Partial Duct Ligation: β-Cell Proliferation and Beyond

Experimentally induced injury is an established strategy for studying mechanisms of tissue remodeling with the final goal of developing new regenerative therapies. Under normal physiological conditions, proliferation and differentiation of progenitor cells, including even canonical stem cell–like activity, can be stimulated in tissues, such as brain and liver, that have a low cellular turnover rate (1,2). The presence of stem/progenitor cells in the pancreas could be relevant to normal homeostatic maintenance of various cell types in this organ, such as endocrine hormone–expressing cells, enzyme-secreting acinar cells, and the less secretory exocrine duct cells. Further, pancreatic stem/progenitor cells may be a possible source for replenishing cells destroyed by autoimmune disease or other stressors. We speculate that proliferative progenitors might be isolated, expanded, and differentiated in vitro to alleviate the donor scarcity in human islet transplantation and may therefore be developed as a therapy for diabetes. However, the existence and exact location of adult stem- or progenitor-like cells that can give rise to functional β-cells is highly controversial. This Perspective focuses on findings from a severe insult model (partial duct ligation [PDL]) with a long history (3). PDL received renewed attention when a 2008 study combined it with genetic reporter strategies now possible in mice to try to identify and isolate cells acting as β-cell progenitors (4). In vivo β-cell neogenesis under PDL was recently substantiated (5,6). Because the outcomes from this technique appear to vary across laboratories, we summarize and discuss some of the reported discrepancies to help identify current limitations and pitfalls of this model as well as opportunities for forward progress.

INJURY MODELS ACTIVATE DIVERSE PROCESSES LEADING TO NEW β-CELL GENERATION IN THE MOUSE PANCREAS

The mechanisms leading to replacement of the endogenous β-cell pool have been studied under several cell ablation paradigms to test for the existence and type of cells in the adult mouse pancreas that are capable of yielding new β (insulin-producing) cells. These paradigms are of varying specificity and efficiency, and they use chemical, genetic, or surgical methods (7–12). Newly emerging β-cells derive mainly from preexisting β-cells in adult mice under normal physiological conditions. This occurs in mothers responding to the increased demands of pregnancy and in the compensatory β-cell expansion that ensues after 50–70% pancreatectomy (8,13,14). Some widely reported studies base this conclusion on a statistical analysis of a pool of β-cells that was deliberately partially labeled, and in which the fraction of labeled cells showed no alteration (dilution) over time by β-cells that were derived from other (unlabeled) cell types. A β-cell population prelabeled to ~20–30% showed the same approximate percentage of labeled cells after the test period or manipulation (8,13).

However, a big caveat here is that even significant “minority seeding” of β-cells from other sources, whether duct/acinar or even other endocrine cell types—even at a 10% rate—would be unlikely to statistically deflect the 20–30% labeling. This scoring issue is exacerbated when combining data from several mice because of the inherent variabilities in tamoxifen (TAM)-induced labeling methods. Conversely, in another type of experiment that is not scored by a lack-of-dilution metric, a dramatic interconversion between cell types was detected. The near-total ablation of β-cells (99.6% loss) using targeted diphtheria toxin–based killing led to spontaneous regeneration of up to 17% of the normal β-cell content by an apparently direct transdifferentiation from preexisting α (glucagon-producing) cells into β-cells, with no involvement of proliferation (9). The latter case remains intriguing in that the type of signal inducing such a conversion is still unknown particularly because the interconversion does not occur when there are slightly lower levels of β-cell destruction (9).
PDL AS A SELECTIVE TYPE OF PANCREATIC INJURY

In the adult pancreas, PDL elicits large alterations in tissue composition, including increased volume of the endocrine compartment, along with several important features that warrant review. This procedure involves tight ligation of one of the main ducts that drains enzymes secreted from acinar cells toward the duodenum. In most cases, the ligature is applied immediately downstream of the splenic or tail part of the pancreas, called “PDL tail.” About 55% of tissue that is distal to the ligation undergoes inflammation and acinar cell atrophy. In contrast, acinar cell death does not occur in the “head region” of the PDL pancreas. First applied in rats to study activated proliferation of duct and endocrine cells, this model also provided circumstantial evidence for the transicional cytodifferentiation between duct and endocrine cells, including the formation of new insulin-producing cells (10). β-Cell neogenesis is defined as the generation of new β-cells by differentiation of cells other than β-cells and could include facultative β-cell progenitors. When applied to reporter mice carrying a genetic cell lineage tracer, Ngn3-expressing cells, which carry β-cell progenitor characteristics, could be isolated from PDL pancreas (4) and were inferred to derive from (near) the duct epithelium.

Although PDL as an experimental model reflects an artificial situation, it has been widely used and has provided novel insights on β-cell formation. This model could be considered useful in mimicking human conditions that promote neoplasia and formation of adenocarcinoma. While the endocrine cells of the islets of Langerhans and the ducts remain largely unaffected under PDL, the majority of acinar cells involute under marked apoptotic death. It is possible that this is simply a damage response to the massive backing up of enzyme-rich secretions from acinar cells. Notably, PDL has been likened to chronic pancreatitis, and there is substantial inflammatory cell migration into the injured tail piece. The reason that some acinar cells can escape death remains unknown. Although a subpopulation may have a currently undefined specific escape competence (15), cell morphology alterations near the termini of the ductal network suggest that some acini lose their intracellular zymogen granules, become much less bulbous and flatter, and become more contiguous with the terminal duct epithelium (16). Perhaps concurrently, many of the postmitotic acinar cells become proliferative again. Conceptually, the postinjury recovery period is very different from pancreatectomy. In the latter procedure, there is surgical removal of large amounts of pancreatic tissue and compensatory growth occurs in the unresected tissue. The PDL model leaves duct, acinar-derived, and islet cells as potential sources for derivation of new endocrine cells or for cell transdifferentiation processes that are induced by the injury remodeling process and influenced by immune cell infiltration.

Immune cells are well known for their release of a variety of potent cytokines. PDL also provides the pancreatic head piece as an internal control in which systemic (i.e., long-range or circulatory) versus local effects can be distinguished. Any increase in β-cell mass induced in the PDL tail is not driven by a physiological need for insulin because the animal usually maintains good glycemic control unless endocrine-cell destruction is deliberately added into the mix. More likely, the stimulus is inflammatory cell–related or it combines locally released signals from the tissue blockage and acinar death in the ligated tail lobe (15,17). Because PDL is often reported to induce increased but discrepant numbers of β-cells, it is important to discriminate the relative contribution of new β-cell production by β-cell proliferation or neogenesis.

The in vivo contribution of various non-β-lineages to β-cell neogenesis in a PDL setting has received more intensive study. Although evidence has been presented for β-cell formation from non-β-cells in PDL, the rate at which this formation occurs can be too small to have a measurable impact on total β-cell mass. That β-cell formation occurs from exocrine acinar cells in the setting of PDL (5) is proof of concept and was important for using cell-type–specific lineage tracing with indelible genetic marking of progeny cells. Nonetheless, in this and in many other cases, the relevance to human pancreas under normal physiological conditions or in diabetes still needs to be resolved. It is essential to explore the issue of derivation of β-cells from non-β-cells, especially nonendocrine, sources in the human pancreas. Moreover, finding ways to turn the process on and off and to increase the lineage flux toward defined and authentic final fates, especially with pharmacological approaches, will be helpful to develop therapeutic β-cell replacement strategies.

INCREASED β-CELL FORMATION IN PDL PANCREAS

The response to PDL has been widely reported to induce not only measurable, but large increases in β-cell mass (4,10). However, other reports (18–20) have raised questions about the possible existence of underappreciated artifacts that could impact morphometric analyses in the earlier reports. It has been suggested that the massive post-PDL tissue modification, characterized by loss of acini, relative “collapse” of the epithelially derived tissue, together with tissue edema, fibrosis, enlarged septa, and adipocyte infiltration, leads to overestimates in PDL tail tissue weight and could skew estimations of β-cell mass.

Given this debate, it is useful to understand how β-cell mass is measured. The method for calculation of β-cell mass involves scoring insulin+ cells over a subset of tissue sections, then using this number to estimate the total number of insulin+ cells throughout the pancreas and then expressing that number as milligrams (mg) of β-cells in the entire organ. If pancreatic tissue mass is overestimated, then β-cell mass can also be incorrect. Some authors suggest that in PDL, determining the fractional β-cell volume per unit of pancreatic tissue (excluding any fibrotic, adipocyte, or other
tissue) is more accurate than β-cell mass, which first requires a determination of mass (18). However, even when using the fractional total β-cell volume as a metric, some laboratories observe quantifiable increases in the β-cell pool under PDL (4,6,10), while others do not (18–20).

Some of the reported failures to observe an increase in β-cell numbers in the post-PDL pancreas also fail to observe increases in total insulin content (18,20) or increased rates of β-cell proliferation (20). The post-PDL recovery time is not the variable explaining these interlaboratory discrepancies because most of the studies report on several postsurgical time points, generally 7 and 14 days after surgery. First, it is possible that some analytical methods are incompatible with estimating increases in β-cell formation. For example, continuous labeling with thymidine analogs used by some laboratories (20) could limit expansion of β-cell numbers without assessing the toxic effects of these analogs and/or effects on proliferation rate (21). Second, PDL-induced expansion of β-cells may be masked if the sham-operated and PDL groups of mice differ in preoperative body weight. Similarly, the effect might be absent in mice that are too young, suggesting that age-matching needs to be included in the experimental design. Third, data from male and female mice should not be pooled because our unpublished data (Y.Y., Y.C., B. Legein, G.L., V.C., N. De Leu, G. Martens, P. Chambon, H.H., and M.V.d.C., unpublished data) suggest that sex hormone physiology may play an important role in PDL. Perhaps most important, surgical procedures may differ across laboratories and have a meaningful impact on results. For example, the increase in post-PDL caspase-3 positivity in β-cells (20) suggests there is more β-cell damage than documented in earlier reports (4), possibly due to differences in surgical technique. These differences can include cleanliness of ligature insertion or tying off the duct too tightly or not tightly enough in ways that damage the vasculature and could result in varying levels of cell death.

We know that it is crucial to monitor the general postoperative health of sham-operated and PDL mice because pain and abdominal infection decrease drinking and food intake, reducing postoperative weight gain. Furthermore, there is likely to be a role for inflammatory signaling in setting up an environment conducive to metaplastic and neoplastic growth. Clearly, given the potency of the PDL model for determining and characterizing interendocrine or acinar to β-cell transformation, the potential influence of these variables mandates development of an agreed-on set of standard methods for PDL and analysis. Accordingly, we suggest promotion of uniformity across laboratories with approaches such as cross-training and/or comparative analysis of the same tissue samples. Agreement would first need to be reached concerning experimental parameters and types of surgery. For example, whether or not to involve the larger blood vessel that runs near the pancreatic duct as a way of inducing a different type or rapidity of injury.

**NGN3 TRANSCRIPT LEVEL AND β-CELL NEOGENESIS**

Following PDL, Xu et al. (4) detected a subpopulation of cells that induced expression of the gene encoding neurogenin 3 (Neurog3 or Nggn3). This was the earliest transcription factor whose transient expression commits embryonic progenitor cells to a postmitotic endocrine cell fate (22,23). Nggn3 expression occurred specifically in cells near the epithelial lining of the exocrine duct. There has been what can be described as an unnecessarily overwrought debate concerning whether or not an embryonic endocrine precursor—like Nggn3-expressing state is intermediate in the appearance of new β-cells. That is, does it reflect the occurrence of some kind of neogenetic process rather than a straightforward replicative derivation from extant β-cells? However, in two mouse models—PDL pancreas (4) and ectopic Pax4 transcription factor expression from a glucagon promoter (24)—short hairpin-mediated knockdown of Nggn3 expression reduced the generation of new β-cells, thereby providing additional support that Nggn3 is necessary for β-cell neogenesis. Moreover, adenovirus-mediated overexpression of Nggn3 in combination with the transcription factors Pdx1 and MafA reprogrammed acinar to β-cells in the pancreas of adult mice (25). Although expression of Nggn3 has been suggested to be important for β-cell generation under these particular experimental conditions, so far there is no solid evidence that new β-cells in PDL pancreas are directly derived from cells expressing Nggn3. This situation is complicated by the fact that some laboratories report expression of Nggn3 well past the embryonic endocrine precursor stage and into mature β-cells. Thus, although Nggn3+ cells may well represent β-cell progenitors in adult pancreas, this cannot be ascertained conclusively by their genetic tracing.

Because we hypothesized that the appearance of new β-cells moves through an intermediate or transitional state associated with the re-expression, or increased expression of Nggn3, we sought circumstantial evidence for this idea (6). We found that the global Nggn3 mRNA level detected by quantitative RT-PCR in the PDL tail showed a strong correlation with 1) number of β-cells derived from Nggn3+ cells; 2) number of islets that showed dilution of prelabeled β-cells by unlabeled β-cells at 14 days post-PDL, particularly small ones containing 20 or fewer β-cells; and 3) the overall dilution of prelabeled β-cells scored at 35 days post-PDL (6). Together, these data support the idea that precursors of at least some new β-cells derived under PDL context are from a non-β-cell source(s).

In our view, one important calculation was based on the dilution of yellow fluorescent protein (YFP) label in islets of RIPCreERT;R26RYFP mice from which we estimated that among PDL animals showing high Nggn3 levels (defined as exceeding 0.6-fold compared with a standard deduced from duodenum-sourced mRNA) about 14% of new β-cells could be derived from non-β-cells. In contrast, we
concluded that neogenesis was insignificant in PDL pancreases because Ngn3 transcript levels were below 0.2-fold of the normalized level seen in the duodenal tissue (6). The hypothesis that the number of Ngn3+ transitional intermediate cells or the individual Ngn3 level per cell is rate-limiting for β-cell neogenesis should be testable. For example, this could be explored by overexpressing Ngn3 in acinar or duct cells and tracing of these cells while also monitoring the dilution of preexisting β-cells in PDL pancreas. Along the same lines, it would also be important to quantify and isolate green fluorescent protein (GFP)-positive cells from the PDL pancreas of Ngn3-GFP mice with inducible and transient GFP expression driven by the Ngn3 promoter and compare the transcript profile of these cells to that of embryonic endocrine progenitor cells. It is likely that the number of Ngn3+ cells in PDL is controlled by signals from a specific combination of cytokines that has not yet been defined.

**HETEROGENEITY AMONG NGN3-EXPRESSING (β) CELLS**

Ngn3+ cells, most of which were located near the lining of duct epithelium (4), seem relevant for in situ β-cell neogenesis in PDL (5). Based on lineage tracing in Ngn3CreERT;R26R-YFP mice, 5–12% of β-cells derive from Ngn3+ cells in PDL (6). As noted above, there are limitations to arguing that Ngn3 expression shows evidence of an embryonic-like transitional proendocrine state. Ngn3 also appears to be expressed in normal β-cells, although at very low levels (26); a consideration that fuels disagreement between laboratories on its detectability. However, the hypothesis that the number of Ngn3+ transitional intermediate cells or the individual Ngn3 level per cell is rate-limiting for β-cell neogenesis should be testable. For example, this could be explored by overexpressing Ngn3 in acinar or duct cells and tracing of these cells while also monitoring the dilution of preexisting β-cells in PDL pancreas. Along the same lines, it would also be important to quantify and isolate green fluorescent protein (GFP)-positive cells from the PDL pancreas of Ngn3-GFP mice with inducible and transient GFP expression driven by the Ngn3 promoter and compare the transcript profile of these cells to that of embryonic endocrine progenitor cells. It is likely that the number of Ngn3+ cells in PDL is controlled by signals from a specific combination of cytokines that has not yet been defined.

Unfortunately, discrepancies among PDL studies have led to discouragement about the validity of this injury model as a valid analytical tool to study the ability of β-cells to return to a proliferative condition or increase their cell-cycling rate or for non-β-cells to undergo redirection toward a β/and/or other endocrine state (4–6,12,29). Although some of this uncertainty pertains to the experimental variability described, it is important to point out that appropriate application of rigorous lineage-tracing tools could help sort through the apparent confusion. We propose that a much more careful application of these methods should be mandated before reaching conclusions on the inherent reprogramming or transdifferentiation potential of the various pancreatic cell types. Such a mandate could help avoid further controversies in the β-cell neogenesis field.

As described, results from insulin/Ngn3-based lineage-tracking technology suggest a fractional response among β-cells. In another approach (28), chimera-based fractional prelabeling of islets in prediabetic NOD mice was used to show a post-PDL decrease in the preponderance of labeled β-cells (over ~40% of scored islets), again supporting the idea that new β-cells can derive from non-β-cells (29). A side point needs to be made here: We assert the importance of robust, extensive quantitative analysis of tissue when conclusions are being based upon statistical
comparisons, and this issue becomes especially relevant when comparing outcomes between independent cohorts of mice. In addition, one of our studies that used lineage tracing of Ptf1a-expressing acinar cells— and in which CreER expression was tightly restricted to acinar cells— provided solid evidence for β-cell generation from nonendocrine acinar cells in PDL (5). This study defined the lack of expression of the lineage tracer in β-cells in detail and presented a transdifferentiation hypothesis that is well aligned with the idea of a (partial) return to an embryonic endocrine cell ontogenetic process. The pancreatic progenitor Ptf1a+-labeled cells in these experiments yielded Sox9+ and/or Hnf1b+ duct cells, of which up to 38% transiently expressed Ngn3 protein. When combined with the parallel recombination of a fluorescent reporter, Ptf1a+ cell-derived acinar-to-ductal transitional intermediates could represent an isolatable, possibly expandable, source of β-cell progenitors. Importantly, this study reported no post-PDL increase

Figure 1—Ngn3 mRNA expression in adult β- and non-β-cells. Eight-week-old MIP<sup>RFP</sup>;Ngn3<sup>CreERT</sup>;R26<sup>YFP</sup> mice underwent PDL or sham surgery and administration of TAM (5 × 4 mg distributed over 10 days) to label Ngn3<sup>+</sup> cells and their descendants. A: β-Cells were isolated by FACS sorting based on MIP-driven expression of RFP (mRFP1), and binding of Zn<sup>2+</sup> chelator TSQ was performed as described (4). Sham or PDL β-cells were either isolated as the RFP<sup>+</sup>TSQ<sup>+</sup> population or PDL β-cells were further sorted into YFP<sup>+</sup> and YFP<sup>−</sup> subpopulations. Non-β-cells from PDL pancreas were selected as RFP<sup>+</sup>TSQ<sup>−</sup> cells. Sorting of YFP<sup>+</sup> and YFP<sup>−</sup> β-cells and of YFP<sup>+</sup> non-β-cells from PDL tail tissue is illustrated (see also RESEARCH DESIGN AND METHODS). From the same mice, Ngn3<sup>+</sup> cell-derived duodenum cells were sorted as YFP<sup>+</sup> duodenum cells (not shown). B: Ngn3 mRNA expression was studied in the indicated cell populations or tissues. All data are normalized to cyclophilin A mRNA and shown relative to the Ngn3 mRNA expression level in total duodenum tissue (normalized to 1.0). Data represent mean ± SEM (n = 5–9; ns: P > 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA with Tukey test). Ngn3 was elevated in the sparse (<5% of total β-cells [A]) YFP<sup>+</sup> β-cells, but not in the YFP<sup>−</sup> β-cells of PDL tail, when compared with sham tail β-cells.
in β-cell number, again reflecting possible interlaboratory technical variations in the protocol (or even between strains of mice). The report concluded that the conversion of acinar cells to new β-cells was both low and slow—requiring between 30–60 days post-PDL. A combination PDL injury approach plus β-cell destruction aimed at inducing β-cell deficit did, however, accelerate acinar-to-endocrine transdifferentiation (5).

Therefore, our findings disagree with tracing studies claiming that no β-cell neogenesis could occur from duct cells (18,27,30,31). We suggest several possibilities concerning why the conversion might simply have been missed in those other studies. First, while we have no explanation regarding the length of time to undergo acinar to endocrine conversion, we feel it is essential to highlight that our cell of origin quantification was thoroughly investigated using extended periods of cell tracing and scored over a large fraction of pancreas volume. These same PtflaCremice were imported to Europe, where we confirmed that similar numbers of β-cells derived from Ptfla-expressing acinar cells post-PDL. However, in some mice they appeared more rapidly following surgery. Given that cytokines likely influence conversion, we speculate that environmental (housing, feeding) or other unmeasured effects could lead to variable timing or efficiency in generating other cell types from the acini. Second, the capacity to progress toward β-cells might have been limited to acinar-derived cells that moved through an intermediate Hnf1b+ Sox9+ duct cell–like condition. It remains possible that tracing of either Sox9+ or Hnf1b+ preexisting duct cells may be less sensitive or less specific in detecting β-cell neogenesis.

In PDL, there is evidence that β-cell neogenesis correlates with the induced level of Ngn3 expression, which is supported by the observation of embryonic duct-like cells that were either Ngn3+CK19+ (4,5) or Ngn3+Sox9+ (5,18). These findings justified reevaluation of the outcome of PDL in Sox9CreERT;R26YFP mice that labels the mature duct and, to some extent, acinar cells (32,33). After administration of TAM and a 2-week washout, PDL surgery was applied and the pancreas was analyzed 14 days later. Sox9CreERT-driven YFP labeling in CK19+ duct cells varied somewhat among animals (44–62% of CK19+ cells being YFP+). In the ligated pancreas, the average Ngn3 mRNA expression level was 0.66 ± 0.2-fold that of duodenum (n = 9), with YFP expressed in 0.60% of β-cells against less than 0.01% in sham pancreas (Fig. 2). This result bolsters the hypothesis that there is PDL-induced derivation of β-cells from Sox9+ nonendocrine cells. We propose that it is unlikely that any highly dysregulated expression of Sox9 in preexisting β-cells is somehow initiated by the PDL process and then leads to spurious YFP labeling because of the extensive pre-PDL TAM washout period.

DIFFERENTIAL PROLIFERATION OF β-CELL SUBPOPULATIONS IN PDL PANCREAS

Connecting several studies that reported increased β-cell proliferation in PDL (4,6,10,17,19,34) with those showing a limited contribution of neogenesis (5,6,18,27,30,31,33) implies that proliferation of extant β-cells is the main mechanism for PDL-related increases in β-cell number. As expected, no or only limited increase in β-cell proliferation was reported under PDL conditions that caused no increase in β-cell number (19,20). Because we were concerned by the conclusion that PDL does not generate β-cells by proliferation (20), we used an alternative approach to study this process. GFP+RFP+ β-cells were isolated from PDL or sham pancreas from Pdx1GFP;MIPRFP mice (Fig. 3A and B). As a large number (>50,000) of β-cells were isolated from each dissociated PDL or sham pancreas tail, measurement of proliferation in the overall β-cell population was favored, rather than a particular islet subset. In addition, the level of Ki67 mRNA was an unbiased estimate of relative cell-cycle activity. Interestingly, the near-fourfold increase of Ki67 mRNA in PDL versus sham pancreas (Fig. 3C) is close to the previously reported increase in percentage of directly counted Ki67+ insulin+ cells (6). The current data corroborate earlier reports of increased proliferation in the average PDL β-cell (4,6,10,17,34) and strongly challenge the idea of a selective proliferation that is activated in <5% of β-cells residing only in small islets (19). Interestingly, mRNA for G2/M phase regulators cyclin B2, CDK1, and CDK regulatory subunit 2 (Cks2)—markers of mouse and human β-cell proliferation (35) and replicative fidelity (36)—were significantly upregulated in PDL tail β-cells (Fig. 3D). The positive correlation between Ki67 and cyclin B2 mRNA expression in PDL tail β-cell samples (Fig. 3E) suggests that the cyclin B2 mRNA level could independently gauge β-cell proliferation beyond the G1-S phase.

It remains difficult to ascertain exactly why several groups failed to observe increased proliferation and major β-cell formation following PDL—there is even nonuniformity within our own laboratories. Perhaps one complicating factor is the age difference of mice used in these experiments. The refractory period (duration between cell divisions) increases as mice age, and older mice have much reduced β-cell proliferation capacity (37–39). In 6-week-old mice (used in [20]), the basal β-cell proliferation is significantly higher than in 8-week-old mice (used in [4,6,30]). Thymidine analog labeling declines between week 6 and 8 in normal or sham-operated mice, and PDL applied at week 6 apparently cannot forestall this decline (20).

However, in our hands at least, when PDL is applied in 8-week-old mice they exhibit increased post-PDL β-cell proliferation in the tail. Although this observation seems counterintuitive, it hints at the idea that different molecular programs control cell-cycle quiescence in normal versus PDL pancreas. These counteracting effects, together with potential variability related to the experimental environment of the study (possibly currently underappreciated influences such as the undefined amount of chronic, systemic low-level inflammation under different animal housing conditions), could unfortunately lead to
cross-cancellation. Nevertheless, even this caveat does not seem to warrant labeling PDL as lacking utility in studying inherent and induced pancreatic cell plasticity. Careful age, sex, and strain matching should allow comparisons between mice in which the natural juvenile stage β-cell expansion has mostly subsided and basal proliferation rate has stabilized.

Despite the conflicting results in recent reports, a number of studies agree on the post-PDL β-cell proliferation in adult rodent pancreas (4,6,10,17,19,29,34),

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**Figure 2**—β-Cells derived from Sox9+ cells in adult mouse pancreas 14 days after PDL surgery. Eight-week-old male Sox9CreERT;R26YFP mice received subcutaneous TAM (5 × 4 mg distributed over 10 days) to label Sox9-expressing duct cells. The mice underwent sham or PDL surgery 2 weeks after washout of TAM, and 14 days later, pancreatic tail tissue was examined by immunofluorescence for cytokeratin 19 (CK19, pink), insulin (INS, red), YFP (green), and DNA (blue). Sham tail pancreas (A–A”) and PDL tail pancreas (B–D”). Arrowheads indicate YFP+ β-cells that were probably derived from Sox9+ (YFP+) exocrine/duct cells. E: A total of 119 YFP+ β-cells out of 19,012 β-cells were observed in nine mice with PDL pancreas, as compared with 1 YFP+ β-cell out of 6,773 β-cells counted in five mice with sham-treated pancreas. Data represent the percentage of β-cells that were YFP+ in individual organs (n = 5–9; *P < 0.02, PDL vs. sham tails by two-tailed t test).
Figure 3—Ki67 mRNA in sorted sham and PDL tail β-cells. Male 8-week-old Pdx1^GFP^,MIP^RFP^ mice underwent sham or PDL surgery and β-cells were isolated 1 week later by sorting GFP^+^RFP^+^ double-positive cells. Representative isolations of β-cells from PDL tail (A) or sham tail (B) pancreas are shown. For each isolation, two to three complete PDL or sham tails were pooled, and seven isolations for PDL and sham were performed. C: Ki67 mRNA in sorted β-cells of PDL pancreas was normalized to cyclophilin A mRNA, and data are expressed relative to sham tail β-cells. **P < 0.01. D: PDL tail β-cell mRNA of cell-cycle genes Ki67, cyclin D1 and D2, cyclin B1 and B2, anagen-promoting complex APC (cdc27), and cyclin-dependent kinase 1 (CDK1, cdc28). Data for PDL β-cells are expressed relative to the same gene in sham β-cells (represented by dotted line = 1) (n = 7; *P < 0.05, **P < 0.01 by two-tailed t test). E: Correlation between the quantities of Ki67 and cyclin B1 or B2 mRNA in preparations of sorted β-cells from PDL pancreas are based on linear regression analysis (R^2, squared correlation coefficient; p, P value for slope deviating from 0).
increased overall islet number (6,10,29,34), and, in some cases, that there is a particular enrichment of these effects within small islets that seem to have a relatively higher proliferation potential (6,10,19,29). Smaller islets could be younger than larger ones, and they could be derived by slightly different ontogenetic processes such that they, or their precursors, are exposed to specific local and systemic environmental influences. In one of our studies, these small islets were markedly enriched for β-cells that were not derived from preexisting ones and β-cells derived from Ngn3+ cells (6). More work is required, but we are very interested in determining if and why the proliferation potential of β-cells in PDL appears heterogeneous and why the mitotic recruitment of β-cells occurs preferentially, but not exclusively, in these smaller islets.

Some authors have taken the unchanged number of small islets, in parallel with the lack of detecting a contribution of Sox9+ cells to β-cells, as evidence against neogenesis (18). We would counter, however, that our observation of the doubling in number of small endocrine cell clusters in PDL pancreas, together with the increased overall volume of tissue represented by β-cells (without appreciably increased β-cell size), strongly supports continuous neogenesis. We therefore advise that the generalized statement that no β-cells are generated in PDL (20) is an oversimplification that neglects the conclusions of several studies demonstrating β-cell proliferation and/or neogenesis in PDL. Perhaps a more useful strategy will be to find the manner in which PDL can be guided so as to yield common outcomes across laboratories and to find the reasons for the current variability. Moreover, we need to seek ways to ensure that the strictest lineage-tracing tools are developed and used to determine the mechanisms underlying the cellular transitions engaged by PDL and the role of specific individual intercellular signals.

NGN3-EXPRESSING CELLS PROVIDE PROLIFERATIVE SIGNALS TO A β-CELL SUBPOPULATION

As only 5–12% of β-cells derive from Ngn3+ cells in PDL pancreas, and because only a partial ablation of Ngn3+ cells by diphtheria toxin at the end of the ligation period reduced β-cell proliferation by over 50% (6), we have theorized a non-cell-autonomous effect of Ngn3+ cells on β-cell proliferation. Both the proliferation and expansion of β-cells in PDL pancreas depend on transforming growth factor (TGF)β-receptor expression (17), suggesting β-cells may respond to a proinflammatory environment (15,40). Intriguingly, null mutation of TGFβ receptor in whole pancreas did not affect Ngn3 gene expression in PDL (17)—a result that is also proposed as evidence that β-cell proliferation, not neogenesis, accounts for most of the induced β-cell area expansion that often results from PDL. Nevertheless, shRNA-mediated Ngn3 knockdown (4) and partial ablation of Ngn3+ cells (6) showed that β-cell proliferation in PDL depends on Ngn3+ cells that are distinct from β-cells (18,26), a concept that is in agreement with the removal of Ngn3 from β-cells not affecting their post-PDL proliferation (6). Taken together, Ngdn gene activation in PDL is necessary but not sufficient for β-cell proliferation, at least for the proportion that also requires TGFβ signaling. The latter ontogenetic pathway seems independent of Ngn3 activation or, alternatively, it could act downstream of the Ngn3+ cell state in stimulating β-cell proliferation. Further investigation may provide clues to the non-cell-autonomous signaling cascade that is necessary for β-cell expansion in PDL.

CONCLUSION

Both β-cell neogenesis and duplication of preexisting β-cells are often, but not always, increased in PDL pancreas, and re-engagement of the mitotic condition in virtually quiescent β-cells is the main driver of β-cell expansion (4,6,17,27,34). Lineage tracing of non-β-cells shows either no (18,27,30) or limited contribution (5,6) (Fig. 2) to the formation of β-cells, but this does not mean that these transformations are irrelevant. Learning about both epigenetic and genetic resistance to plasticity and how to enhance and lineage-direct the acinar-to-ductal-to-endocrine processes could have substantial application to cell-based therapies. A complementary goal would be ensuring the adoption of a final, determined, and differentiated cell state. For instance, the physiologic need for insulin that results from chemical ablation of preexisting β-cells or from autoimmunity in chimeric NOD mice can substantially increase the extent of β-cell neogenesis in PDL (5,29,41). However, the identity of components of the innate immune response that are key to efficient reprogramming of pancreatic cells remains elusive (42). The membrane Tomato/membrane Green (mTmG) technology, which switches from membrane-bound red to green fluorescence and that was previously used to suggest an absence of β-cell neogenesis in various (re)generation models (27), provides a powerful flow-sorting tool to identify conditions, signaling factors, and target cells for increased in vitro or in vivo β-cell formation from non-β-cells. Identifying those signals and cells will in turn help to boost endocrine progenitor cell numbers, ultimately eliminating the need for PDL surgery.

RESEARCH DESIGN AND METHODS

Mouse Manipulation

All mice experiments were performed according to the guidelines of our institutional Ethical Committee for Animal Experiments and national guidelines and regulations. Pancreatic duct ligation and sham operation were performed on 8-week-old male mice, as described (4,10).

Cell Tracing

TAM (Sigma-Aldrich, Diegem, Belgium) was dissolved at 10 mg/mL in corn oil (Sigma-Aldrich). For conditional YFP-labeling of Ngn3+ cells in pancreas or duodenum of MIPRFP;Ngn3CreERT;R26RYFP mice, surgery was performed...
and a total of 20 mg of TAM was given subcutaneously in five doses (each 4 mg) distributed over the 2-week post-surgery period, as described (6). For conditional YFP labeling and tracing of preexisting Sox9+ cells in Sox9CreERT2;R26R-YFP mice (33), the same dose of TAM was administered, but was followed by a 2-week washout period and then surgery and YFP+ cells were studied 2 weeks later.

**Histochemical Procedures and Image Analysis**

Samples for immunohistochemistry were fixed in 4% formaldehyde overnight and embedded in paraffin. Paraffin sections (4–5 μm) were incubated with antisera specific for insulin (1/3,000, guinea pig, generated at the Diabetes Research Center, Brussels, Belgium), CK19 (1/100, rat, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), or GFP (1/100, goat, Abcam, Cambridge, U.K.). Secondary antibodies for detection of guinea pig, goat, or rat antibodies were labeled by fluorescence (Cy3, Cy2, Cy5, or AMCA) (Jackson Immunoresearch Laboratories, Suffolk, U.K.). Nuclei were labeled by Hoechst 33342 (4 mg/mL, Sigma-Aldrich). CK19 or insulin-positive cells were counted separately, and localization of YFP expression within insulin+ (β) cells or CK19+ (duct) cells was checked individually. At least 1,300 β-cells per sample were evaluated. Images were acquired and analyzed as described (6).

**Cell Isolation**
PDL or sham-operated pancreas and duodenum tissue were dissociated and total cells sorted by FACS (4). β-Cells were isolated by two consecutive rounds of sorting on a FACSVantage SE (Becton Dickinson, Erembodegem, Belgium) as GFP+RFP+ cells from dissociated pancreas of Pdx1Cre;MIPGFP mice (43,44). mRFP1 protein has minimal emission when excited at wavelengths optimal for GFP (44) and was excited using a 561-nm 50 mW Excelsior laser (Spectra-Physics, Santa Clara, CA); emission was detected using a 625/35 band-pass filter. From dissociated pancreas of TAM-induced MIP-GFP;Ngn3CreERT2;R26R-YFP mice (6,44), β-cells were isolated by sorting RFP+ cells as above, followed by a second round of sorting on a FACSDiva cell sorter (Becton Dickinson) using Zn2+ chelator 6-methoxy-8-p-toluensulfonyl-amidino-triazole (TSQ) as β-cell probe (4). YFP+ cells were sorted as before (6).

**RNA Analysis**
RNA was isolated from pancreas or duodenum tissue (RNasey, Qiagen, Venlo, the Netherlands) or from sorted cells (RNasey Micro Kit, Qiagen). cDNA synthesis and RT-PCR were performed as described (4) with TaqMan Universal PCR Master Mix on an ABI Prism 7700 sequence detector, and data were analyzed using the Sequence Detection System Software, version 1.9.1 (all Applied Biosystems, Life Technologies, Ghent, Belgium). Mouse-specific primers and probes recognizing Ngn3 (6), cyclinophilin A (Mm02342429g, K67 (Mm01278617_m1), Cnd1 (Mm. PT.49a.17169504.gs), Cnd2 (Mm. PT.49a.15761571), Ccnb1 (Mm. PT.49a.6999464.gs), Ccnb2 (Mm. PT.49a.7031564.gs), Cks1b (Mm.PT.53a.13780725), Cks2 (Mm.PT.53a.30578885), CDK1 (Mm.PT.53a.5595112), and Cdc27 (Mm.PT.53a.15804595) were all from Integrated DNA Technologies (Heverlee, Belgium). Quantitative PCR data were normalized to cyclophilin A before comparison between tissues or cells.

**Statistics**
Data are expressed as mean ± SEM of five to nine independent experiments. Differences between data were analyzed by unpaired two-tailed Student t test or one-way ANOVA with Tukey test for multiple comparisons and were considered statistically significant when P < 0.05.

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**References**