$) did not differ from that of equivalent parts of control animals ($n = 7$). Abbreviations: B, β -cells; A, α -cells, PP, PP cells, L, ligated portion; UL, unligated portion; L EQ, ligated equivalent of control animals; UL EQ, unligated equivalent of sham control animals. ns, $P > .05$; *, $P < .05$; **, $P < .01$; ***, $P < .001$.

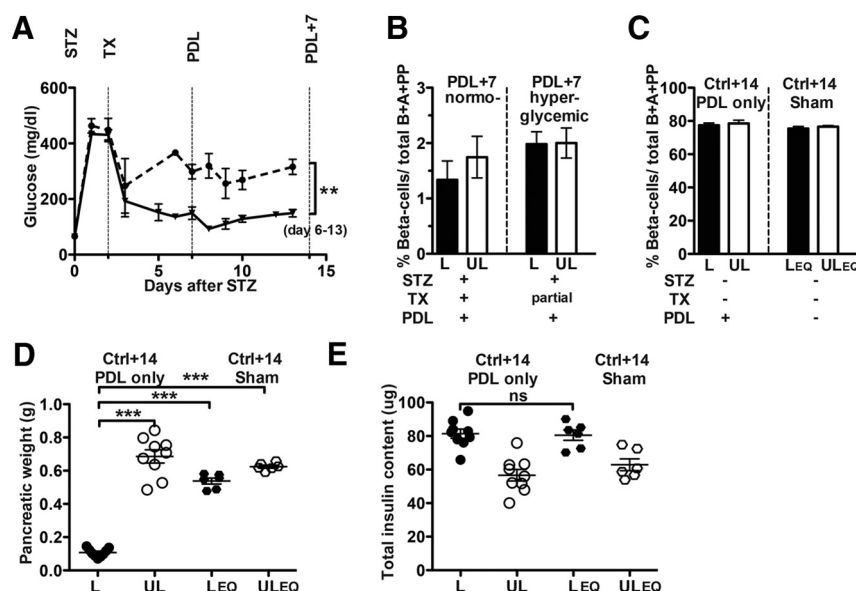


Figure 3. Subtherapeutically Transplanted Rats and Nondiabetic Controls With PDL Have No Increase in β -Cell Measures in the Short Term. A, To exclude normoglycemia inhibition of β -cell regeneration, optimally (2000 IEs per rat) ($n = 8$, solid line) or subtherapeutically transplanted (1300 IEs per rat) STZ+TX+PDL+ rats ($n = 6$, dashed line) were compared and showed significantly different blood glucose levels between days 6 and 13. B, At PDL+7 days, no difference in proportion of β /islet cells between normo- and hyperglycemic animals was observed (L, $P = .2$; UL, $P = .6$). C, After PDL in nondiabetic rats (Ctrl+14 PDL only, $n = 8$), there was no change in the proportions of β /islet cells compared with nondiabetic sham-operated rats (Ctrl+14 Sham; $n = 6$). Nondiabetic controls with PDL (Ctrl+14 PDL only) had reduced pancreatic weights (panel D), but unchanged insulin content (panel E) compared with nondiabetic controls with sham operation (Ctrl+14 Sham). ns, $P > .05$; **, $P < .01$, days 6–13; ***, $P < .001$.

sulin concentration was increased in L portions, no increase of insulin content was found: $0.38 \pm 0.10 \mu\text{g}$ in L portions ($n = 12$) compared with $0.52 \pm 0.10 \mu\text{g}$ in equivalent portions of controls ($n = 7$) (Figure 2D). Of note, these were far lower than the insulin content of whole untreated pancreas ($103 \pm 12.4 \mu\text{g}$; $n = 5$). The weights of UL portions were exactly the same at $0.64 \pm 0.02 \text{ g}$ in both L and control animals (Figure 2C), confirming the comparable dissection of portions.

To exclude normoglycemia inhibiting β -cell regeneration, we asked whether subtherapeutic transplantation of islets would enhance β -cell regeneration. Animals transplanted with only 1300 IEs had higher glucose levels than animals transplanted with 2000 IEs ($P < .01$ days 6–13; Figure 3A). However, no significant increase in their β /islet cell proportions was observed 7 days after PDL ($P = .2$; Figure 3B and Table 1).

Nondiabetic rats receiving PDL

As additional controls, rats not given STZ received either PDL (Ctrl+14 PDL only) or sham surgery (Ctrl+14 Sham). After 14 days, β /islet cell proportions did not differ in both L and UL portions (Figure 3C). The extensive pancreatic weight loss led to an increase in insulin con-

centration but notably not in total insulin content (Figure 3, D and E, and Supplemental Figure 1B).

Lack of meaningful β -cell regeneration after extreme β -cell loss combined with PDL in the long term

Long-term observation of animals that received STZ, TX, and PDL displayed sustained normoglycemia with islet cell transplants. Removal of the transplanted islets by nephrectomy 5 and 10 months after PDL resulted in hyperglycemia within 1 day (Figure 4A). The atrophic islets looked the same as those examined at earlier time points but were now embedded in adipocytes with nearby patches of acinar and duct cells (see below for further description). β /islet cell proportions remained very low (5 and 10 months: 1.5 ± 0.2 and $1.1 \pm 0.2\%$, respectively) in the L portions and were similarly low in the UL portions (5 and 10 months: 2.1 ± 0.4 and 1.7 ± 0.4 , respectively) (Figure 4B and Table 1), again indicating no evidence

for meaningful β -cell regeneration or conversion of α -cells to β -cells.

Over time, pancreatic weights of L portions increased, yet remained lower than their corresponding UL portions (Figure 4C). The insulin content of the L portions at 5 months remained very low ($0.39 \pm 0.1 \mu\text{g}$; $n = 4$) and had not increased from the 2-week values ($0.38 \pm 0.1 \mu\text{g}$; $n = 12$) (Figure 4D).

No evidence for neogenesis after PDL with prior STZ treatment

Acinar degeneration with shrinkage of the exocrine tissue caused increased density of atrophied islets per section in the L compared with the UL portions (Figure 5, A and C). However, there was no evidence of endocrine regeneration through any pathway including neogenesis. In these β -cell-depleted pancreases, most β -cells were found in islets in both L and UL portions (short term: UL, $55.5 \pm 3.9\%$; L, $63.7 \pm 3.8\%$ [Figure 5B]; 5 months: UL, $58.4 \pm 3.9\%$; L, $71.5 \pm 4.2\%$; 10 months: UL, $63.8 \pm 6.4\%$; L, $65.2 \pm 7.0\%$ [Figure 5D]). There were no discernible increases of β -cells or non- β -cells as small clusters or single cells as would be expected with neogenesis; instead, from

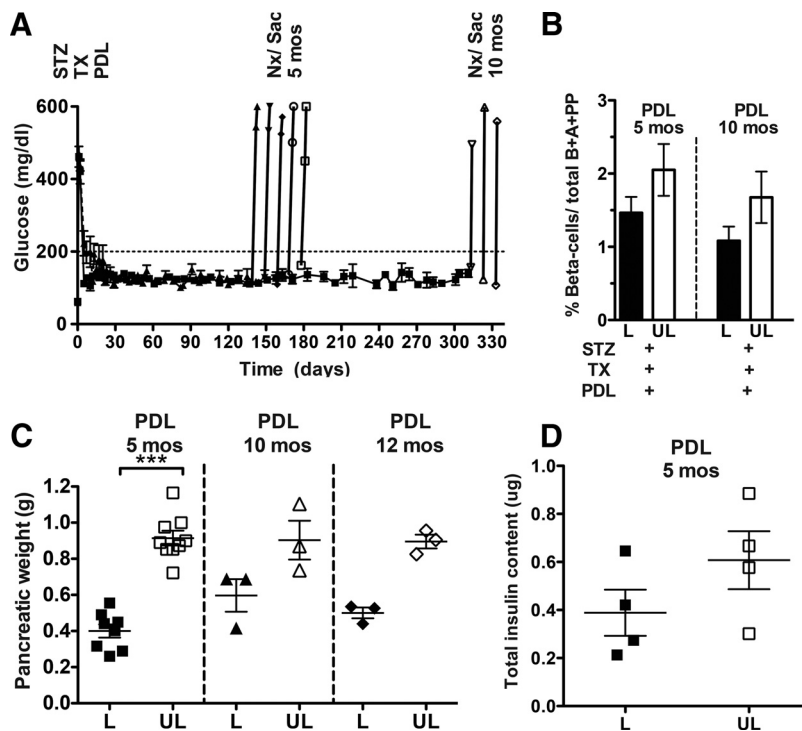


Figure 4. Long-Term Changes (5 and 10 Months) After Extreme β -Cell Loss Combined With PDL: Blood Glucose, Histology, and Pancreatic Insulin Content. **A**, Removal of the transplanted islets by nephrectomy after 5 or 10 months led to severe hyperglycemia within 1 day. **B**, After 5 and 10 months, the proportion of β /islet cells remained very low at $1.3 \pm 0.2\%$ in L and $1.9 \pm 0.3\%$ in UL portions. **C**, Weights of L pancreatic portions remained lower than their corresponding UL portions. **D**, At 5 months ($n = 4$) insulin content of the L portions remained very low with no increase compared with 2-week values (L, $P = .9$; UL, $P = .2$). Nx, nephrectomy. $***, P < .001$.

day 10 onward the highest percentage of endocrine cells in the L portions was found in islets (Supplemental Figure 2, A–D). Although we could not find evidence of enhanced neogenesis after PDL, we cannot exclude the presence of some low level of activity.

New acinar formation develops with time after PDL

Exocrine tissue changes were followed over time. Shortly after PDL (PDL+3, PDL+7 days), the acinar cells had largely disappeared and ductular complexes were the dominant structures in the L portions (Figure 6B and Supplemental Figure 3A). Occasional acinar cells could be seen, and some were apoptotic (inset Figure 6B). In the second week after PDL (PDL+10, PDL+14 days), some cells with acinar characteristics appeared to bud from ducts (Figure 6C, inset). At 5 and 10 months after PDL, the lobes, still definable by interlobular connective tissue (Supplemental Figure 3B), consisted mainly of adipocytes with tree-like ducts and patches of acini and islets (Figure 6, D–E). At 12 months after PDL, normal-sized lobes were filled with acini and only sparse adipocytes (Figure 6F). These changes of exocrine tissue were observed exclusively

in the L portions, whereas the UL portions were not distinguishable from untreated controls at any time (Figure 6A).

At PDL+7 days, proliferation (as assessed by Ki67 positivity) of ductal cells markedly increased in the L compared with the UL portion (L, $12.0 \pm 1.7\%$; UL, $1.4 \pm 0.6\%$; $P = .003$; $n = 7$), whereas that of acinar cells was less pronounced (L, $6.1 \pm 1.4\%$; UL, $1.2 \pm 0.4\%$; $P = .005$; $n = 6$) (Figure 6, G, J, and K). However, at PDL+14 days, the acinar cells were highly replicative in the L portions (L, $26.5 \pm 4.0\%$; UL, $0.4 \pm 0.2\%$; $P = .004$; $n = 5$), whereas proliferation in ductal cells had diminished (L, $1.9 \pm 0.7\%$; UL, $0.5 \pm 0.2\%$; $P = .03$; $n = 6$) (Figure 6, H, J, and K). At 5 and 10 months after PDL, replication in duct and acinar cells of the L portions was still evident, yet to a lower extent (5 months ducts: L, $2.0 \pm 0.5\%$; acini L, $1.0 \pm 0.2\%$; $n = 5$; 10 months ducts: L, $3.6 \pm 1.4\%$; acini L, $2.8 \pm 1.1\%$; $n = 3$). In contrast, ducts and acini of the UL portions had almost negligible proliferation (5 months duct: UL, $0.3 \pm 0.2\%$; acini UL, $0.2 \pm 0.07\%$; $n = 5$; 10 months duct: UL, $0.2 \pm 0.1\%$; acini UL, $0.1 \pm 0.07\%$, $n = 3$). At 12 months, 2 of 3 animals showed very low proliferation in ductal cells in L portions comparable to the UL portions. The one rat with prolonged proliferation at 12 months in the L portion seemed to lag behind the other two rats in the extent of exocrine tissue regeneration, having fewer acini and more adipocytes.

Discussion

β -Cell regeneration was studied in a model of extreme β -cell loss and PDL in rats. We found no evidence of meaningful β -cell regeneration, be it by replication, neogenesis, or conversion from α - to β -cells. However, the exocrine pancreas showed extensive regeneration by 12 months.

Susceptibility to STZ correlates with GLUT2-expression (27); therefore, we suspected less competition between STZ and glucose for transport when glucose was lowered prior to STZ administration. Indeed, lower glucoses resulted in more severe β -cell destruction from STZ,

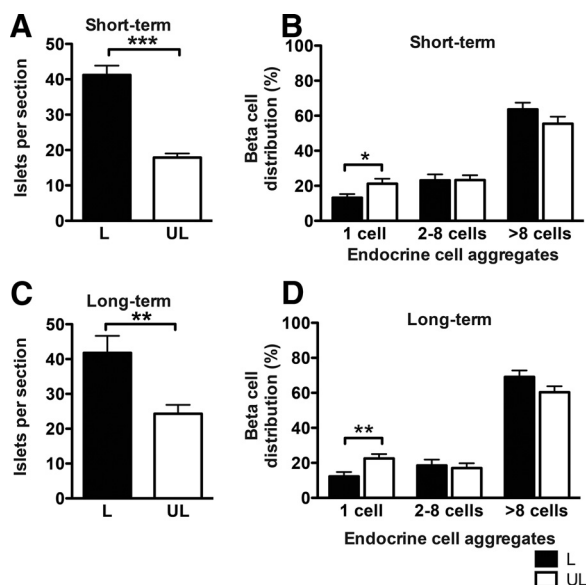


Figure 5. Distribution of β -Cells After PDL. With acinar degeneration after PDL, more atrophied islets (>8 endocrine cells per aggregate) were seen in each section in the short-term (A) ($P < .001$) and long-term (C) ($P = .002$) L portions compared with UL portions. In both portions, the highest percentage of the entire population of β -cells was found in islets (>8 endocrine cells/aggregate) with fewer being found in single β -cells (1 endocrine cell per aggregate) or in clusters (2–8 endocrine cells per aggregate) (short-term [panel B]; long-term [panel D]). *, $P < .05$; **, $P < .01$; ***, $P < .001$.

although extreme hypoglycemia from insulin injection made no further difference.

The percentage of insulin-positive cells as proportion of all endocrine cells, pancreatic insulin content, and actual cell counts served as rough surrogates for β -cell mass. However, insulin content may have underestimated β -cell mass due to hyperglycemia-induced degranulation. For example, insulin content after our fasting/STZ regimen decreased to about 0.2% of normal compared with a remaining β /islet cell proportion of 1.4%.

Two studies reported that extreme β -cell depletion led to conversion of α - to β -cells. Thorel and colleagues (21), using mice with genetically marked α -cells and diphtheria-toxin induced near-total β -cell ablation, observed α -cells being converted into β -cells after several months. Another study applied PDL after alloxan and reported conversion of α - to β -cells within 2 weeks (22). We achieved comparable β -cell depletion with fasting and STZ, but we found no evidence of α - to β -cell conversion, as demonstrated by no increase of β /islet cell proportion or insulin content. Potential explanations for the discordant findings include species differences (mouse vs rat), different means to achieve glycemic control (insulin pellets vs islet transplantation), and a potentially harmful effect of STZ on α -cells. Nonetheless, α - to β -cell conversion does not seem to depend on extreme β -cell depletion.

Importantly, no evidence of significant β -cell regeneration of any kind was found after PDL, because both the β /islet-cell proportion and insulin content stayed low. Additional control experiments with rats not treated with STZ corroborated these results, thus excluding STZ damage to progenitor cells as an explanation. This is in line with recent studies using lineage tracing in mice (19, 20): one study used insulin promoter-based lineage tracing and showed lack of dilution of β -cells after PDL (19). The other, more recent, study employed a time-sensitive lineage approach to track neogenesis, which was not found to occur after PDL (20). Conflicting data exist on the role of Ngn3-up-regulation after PDL. Although some claim it plays a role in driving differentiation of Ngn3+ cells to β -cells after PDL (12), others had contrary findings (16, 28). Our study expands these findings as our model 1) provides a clean approach avoiding potential misinterpretation of preexisting β -cells as new β -cells and 2) extends the follow-up period up to 10 months after PDL. Earlier studies using PDL have shown increased numbers of small β -cell clusters or single cells (10, 12), increased β -cell mass (10–12, 17), or acinar-to-endocrine conversion after PDL (29). Results on insulin content have been mixed, with levels being increased (11) or unchanged after PDL (10, 16, 19). Our inability to stimulate significant neogenesis with PDL agrees with concerns by others about measurements of β -cell mass after PDL (16, 18). Because profound tissue changes with edema and inflammatory infiltrates are accounted for in the weight but not necessarily in morphometric measurements of total tissue area, β -cell mass can be overestimated. A recent study suggested that direct β -cell counts were preferable and avoided the changes in pancreatic composition after PDL (18). Species (mouse vs rat) and strain differences might explain some of the discrepancies found by others. For example, pancreatic weight loss in mice (18) is more pronounced than in rats after PDL (10), perhaps due to differences in inflammation. Whether other tissue changes, such as blood supply, also might be responsible for differences seen between PDL results are not known, these changes could be addressed with future studies.

Formal determination of β -cell mass was not practical due to low numbers of β -cells remaining after STZ. In addition to the low β /islet-cell proportion, the absolute numbers of β -cells, as determined by cell counts, remained very low (Table 1). Thus, the unchanged β /islet-cell proportion is unlikely due to simultaneous increases of both α - and β -cells. Further evidence for maintained severe β -cell deficiency was demonstrated by rapid hyperglycemia after graft removal.

Because glucose metabolism influences β -cell replication (30–32), we examined whether subtherapeutic islet transplants might enhance β -cell regeneration. No evidence of increased β -cell number was found over the short term, but because these cells were so few to begin with, the changes

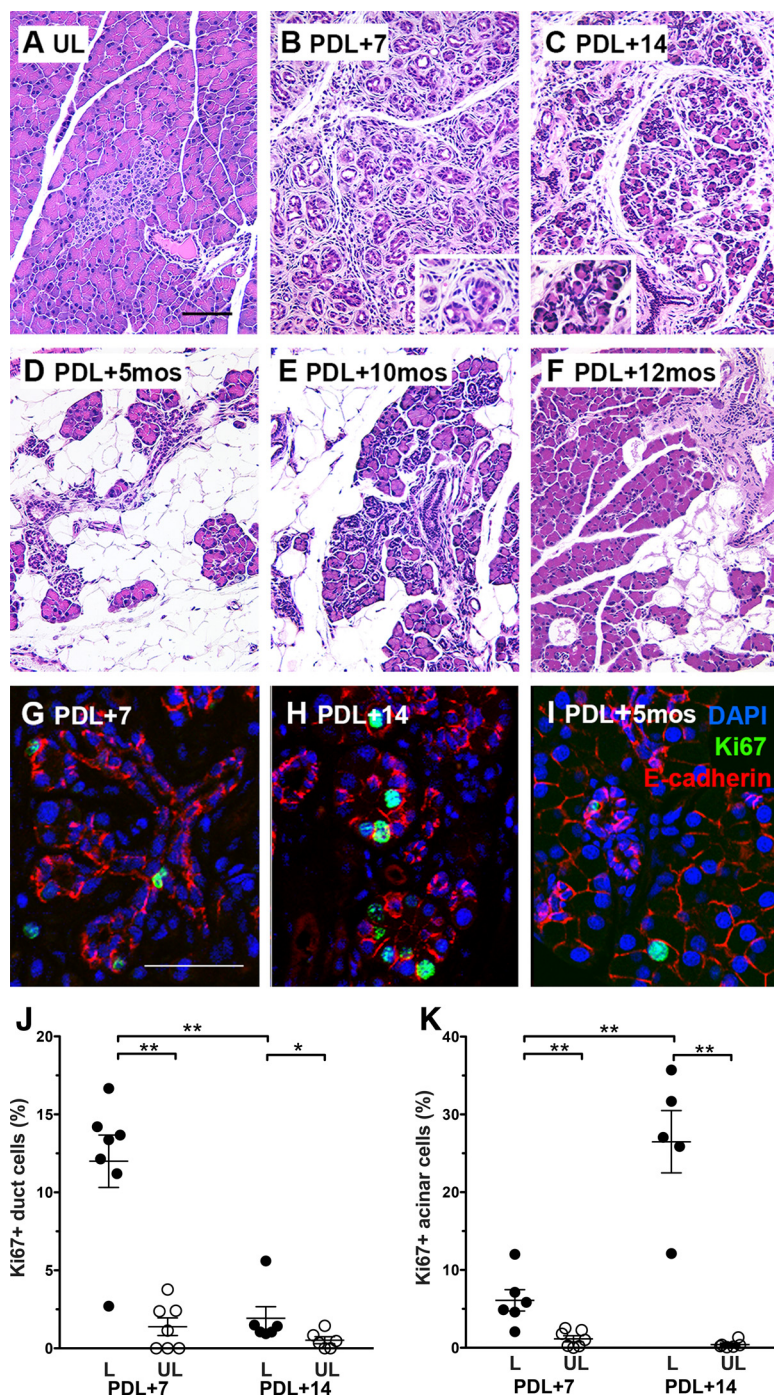


Figure 6. Rapid loss of Acinar Tissue After PDL and Later Gradual Regrowth. In contrast to UL pancreas (A), at PDL+7 days (B) L pancreas consisted mainly of ductular complexes with a few degenerating acinar cells remaining (inset: apoptotic acinar cells). C, By PDL+14 days, acinar cells were seen budding from ductular complexes (inset at higher magnification). At PDL+5 (panel D) and +10 months (panel E), lobes of pancreas consisted mainly of adipocytes with patches of duct tree-like structures with islets and acinar cells. F, At PDL+12 months, lobes within L portions had regained normal architecture of acinar tissue with only a few adipocytes left. Immunostaining for Ki67 (green) showed replicating duct cells at PDL+7 days (panel G) and acinar cells at PDL+14 days (panel H). I, At PDL+5 months, duct and acinar cells were still Ki67-positive. Quantification of replication showed a first wave in duct cells at PDL+7 days (L vs UL, $P < .01$) (panel J), followed by active acinar cell replication at PDL+14 days (L vs UL, $P < .01$) (panel K). A–F: Hematoxylin and eosin; magnification bar, 100 μm . G–I: immunofluorescence for Ki67 (green), E-cadherin (red), and 4',6-diamidino-2-phenylindole (blue) for nuclei; magnification bar, 50 μm . *, $P < .05$; **, $P < .01$; ***, $P < .001$.

might have been too small to measure. It is possible that a longer period of hyperglycemia may have led to some regeneration.

In contrast to the lack of β -cell regeneration, regeneration of the exocrine pancreas was found. As is known from acinar regeneration after cerulein-induced pancreatitis, some regeneration of acinar cells can occur within just 1 week (33). This regeneration has been thought to result from replication of acinar cells (34) or through acinar cell dedifferentiation to “metaplastic epithelium” with subsequent redifferentiation to acinar cells (33). Acinar regeneration after partial pancreatectomy also has been linked to replication (35), and there is evidence of postnatal new acinar cell formation from duct cells in some studies (6, 9, 13, 14) but not others (16, 17). Work by Crisimanna et al found regeneration in mice after genetic depletion of both acinar and endocrine cells. Acinar regeneration in that study was similar to ours. The differences seen in endocrine regeneration, however, are not yet understood but may be due to varying inflammation after PDL and genetic ablation.

In our model, the vast majority of acinar cells were lost soon after PDL. Some of the regeneration could have been from acinar dedifferentiation upon injury followed by redifferentiation to acinar cells as suggested by the cerulein model (33). An alternative explanation is that duct cells after injury regress to a less differentiated state and then serve as facultative stem cells (9, 36) that give rise to new acinar cells that then replicate.

Extensive pancreatic fat replacement with interspersed tree-like structures of duct, islet, and acinar cells was a predominant feature 5 and 10 months after PDL. Acinar regeneration was only near complete at 12 months. Accumulation of adipocytes, termed “lipomatosis,” has been de-

scribed in different disease states and experimental models. Pancreatic lipomatosis in patients has been associated with pancreatic stones (37), leiomyosarcoma in the pancreatic head (38), neoadjuvant radiochemotherapy for pancreatic adenocarcinoma (39), pancreatic pseudocysts (40), cystic fibrosis (41), the rare autosomal recessive Shwachman syndrome (42), and a newly described maturity onset diabetes of the young (MODY 8) caused by a heterozygous carboxyl-ester lipase mutation (43). Experimental animal models of pancreatic lipomatosis include copper depletion in rats (44) and different genetic mouse models including pancreatic cilia deletion (inactivation of Kif3a (45)), genetic manipulations of acinar cell growth and differentiation (inactivation of c-Myc (46), or serum response factor (47); knock-outs of E2F2/F1 (48) or TGF- β type II receptor (49); knock-ins of activin β -E in mice (50)) or of acinar cell polarity (inactivation of LKB1 (51)). Although the etiologies of these conditions seem to be very different, their underlying pathology may result from impairment of the pancreatic drainage or disruption of the acinar cells. In the case of the newly reported carboxyl-ester lipase mutation, the mutated protein forms aggregates (52), which raises the question of whether these aggregates might increase the viscosity of pancreatic fluid in small ducts leading to impaired flow similar to cystic fibrosis. In the experimental model of c-Myc inactivation in pancreatic progenitor cells, lineage tracing showed adipocytes deriving from acinar cells (46). Thus, acinar cells degenerating after pancreatic obstruction might convert to adipocytes.

While the acinar cell compartment was regenerating, there was presumably restoration of pancreatic drainage. However, the drainage of the new exocrine tissue seen at 12 months presents a puzzle because the original ligature remained intact. Perhaps the ducts find new pathways separate from the old ligated duct to allow fluid to enter the gastrointestinal tract or be directed elsewhere. Answers to these questions await future experiments.

In summary, after almost complete β -cell loss following STZ, we found no evidence of significant β -cell regeneration from replication, neogenesis, or α - to β -cell conversion. However, acinar cell regeneration occurred after PDL, although near-complete acinar regeneration was only seen after 12 months. Thus, the regenerative capacities of endocrine and exocrine cells in our PDL model in adult rats are very distinct.

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