Intraislet Pancreatic Ducts Can Give Rise to Insulin-Positive Cells

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A key question in diabetes research is whether new β-cells can be derived from endogenous, nonendocrine cells. The potential for pancreatic ductal cells to convert into β-cells is a highly debated issue. To date, it remains unclear what anatomical process would result in duct-derived cells coming to exist within preexisting islets. We used a whole-mount technique to directly visualize the pancreatic ductal network in young wild-type mice, young humans, and wild-type and transgenic mice after partial pancreatectomy. Pancreatic ductal networks, originating from the main ductal tree, were found to reside deep within islets in young mice and humans but not in mature mice or humans. These networks were also not present in normal adult mice after partial pancreatectomy, but TGF-β receptor mutant mice demonstrated formation of these intraislet duct structures after partial pancreatectomy. Genetic and viral lineage tracings were used to determine whether endocrine cells were derived from pancreatic ducts. Lineage tracing confirmed that pancreatic ductal cells can typically convert into new β-cells in normal young developing mice as well as in adult TGF-β signaling mutant mice after partial pancreatectomy. Here the direct visual evidence of ducts growing into islets, along with lineage tracing, not only represents strong evidence for duct cells giving rise to β-cells in the postnatal pancreas but also importantly implicates TGF-β signaling in this process. (Endocrinology 157: 166–175, 2016)

The incidence of diabetes mellitus continues to rise, with no curative treatment currently available. The disease is caused by a functional deficit in the insulin-producing β-cells in the pancreas. The best avenue for treatment is β-cell replacement therapy, ideally using the patient’s own endogenous cells. However, inducing noninsulin-producing cells to undergo transdifferentiation to form β-cells in vivo has proven challenging. Although several studies have concluded that the neogenesis of β-cells can occur under certain conditions (1–5), numerous other studies, including our own recent study, have concluded that significant neogenesis of β-cells does not typically occur in adult mice (6–10). Many potential sources for β-cell neogenesis have been proposed (1–3, 11–16). Historically, a prime candidate source for neogenesis of β-cells has been pancreatic ducts. An intimate anatomic relationship be-

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Abbreviations: BrdU, bromodeoxyuridine; 3-D, three dimensional; DBA, Dolichos biflorus agglutinin; DNT, dominant negative TGF-β receptor II; FACS, fluorescence-activated cell sorting; HNF1, hepatocyte nuclear factor-1; PDX-1, pancreatic duodenal homeobox factor-1; Sox9, Sry-type high-mobility-group box transcription factor-9; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.
tween pancreatic ducts and pancreatic islets of Langerhans has been known for more than 100 years (17). Conversion of duct cells into islet cells seems plausible. In the embryo, neogenic islet cells derive from duct-like tubular epithelial structures as proendocrine cells break away from the epithelial lining (18). Some lineage-tracing studies have suggested that a duct-to-islet cell transdifferentiation can occur in the regenerating adult pancreas (1, 4), but if so, any specific duct cell subpopulation that acts as the endocrine progenitor is unknown. Analysis of static histological sections showing islet cells or endocrine clusters apparently budding out of a duct are suggestive (19, 20) but are not definitive. If this budding does represent islet cell neogenesis from ducts, it still remains unclear how, anatomically, these new cells may work their way into an existing islet, as suggested by Inada et al (1).

We recently developed a whole-mount imaging technique for the juvenile and adult pancreas that allows clear three-dimensional visualization of the pancreatic ducts and islets (20). Using this technique on the pancreas from normal juvenile mice and from children, we were able to detect small ductal branches off larger pancreatic ducts that penetrated and branched within the islets. This branching pattern was not seen in the pancreas of normal adult mice and adult humans. However, in certain TGF-β signaling mutant mice, we saw a dramatic enhancement in the formation of these intraislet ducts after a nondiabetogenic 60% partial pancreatectomy (PPx). These regenerative intraislet ducts were first seen 1 week after partial pancreatectomy, peaking at 4–5 weeks but then were surprisingly absent after 10 weeks. To lineage trace these intraislet ducts, we used our recently described viral duct infusion technique (21) that allows for duct-specific lineage labeling without dependence on tamoxifen. We show here that many new β-cells had formed from the duct cells in these islets.

Materials and Methods

Transgenic animals

Animal experiments were performed as approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Cadaveric human samples were analyzed with approval from the Committee for Oversight of Research Involving the Dead. TGF-β type II receptor (DNTBRII) transgenic mice were generous gifts from Dr Erwin Bottinger (Mt Sinai School of Medicine) and Lalage Wakefield (National Institutes of Health, Bethesda, Maryland). DNTBRII mice were maintained on zinc water to enhance their expression of the transgene at least 1 month prior to beginning an experiment or from birth in the case of young animals. Sox9CreERT2 mice (8) were generous gifts from Professor Y. Kawaguchi (Department of Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan). FVB mice, the background strain of the DNTBRII mice, were used as the controls for our experiments. Animal tissues from the male and female mice were obtained and analyzed without regard to sex.

Partial pancreatectomy

The procedure was performed as previously described (22). Briefly, a midline upper abdominal incision was made and the tail of the pancreas and spleen was delivered into the operative field. The pancreas was ligated with a suture, divided, and the pancreatic tail and spleen were removed. Forty to 50% of the pancreas remains after the procedure. The abdomen is then closed.

Metabolic testing

Glucose and insulin tolerance testing was performed consistent with protocols published by the National Institutes of Health Mouse Metabolic Phenotyping Centers (23, 24). Briefly, the ip insulin tolerance test was performed by obtaining a baseline fasting blood glucose and then an ip injection of 0.5 U/kg insulin was administered. Blood glucose measurements were obtained at 15, 30, 45, 60, and 120 minutes after injection. Intraportal glucose tolerance testing was performed similarly except the fasting time was increased to 16 hours and, instead of insulin, 2 g/kg of glucose was injected after the baseline serum glucose level was obtained.

Whole-mount imaging and three-dimensional (3-D) reconstruction

The procedure was performed as previously described (20). Briefly, the pancreas is minced into small pieces and then briefly fixed in 4% paraformaldehyde. The tissues are washed several times and then passed through a methanol gradient before incubation in primary and then secondary antibodies. After an additional series of washing, the tissue fragments are then mounted onto a glass slide.

Immunohistochemistry

Polyclonal guinea pig anti-insulin 1:500 (Dako), polyclonal goat antipancreatic duodenal homeobox-1 (PDX-1), 1:1000 (Abcam), fluorescein isothiocyanate-conjugated or biotin-conjugated Dolichos biflorus agglutinin (DBA), 1:100 (Vector Laboratories), monoclonal rat antibromodeoxyuridine (BrdU) antibody, 1:400 (Abcam), polyclonal rabbit antihepatocyte nuclear factor-1 (HNF1)-β, 1:200 (Santa Cruz Biotechnology), polyclonal rabbit anti-Sry-type high-mobility-group box transcription factor-9 (Sox9), 1:1000 (Chemicon), polyclonal rabbit anti-pan-cytokeratin, 1:100 (Dako), and polyclonal rabbit anti-CD31, 1:100 (Abcam) (Table 1). Antibody dilutions used for whole-mount staining were halved. Tissue sections were viewed on an upright Axio Imager Z1 microscope. Images were captured with an AxiosCam MRc5 camera and processed using AxioView version 4.8.2.0 software. Confocal images were obtained using an Olympus Fluoview 1000 microscope running Fluoview imaging software.

BrdU incorporation and pulse-chase experiment

Adult mice were injected with BrdU (Sigma) 200 mg/kg ip after performing PPx once daily for 7 consecutive days. The pancreas was harvested at serial time points after surgery, fixed...
in 4% paraformaldehyde overnight at 4°C, and then placed in 30% sucrose overnight at 4°C. Antigen retrieval was performed by treating slides with 2 M HCl for 35 minutes, followed by overnight incubation with primary antibodies.

Tamoxifen injection
In experiments involving the cre-ER system, tamoxifen (Sigma) was dissolved at 20 mg/mL in corn oil (Sigma) and was administered into adult mice ip, 2 mg per day for 5 days, to induce cre recombination. PPx was performed 7 days after tamoxifen injection.

Fluorescence-activated cell sorting (FACS) DBA⁺ cells
This was performed as previously described (10).

Sox9-cre virus infusion
This was performed as previously described (21). Briefly, an upper midline abdominal incision was performed and the common bile duct was identified at the point in which it enters the duodenum. A catheter was introduced through the duodenal wall and into the common bile duct via the ampulla. The virus was administered by an infusion pump. When the infusion was complete, the catheter was removed and the abdomen was closed.

Pancreatic islet counting
The pancreas of DNTβRII mice were injected with the AAV6-Sox9-Cre virus, had PPx performed 1 week later, and were then harvested 4 weeks after PPx. The pancreatic remnant was fixed and mounted, and the slides were cut across the main body of the tissue. Five slides were chosen at equal intervals across the pancreas and stained. Random islets were quantified across each pancreas until at least 1000 ß-cells and 500 ductal cells had been quantified.

Statistical analysis
Statistical significance was determined as a value of P < .05 by a two-tailed Student’s t test.

### Results

**Metabolic characterization of the animal models**

DNTβRII mice are known to develop intralobular pancreatic fibrosis and replacement of acinar tissue by adipose at 5 months of age (25). The mice in our experiment completed the experiment before these histological features were to take place. Despite this, the DNTβRII mice were found to weigh less than their background FVB mouse counterparts (Supplemental Figure 1A). There was no significant difference between fasting blood glucose, insulin tolerance tests, or glucose tolerance tests between the DNTβRII and FVB mice at baseline (Supplemental Figure 1, B–D). When zinc was added to the DNTβRII drinking water to enhance transgene expression (25), mild elevations in the mouse’s fasting glucose were seen compared with the wild-type FVB control mice (Supplemental Figure 1, F and G). Interestingly, on zinc water, the DNTβRII mice weighed as much as the wild-type FVB mice (Supplemental Figure 1H).

After a nondiabetogenic 60% PPx, we reassessed the FVB and DNTβRII metabolism. Of note, there were slight alterations in the insulin and glucose tolerance tests in the control FVB mice, suggesting the development of a slight degree of glucose intolerance in these mice (Supplemental Figure 1, I and J). This effect was not seen in the DNTβRII mice on either zinc or standard water (data not shown). Fasting serum glucose was no longer significantly different between the FVB and DNTβRII mice after PPx (data not shown). Serum insulin levels measured before and after partial pancreatectomy were similar for FVB mice receiving standard water and DNTβRII mice receiving zinc water (Supplemental Figure 1K) or standard water (Supplemental Figure 1L).

### Table 1. Antibody Table

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<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
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Abbreviation: FITC, fluorescein isothiocyanate.
Ontogeny of intraislet pancreatic ducts in juvenile mice and children

Islets have long been known to be intimately associated with pancreatic ducts (17). We revisited this association using immunohistochemistry and whole-mount immunostaining (20, 26), specifically in young mice. Histological analysis showed pancreatic duct cells that appeared to be within islets of young wild-type mice as early as 2 weeks of age. Although in only two dimensions, it appeared that the intraislet ducts may be arising from larger ducts outside the islet (Figure 1, A–C, and Supplemental Figure 2, A–C). We then applied our whole-mount system (20, 26) to better visualize the ducts within the islets. Here it became clearer that these intraislet pancreatic ducts did in fact arise from larger ducts outside the islet (Figure 1, A–C, and Supplemental Figure 2, A–C). We then applied our whole-mount system (20, 26) to better visualize the ducts within the islets. Here it became clearer that these intraislet pancreatic ducts did in fact arise from larger ducts outside the islet (Figure 1D and Supplemental Figure 2D). 3-D reconstruction and rotating movies of those reconstructions confirmed that the intraislet ducts penetrate deeply into the islets (Figure 1E, Supplemental Figure 2D, inset, and Movie 1). The presence of pancreatic ducts within islets was rare in mice less than 2 weeks of age, still infrequent at 2 weeks of age (3.6% ± 1.3% of islets), but then progressively became more frequent with increasing age, peaking at 5 weeks of age (43.8% ± 5.9%), and then gradually declining with age (15.3% ± 3.1% at 12 wk) (Supplemental Figure 2E). To determine whether intraislet ducts were present in humans, we analyzed three small pieces from three young cadaveric human pancreases through institutional review board approval, aged 2 months, 3 years, and 16 years. We confirmed the presence of intraislet ducts in pancreatic tissue histologically from a 2-month-old human pancreas (Supplemental Figure 3, A and B) in which 33 of 71 islets (46.5%) had intraislet ducts and by whole-mount-stained pancreas of a 3-year-old child (Supplemental Figure 3C), in which 26 of 216 islets (12%) had intraislet ducts. Three-dimensional reconstruction from this latter pancreas confirmed that the duct branches were within the islet (Supplemental Figure 3D). In the 16-year-old pancreas, only one intraislet duct of hundreds of islets examined was seen.

Intraislet pancreatic ducts after partial pancreatectomy

Based on our finding of intrapancreatic ducts specifically in juvenile mice and children, we hypothesized that a similar process may reoccur in a regeneration model, here using a nondiabetogenic 60% partial PPx in mice. However, in both sham-operated and 1 week after PPx in 10-week-old mice, we saw few intraislet ducts by whole-mount analysis in wild-type FVB mice, similar to the un-perturbed mice (Supplemental Figure 2E and Figure 2, A and B).

TGF-β superfamily signaling has been shown to play a critical role in regulating pancreatic endocrine maturation and development (27–34). We showed previously that TGF-β signaling can suppress the neogenesis of endocrine cells from pancreatic embryonic ducts (34). Here we hypothesized that TGF-β signaling may be an important suppressor of the formation of these intraislet ducts, and...
therefore, we analyzed the ducts of adult mice (background strain FVB) expressing a dominant-negative TGF-β type II receptor under the metallothionine-1 promoter (DNTβRII) (25). The metallothionine-1 promoter leads to a strong up-regulation of expression of the transgene when zinc is added to the drinking water. The adult mice from this strain were previously shown to have primarily a pancreatic phenotype and in particular were found to have acinar atypia and acinar-ductal metaplasia, particularly at older ages, eventually developing acinar tumors (25). We analyzed the pancreas of zinc-treated DNTβRII mice, with or without PPx, but at 10 weeks of age, an age well before the onset of the exocrine phenotypic changes seen in the older DNTβRII mice (25). At baseline, without PPx, no intraislet ducts were seen (Figure 2C). Of note, however, we noticed that the baseline ductal network of the DNTβRII mice had a marked pruned-tree appearance that was not described in the original paper and might be difficult to appreciate by two-dimensional histology alone.

In contrast, the wild-type FVB pancreas showed a network of fine ducts (Figure 2A). The pruned-tree appearance may also explain the complete absence of intraislet ducts at baseline, compared with the occasional intraislet ducts we saw at baseline in the background strain FVB mice (Supplemental Figure 2E). When we performed a 60% PPx as a stimulus for regeneration, 1 week later we found numerous islets with small intraislet ducts in the DNTβRII mice (Figure 2D) but not in the FVB mice (Figure 2B). Strikingly, by 4 weeks after PPx in the DNTβRII mice, we saw a dramatic expansion of the intraislet ducts with extensive branching (Figure 2E). Confocal 3-D reconstruction confirmed that these ducts were penetrating deeply throughout the islets (Movie 2). On gross whole-mount analysis, approximately 60%–80% of islets contained ductal structures. Surprisingly, 10 weeks after PPx, there were once again no intraislet ducts seen in the DNTβRII mice (Figure 2F). To determine whether this disappearance of ductal structures was due to apoptosis of the intraislet ducts, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining in DNTβRII mice 4 and 10 weeks after PPx was performed but showed no evidence of apoptosis of ductal cells.

**Characterization of post-PPx intraislet ducts**

To determine whether the observed formation of intraislet ducts seen after PPx in the DNTβRII mice entailed ductal proliferation, we gave daily ip injections of BrdU for only the first 7 days after PPx and then harvested at serial time points (Figure 3, A–D, and Supplemental Figure 4A). β-Cell replication through the first week after PPx...
was less frequent in 
DNTβRII mice than in the wild-type FVB background mice (Supplemental Figure 4B). When the DNTβRII pancreata were harvested 4 weeks after PPx, numerous BrdU+/insulin+ cells were present within the islet, including BrdU+/insulin+ cells by confocal imaging (inset) and BrdU+ duct cells within the islet (intraislet duct cells in dotted circle). E and F, Intraislet pancreatic ducts observed 4 weeks after partial PPx in DNTβRII mice costain for another ductal marker, pan-cytokeratin (E, arrow) but do not costain with the endothelial marker platelet endothelial cell adhesion molecule-1 (F). Scale bar, 20 μm.

DBA with pan-cytokeratin but not with CD31. Furthermore, these intraislet pancreatic ducts stained positive for the duct cell markers HNF1β and Sox9 (Supplemental Figure 5, A and B). Both HNF1β and Sox9 colocalized with DBA in the pancreatic ducts and in the intraislet pancreatic ducts. Pdx1 was also expressed in the duct branches penetrating the islets (Supplemental Figure 5, C and D), consistent with the previous findings of Pdx1 in regenerating ducts (5, 35).

To quantify the amount of duct cells within the islet of the DNTβRII mice 4 weeks after PPx, we performed a FACS analysis of a purified islet fraction from these mice and control mice to determine the number of DBA+ cells within the islet. Whereas control islets had nearly undetectable DBA+ cells (0.3% ± 0.2%, n = 3) in the islet fraction, 5.7% ± 1.0% (n = 3) of cells in the purified islet fraction of DNTβRII pancreata 4 weeks after PPx were DBA+ (Supplemental Figure 4, E and F).

**Lineage Tracing**

We suspected that the intraislet ducts that developed in the DNTβRII mice may give rise to islet cells by transdifferentiation. This possibility was partially supported by the BrdU pulse-chase experiments described earlier as well as by the fact that the intraislet ducts disappeared by 10 weeks after the PPx, and we saw no TUNEL+ cells during that time frame. Clearly the BrdU and TUNEL results are suggestive of only a duct-to-islet cell transformation, so therefore, we moved to more rigorous methods for lineage tracing. In an attempt to specifically trace the fate of these intraislet ducts, we first crossed Sox9CreERTM knock-in mice (expressing the tamoxifen sensitive Cre-recombinase in the Sox9 locus [8]) with R26R<sup>Tm-RED</sup> tomato red reporter mice (36) as well as with DNTβRII mice to create a triple transgenic Sox9CreERTM;DNTβRII; R26R<sup>Tm-RED</sup> mouse. Most intestinal crypts and pancreatic ducts (Supplemental Figure 6, A and B) were lineage tagged with red fluorescent protein after tamoxifen administration. However, some aci-
and duct cells were prelabeled when no tamoxifen was given (Supplemental Figure 6, C and D), but no labeling of islet cells was seen before, or within 3–5 days, after tamoxifen (Supplemental Figure 6, C and E). When harvested 4 weeks after PPx, \( \text{Sox9CreERTM}\); \( \text{DNT}\)/H9252RII; \( \text{R26RTm-RED} \) triple-transgenic mice demonstrated co-localization of the tomato red reporter in some \( \text{DNT}\)/H9252RII-cells (Supplemental Figure 6F), whereas this colocalization was not seen in \( \text{Sox9CreERTM}\); \( \text{R26RTm-RED} \) mice not bearing the \( \text{DNT}\)/H9252 transgene (Supplemental Figure 6G). Due to concerns here regarding inconsistent lineage tagging, we did not quantify these data and focused on what became a more robust viral tagging system (below) (21).

Because of the numerous potential pitfalls of the transgenic cre-ERT system used above, such as tamoxifen dosing, cre leakiness, etc, we attempted to lineage tag pancreatic ducts using our previously described system of infusion of an AAV6-Sox9-cre virus into the pancreatic ductal tree of \( \text{R26RTm-RED} \) mice (21). We previously showed that 89% of the main pancreatic duct cells, 70% of the intermediate duct cells, and 50% of the small ducts were labeled by this virus at baseline (Figure 4, A–C [21]). DBA-weak or DBA-negative duct cells were also frequently lineage tagged (Figure 4, D and E), suggesting a greater sensitivity of this viral cre-mediated labeling system for detecting ducts compared with DBA staining. Sham-operated (no PPx) \( \text{DNT}\)/H9252RII; \( \text{R26RTm-RED} \) mice harvested 5 weeks after Sox9-cre viral infusion displayed persistent duct-specific tomato red labeling (Figure 4F), without islet cell labeling. However, if PPx was performed in the \( \text{DNT}\)/H9252RII; \( \text{R26RTm-RED} \) mice (1 wk after viral infusion), as expected, lineage-tagged ducts could be seen within islets 4 weeks after PPx (5 wk after viral infusion). Importantly, those islets containing ducts showed ductal lineage-tagged, tomato red, insulin-positive cells, indicating that the insulin-positive cells had likely originated from duct cells (Figure 5, A–E). Also, and in contrast, islets with no visible intraislet pancreatic ducts rarely showed tomato red labeling of insulin-positive cells (Figure 5D). This specificity of ductal lineage-tagged insulin-positive cells localizing predominantly to islets with intraislet ducts suggests that those lineage-tagged, insulin-positive cells derived from ducts, rather than perhaps from \( \beta \)-cells that may have transiently expressed Sox9 after PPx, which would also have caused them to become lineage tagged. Overall, 51.1% ± 2.7% of islets (n = 6 mice, minimum of 25 islets per mouse) contained lineage-tagged tomato red cells at 4 weeks after PPx. In virus-injected \( \text{DNT}\)/H9252RII; \( \text{R26RTm-RED} \) mice 4 weeks after PPx, labeled \( \beta \)-cells accounted for 1.5% ± 1.0% of all \( \beta \)-cells, whereas intraislet duct labeling efficiency was 14.5% ± 1.2% (unexpectedly low compared with normal small ducts). Thus, by extrapolation, approximately 11% of the \( \beta \)-cells in these \( \text{DNT}\)/H9252RII; \( \text{R26RTm-RED} \) mice derived from intraislet ducts 4 weeks after PPx. In sham-operated \( \text{DNT}\)/H9252RII; \( \text{R26RTm-RED} \) mice (no PPx), less than 0.1% of \( \beta \)-cells were labeled. Of the 343 tomato red-positive islets counted from the pancreatic remnants of six different \( \text{DNT}\)/H9252RII;
R26R<sup>Tm-RED</sup> mice 4 weeks after PPx, 72 islets (21%) had no visible intraislet pancreatic ducts. Whereas the physiological profile of these lineage-labeled cells was not able to be tested, 95% of cells were monohormonal for insulin, 4% were monohormonal for glucagon, and less than 1% of the cells were bihormonal for insulin and glucagon (data not shown).

To determine whether the intraislet ducts seen in juvenile animals (Supplemental Figures 1 and 2) also contribute to β-cell neogenesis, we infused Sox9-cre virus into the pancreatic duct of 5-week-old juvenile R26R<sup>Tm-RED</sup> mice (Supplemental Figure 7, A and B) and harvested 2 weeks later (n = 3); 1.9% ± 0.1% of insulin<sup>+</sup> cells expressed the tomato red, and 37.5% ± 11.9% of DBA<sup>+</sup> duct cells were tomato red. Therefore, by extrapolation, approximately 6% of β-cells at 5 weeks are derived from the ducts labeled by virus 2 weeks earlier. Of a total of 134 tomato red-positive islets counted from R26R<sup>Tm-RED</sup> pancreases, 42 (31%) had no intraislet ducts (Supplemental Figure 7, C and D). Thus, similar to DNT<sup>BRII</sup> mice after PPx, it appears that the intraislet ducts in juvenile mouse islets can undergo transdifferentiation into β-cells.

Discussion

Although it was generally accepted for many years that duct cells may, in some situations, give rise to new islet cells, more recently this question has become controversial, with strong scientific arguments being made on both sides (1–16, 37). Here our results show how small ductal branches, originating from larger ducts, appear to exist within juvenile mouse and young human islets. In addition, this process appears to be recapitulated during pancreatic growth after PPx in the setting of inhibited TGF-β receptor II signaling. It is possible that rather than growing into islets, these small intraislet duct branches predated the islets, and the islets formed around them. This latter possibility seems unlikely because at only 1 week after PPx in the DNT<sup>BRII</sup> mice, we saw very small duct branches within relatively large islets (Figure 2D). For a large islet to have sprung up within a week from a small duct branch seems unlikely.

One key question is why the presence of these intraislet ducts was not detected in several previous studies using genetically regulated, tamoxifen-inducible cre expression for lineage tagging. First, it appears from our data that the level of intraislet duct growth and conversion to islet cells in young mice is fairly low and thus could easily have been missed. To that point, the specific use of our whole-mount imaging seems to greatly enhance sensitivity for these fine ductal structures that might be missed on histological sections. Second, based on the robust lineage labeling we saw in ducts after viral Sox9-cre infusion in the R26R<sup>Tm-RED</sup> mice, it seems likely that the expression level of cre in genetically engineered mice may be significantly lower than after viral-induced expression, and therefore, the ge-
differently engineered cre expression may inadequately label the duct cells that give rise to these new intraislet branches. This possibility is further underscored by the fact that lineage labeling of intraislet ducts was only 14%, compared with 50% for normal small ducts. Perhaps the specific subpopulation of duct cells that gives rise to intraislet ducts and insulin-positive cells express Sox9 at a lower level than other duct cells. It seems plausible that these small ducts may specifically express the endogenous Sox9 promoter more weakly than the viral Sox9 promoter, perhaps due to the higher gene copy levels with the virus.

In our own previous study, using a rat insulin promoter to drive a nontamoxifen-sensitive cre, we also saw no neogenesis (10). However, in that study we analyzed only embryonic and neonatal mice up to 7 days old and then after that only in 8-week-old mice, so we may have missed the age window when the most intraislet ducts were seen. Here in the current study, we found that 3-week-old mice had infrequent intraislet ducts, and thus, few or no insulin-positive cells derived from intraislet ducts would be expected in 7-day-old mice. Similarly, by 8 weeks of age, the number of islets with intraislet ducts was low, and the size of the ductal network in those islets was small. This age-related pattern of intraislet ducts could explain why we saw no sorted new insulin-positive cells in the 7-day-old and 8-week-old adult mice in our previous study (10).

Also, in our previous study, we used a whole pancreas digestion followed by FACS analysis, which is likely to be less sensitive than direct visual analysis. Lastly and perhaps most importantly, in that study we were detecting only a small subset of the insulin-positive cells, i.e., only those insulin-positive cells that had been insulin positive for 48 hours or less.

Interestingly, a recent study showed that when a mutant form of K-ras was induced in Pdx1-expressing pancreatic cells, specifically in young mice, the characteristic duct-derived pancreatic intraepithelial neoplasias were seen to form, but frequently these particular pancreatic intraepithelial neoplasias were found within islets (38). The fact that this phenomenon was specific to young mice could be due to the presence of Pdx1-expressing intraislet ducts only in young mice.

It is possible that the presence of these intraislet ducts somehow mirrors an embryonic process. Whereas it is clear that pancreatic endocrine cells arise in the embryo from duct-like epithelial cells, that process does not seem to resemble the postnatal process that we observed here. In addition, the general concept of duct cells giving rise to new islet cells was thought to occur through a budding process of neogenic endocrine cells off ducts (19). However, it remained unclear how those newly budded cells could incorporate into existing islets. Our data suggest an answer to this question.

The fact that TGF-β receptor II signaling seems to play an important inhibitory role in the reactivation of this pancreatic ductal growth mirrors a similar role for TGF-β receptor II signaling in the embryo. We showed previously that the delamination and proliferation of new endocrine cells in the embryonic pancreas was greatly enhanced by the inhibition of TGF-β receptor II signaling (34, 39). Given the accepted role of TGF-β signaling in generally inducing and maintaining a differentiated state in epithelial cells (40), it is not surprising that TGF-β receptor II signaling inhibition allows for a greatly altered ductal behavior and phenotype. It is interesting that these intraislet ducts formed in the setting of a specific deficit in β-cell proliferation after PPx (Figure 3). Thus, the formation of intraislet ducts may be an important backup mechanism in the event of inadequate β-cell proliferation. A key factor in the pathogenesis of diabetes mellitus is that β-cell proliferation is not able to compensate for the insulin insufficiency. It is possible that inducing the growth of these intraislet duct structures in diabetic patients may help to improve their β-cell mass. Furthermore, we have been successful in FACS sorting the intraislet duct cells of DNTβRII mice after PPx, and the ability to purify and study these cells may allow us to exploit them therapeutically in a more direct manner.

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