Conversion of α-Cells to β-Cells in the Postpartum Mouse Pancreas Involves Lgr5 Progeny

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In contrast to the skin and the gut, where somatic stem cells and their niche are well characterized, a definitive pancreatic multipotent cell population in the adult pancreas has yet to be revealed. Of particular interest is whether such cells may be endogenous in patients with diabetes, and if so, can they be used for therapeutic purposes? In the current study, we used two separate reporter lines to target Cre-recombinase expression to the Lgr5- or glucagon-expressing cells in the pancreas. We provide evidence for the existence of a population of cells within and in the proximity of the ducts that transiently express the stem-cell marker Lgr5 during late gestational stages. Careful timing of tamoxifen treatment in *Lgr5^EGFP-IRES-CreERT2*;R26^Tomato^ mice allowed us to show that these *Lgr5*-expressing progenitor cells can differentiate into α-cells during pregnancy. Furthermore, we report on a spontaneous lineage conversion of α- to β-cells specifically after parturition. The contribution of Lgr5 progeny to the β-cell compartment through an α-cell intermediate phase early after pregnancy appears to be part of a novel mechanism that would counterbalance against excessive β-cell mass reduction during β-cell involution.

β-Cell replication is considered to be the primary mechanism for β-cell regeneration in the adult pancreas under physiological conditions (1,2), and thus, considerable effort has been put into finding ways to stimulate endogenous β-cell proliferation (3). One obstacle in generating new β-cells is that the overall regenerative capacity of the adult pancreas is limited during homeostasis. The low proliferative rate of pancreatic cells in general, and in β-cells in particular, has led to the search for other sources of β-cell generation, such as progenitor-like cells, facultative stem cells, and terminally differentiated cells. Several studies have demonstrated that in the regenerating pancreas, depending on the injury model, cells residing either within the ducts (4–8) or in proximity to the ductal network (7) can contribute to β-cell neogenesis, whereas other studies have challenged the notion of progenitor-like cells existing within pancreatic ducts (9,10). Nevertheless, more recent reports using pancreatic ducts to generate acinar or endocrine cells have once again focused the search for progenitor cells to within or in proximity to ductal structures (11–14). For example, purified pancreatic Lgr5-expressing cells have been shown to form organoids and undergo unlimited expansion in defined pancreas culture medium (11). Cells derived from these organoids can differentiate into endocrine and exocrine cells in vitro or under the renal capsule (11); however, direct evidence that demonstrates the ability of Lgr5 progeny to commit to different pancreatic lineages in vivo is still lacking.

While a definitive pancreatic multipotent cell population in the adult pancreas has yet to be revealed, the plasticity of the adult pancreas, as evidenced by the lineage conversion of pancreatic exocrine or endocrine cells into insulin-producing β-like cells, is gaining more acceptance

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(6,15–25). Among these terminally differentiated cells, the ability of mouse and human α-cells to transdifferentiate into β-cells has been extensively studied in recent years. Conversion of α- to β-cells can be achieved genetically through the combined expression of the transcription factors PDX1 and MAFA (20,24) or PAX4 alone in glucagon-expressing cells (6). Alternatively, *Pax4* expression can be stimulated in α-cells chemically by using γ-aminobutyric acid or artemisinin (21,22). Similarly, near-complete ablation of β-cells mediated by diphtheria toxin has been demonstrated to induce transdifferentiation of α- to β-cells (16,18,26). Nevertheless, an ongoing α- to β-cell conversion appears to occur in healthy islets without the aforementioned genetic or chemical stimuli. This spontaneous transdifferentiation, which takes place mainly in the periphery of the islets, involves a subset of intermediate immature insulin-producing β-cells that lack urocortin-3 (UCN3) expression (19).

Pregnancy is a condition known to affect the number of β-cells in rodents. β-Cells adapt to the physiological demands associated with pregnancy through a three- to five-fold mass expansion (27,28). This temporary expansion during pregnancy is followed by apoptosis, which reduces the β-cell mass after parturition back to nonpregnant (NP) levels (27,29–32). Given the ability of α-cells to differentiate into β-cells under normal conditions or following specific stimuli, we sought to determine whether normal physiological conditions involving increased pancreatic workload, such as pregnancy, would augment the spontaneous α- to β-cell conversion in the adult pancreas.

Here, we report that in pregnant dams, α-cells increase in number around the time of parturition but then return to NP levels by weaning. It is further demonstrated that the observed expansion of α-cells is not due to a higher proliferation rate but rather to α-cell neogenesis from Lgr5 progeny. Finally, our lineage-tracing studies show that conversion of α- to β-cells is augmented in the postpartum pancreas. In summary, under normal conditions these multipotent cells are quiescent but then become involved in maintaining β-cell mass homeostasis during postpartum β-cell involution.

RESEARCH DESIGN AND METHODS
Mice
Mice used in these studies were maintained according to protocols approved by the University of Pittsburgh institutional animal care and use committee. The Gcg^{iCre} strain (33) was generated in the laboratory of Dr. Gittes at the University of Pittsburgh. Rosa26^{CAGTomato} (34), $Lgr5^{EGFP-IRES-CreERT2}$ (35), and wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild-type CD-1 mice were purchased from Charles River Laboratories.

Immunofluorescence Analysis
Tissue processing and immunostaining were performed as previously described (5). For immunolabeling on cryopreserved sections, harvested pancreata were fixed overnight at 4°C in 4% paraformaldehyde, incubated in 30% sucrose solution overnight at 4°C, and subsequently embedded with optimal cutting temperature compound. Cryosections (8–10 μm) were collected serially so that each slide would contain semiadjacent sections across the entire tissue. Sections were permeabilized with 0.1% PBS/Triton X-100, washed in PBS, and blocked for 30 min in 10% normal donkey serum in 0.1% PBS/Triton X-100. For BrdU staining, slides were pretreated with 2 mol/L HCl for 30 min at room temperature before permeabilization and blocking. Primary antibodies were incubated overnight at 4°C, while secondary antibodies were incubated for 1 h at room temperature. The sources of antibodies and dilutions used are summarized in Supplementary Table 1.

Isolation of Aldefluor and Duct Cells
Total mouse pancreas was used for isolation of duct and Aldefluor-positive (Alde$^+$) cells as previously described (36). The aldehyde dehydrogenase (ALDH) activity was detected using the ALDEFLUOR assay kit (STEMCELL Technologies) per the manufacturer's instructions. Duct cells were isolated using Dolichos biflorus agglutinin.

Tamoxifen Treatment
$Lgr5^{EGFP-IRES-CreERT2}$;Rosa26^{CAGTomato} dams were injected once or on 2 consecutive days intraperitoneally with 2 mg tamoxifen at indicated gestational or postpartum days.

Flow Cytometry Analysis
For surface staining, cells were incubated with primary conjugated antibodies for 20 min at 4°C in the dark. For intracellular cytokine staining, BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Cat. No. 554714; BD Biosciences) was used according to the manufacturer's instructions. Cells were then incubated with conjugated intracellular antibodies for 20 min at 4°C in the dark. Data were acquired with a BD FACSAria Cell Sorter and analyzed with FACSDiva software (BD Biosciences).

Serum Glucagon Measurement
Serum from indicated animals was harvested and used to measure glucagon by ELISA (Mercodia) per the manufacturer's instructions. Samples were assayed in quadruplicate and graphed as the mean ± SEM. ELISA measurements were read on a SpectraMax M2 microplate reader (Molecular Devices), and data were analyzed using SoftMax Pro version 7.0.2 software (Molecular Devices).

BrdU Labeling
BrdU (0.8 g/L) was provided in drinking water at time point gestational day (GD) 10.5 and postpartum day 1

(PP1), with water changed daily. NP Gcg^{iCre};R26^{Tomato} females were given BrdU for the same window of time.

Quantification Analysis

To estimate the percentage of tomato-labeled insulin-expressing cells or the percentage of proliferating tomato-labeled cells in NP, pregnant, or postpartum Gcg^{iCre};R26^{Tomato} dams, whole pancreata were sectioned, and sections separated by 200–300 µm were stained for insulin, glucagon, or BrdU detection. Data were obtained by analyzing ?50 islets per mouse in 4–5 mice. A similar approach was applied to estimate the percentage of α-cells, UCN3-expressing cells, or apoptotic cells in NP, pregnant, or postpartum in wild-type dams. To estimate the contribution of Lgr5-expressing progenitor cells to different pancreatic endocrine cell types, sections at 200–300-µm intervals of the whole pancreas were stained for insulin, glucagon, somatostatin, or pancreatic polypeptide expression. Data were obtained by analyzing between 70 and 80 islets per mouse in three PP21 $Lgr5^{EGFP-IRES-CreERT2}$;Rosa26^{CAGTomato} dams treated with tamoxifen on PP1. To estimate the relative number of GFP$^+$ cells in $Lgr5^{EGFP-IRES-CreERT2}$ mice, 10 sections at 200–300 µm of the whole pancreas were stained with GFP and DAPI. Captured images of entire sections were analyzed using ImageJ software.

Measurements of α- and β-Cell Mass

For these measurements, 50 islets obtained from ?15 sections at 200–300-µm intervals of the whole pancreas were stained for insulin and glucagon. The cross-sectional area of α- or β-cells and cross-sectional area of total tissue were measured using ImageJ software. The α- or β-cell mass of each pancreas was calculated by multiplying the average value of relative cross-sectional area of each cell type and the weight of the pancreas.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software). Specific tests performed are described in the figure legends. $P < 0.05$ was considered significant.

Data and Resource Availability

All data generated or analyzed during this study are included in the published article and its supplementary material.

RESULTS

Expansion of α-Cells in the Pregnant Pancreas

While it is well accepted that in rodents β-cell mass expands during pregnancy and then returns to the NP state following parturition (27,29–32), less is known about the potential changes in α-cell numbers under similar conditions. In this study, we compared the number of glucagon-expressing α-cells in NP, GD17.5, and PP1 and PP21 mice. We found that along with β-cell mass, the percentage of α-cells and α-cell mass increased significantly between GD17.5 and PP1 (Fig. 1A–F) but then was reduced back to baseline levels in the PP21 pancreas (Fig. 1C and D). However, α-cell expansion did not result in increased serum glucagon levels (Fig. 1G).

The decline in the glucagon-positive cells could stem from either loss of α-cell identity and the subsequent cessation of glucagon expression or α-cell apoptosis. We pursued two independent approaches to distinguish between these two scenarios. First, mice expressing Cre-recombinase under the glucagon promoter were crossed into the tomato-red–harboring reporter strain to generate Gcg^{iCre};R26^{Tomato} mice, which labels ~94% of α-cells in the adult pancreas (33). We reasoned that a reduction in the number of tomato-positive cells between PP1 and PP21 would indicate that apoptosis is responsible for the observed α-cell reduction. Conversely, a similar number of tomato-labeled cells between PP1 and PP21 would argue for loss of glucagon expression among a subset of α-cells. We next examined the number of tomato-labeled cells in NP, PP1, and PP21 Gcg^{iCre};R26^{Tomato} dams and found that the numbers of tomato-labeled cells per islet were significantly higher in the PP1 pancreas compared with NP (Fig. 2A, B, and D). Of note, unlike the glucagon-expressing cells, we did not find a decline in tomato-positive cells in the PP21 pancreas (Fig. 2C and D). In addition, we observed an increase in the number of tomato-positive/glucagon-negative cells in PP21 islets (Supplementary Fig. 1).

β-Cell but Not α-Cell Apoptosis in the Postpartum Pancreas

Next, we looked for the presence of cleaved caspase-3 within NP, GD17.5, PP1, and PP21 islets (Fig. 2E–H). β-Cell mass expands during pregnancy but then declines to the nonpregnant state soon after parturition. A number of studies have shown that the mechanism behind the observed reduction of β-cell mass is apoptosis (29–32). Accordingly, while we were not able to find any apoptotic endocrine cells in the NP, GD17.5, or PP21 dams, we could detect cleaved caspas-3–positive cells specifically in insulin-positive cells in the PP1 pancreas (Fig. 2E, F, and H). Our quantification analysis revealed that in the PP1 pancreas, approximately half of the islets contained caspase-3–positive cells and ?0.4% of the total β-cells were undergoing apoptosis on PP1 (Fig. 2G and H). Since macrophages infiltrate tissues following injury or pancreatic cell death (37), the presence of macrophages in the pancreas of NP, pregnant, PP1, or PP21 dams was analyzed (Fig. 2I–L). Conversely, while macrophages were hardly detected in the pancreata of NP, GD17.5, or PP21 dams (Fig. 2I, J, and L), a prominent presence of macrophages in the vicinity of the islets in the PP1 pancreas was found (Fig. 2K).
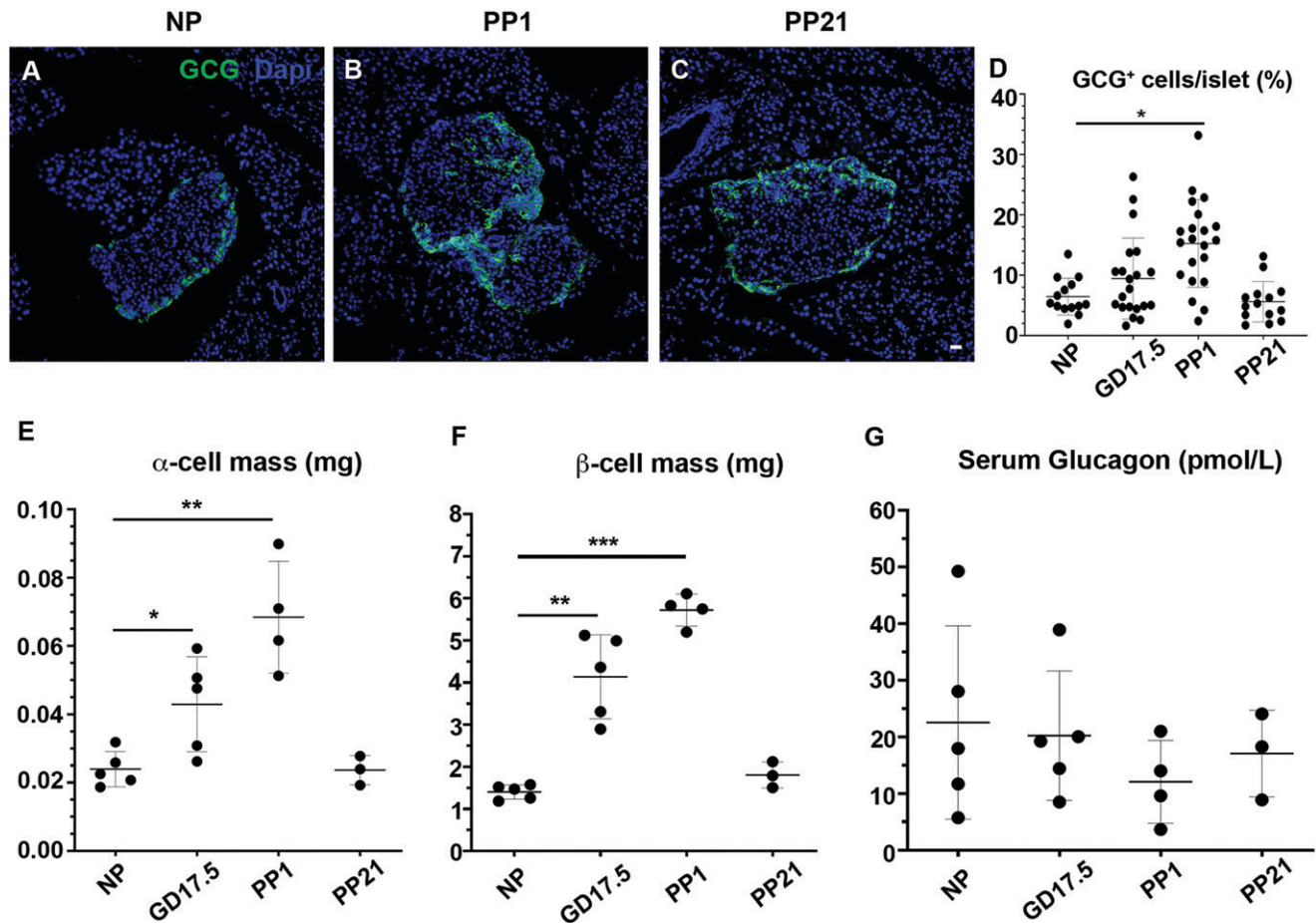
Figure 1—α-Cells expand during pregnancy. *A–C*: Immunostaining of sections obtained from NP, PP1, and PP21 wild-type dams for detection of glucagon (green) and DAPI (blue). Scale bar = 20 μm. *D–G*: Quantification of the percentage of glucagon-positive cells among islet cells, α-cell mass, β-cell mass, and serum glucagon (GCG) levels at the indicated time points during or after pregnancy. Data are mean ± SD of three to five biological replicates. *$P < 0.005$, **$P < 0.001$, ***$P < 0.0001$ by one-way ANOVA.
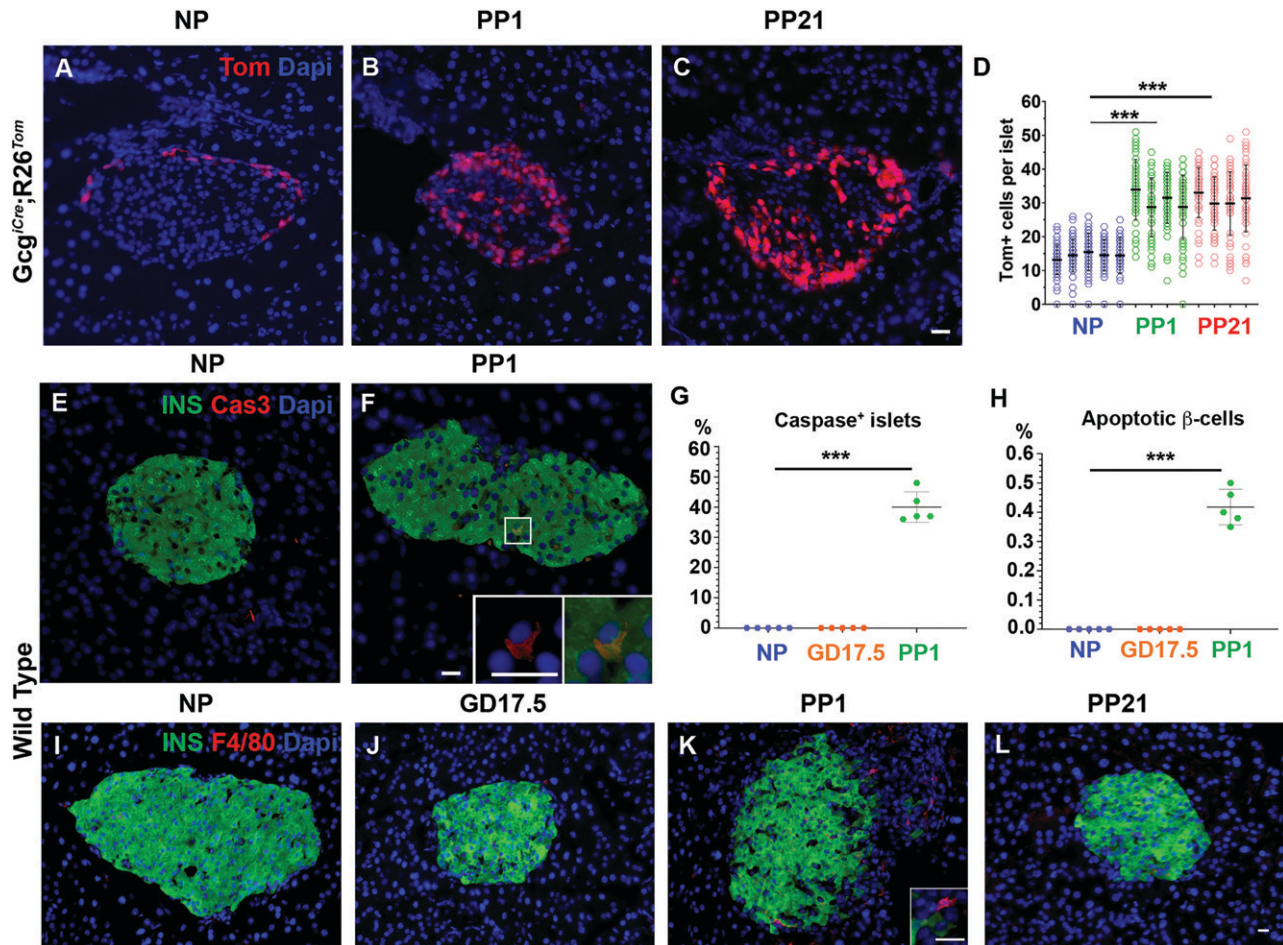
Conversion of α- to β-Cells in the Postpartum Pancreas

The data in Figs. 1 and 2 indicate that the observed postpartum reduction in glucagon-expressing cells was unlikely the result of α-cell apoptosis. To determine whether the described decline occurred as a result of loss of α-cell glucagon expression with possible lineage conversion of α-cells to β-cells, NP, GD17.5, and PP21 pancreata harvested from Gcg^{iCre};R26^{Tomato} dams were stained for insulin (Fig. 3). Previous studies have shown that <1% of β-cells in 2-month-old Gcg^{iCre};R26^{Tomato} mice express tomato-red (33). Since β-cell proliferation is augmented in rodents during pregnancy, we instead opted to quantify the percentage of insulin-expressing cells within the tomato-labeled population. In the NP Gcg^{iCre};R26^{Tomato} islets, insulin could be detected in 4% of tomato-positive cells (Fig. 3A and D). This rate persisted until parturition but then nearly doubled in the PP21 islets (Fig. 3B–D). These results indicate that enhanced conversion of α- to β-cells may explain the postpartum reduction of glucagon-expressing cells back to baseline levels.

Pregnancy Does Not Enhance α-Cell Proliferation

As demonstrated in Fig. 1, α-cell numbers expand significantly toward the end of gestation. We next examined whether α-cell proliferation would account for this observed expansion. To do so, BrdU was administered through drinking water to timed pregnant Gcg^{iCre}; R26^{Tomato} mice between GD10.5 (when pregnancy can be confirmed) and PP1, at which time the mice were euthanized. NP Gcg^{iCre};R26^{Tomato} females that were given BrdU for the same window of time served as controls for baseline BrdU incorporation (Supplementary Fig. 2A). While pregnancy led to increased proliferation among β-cells, it did not appear to enhance proliferation in α-cells (Supplementary Fig. 2B–D). Together, these results imply that α-cell expansion during pregnancy does not rely on proliferation of preexisting α-cells.

## Appearance of Lgr5-Expressing Cells in the Pancreas During and After Pregnancy

Since the increase in α-cell number during pregnancy does not involve replication, α-cell neogenesis was

Figure 2—Postpartum decline in α-cell mass is not due to α-cell apoptosis. *A–C*: Representative images of tomato-red–labeled cells and DAPI (blue) in the NP, PP1, and PP21 Gcg^{iCre};$R26^{Tomato}$ dams. *D*: Quantification of the number of tomato (Tom)-positive cells in islets at the indicated time points. Data are mean ± SD of at least four biological replicates. *E* and *F*: Immunostaining of sections obtained from NP and PP1 wild-type dams for detection of insulin (INS) (green), cleaved caspase-3 (Cas3) (red), and DAPI (blue). *G* and *H*: Quantification of the percentage of apoptotic β-cells at the indicated time points. Data are mean ± SD of five biological replicates. *I–L*: Immunostaining for detection of INS (green), F4/80 (red), and DAPI (blue) on sections obtained from NP, GD17.5, PP1, and PP21 showed F4/80$^+$ cells within and around the islets specifically in PP1 pancreas. Scale bars = 20 μm. ***$P < 0.0001$ by one-way ANOVA.

considered as an alternative mechanism for this observed expansion. The enzymatic activity of ALDH allows isolation by FACS using a fluorogenic ALDH substrate known as Aldefluor. In a previous report, we showed that ALDH-expressing cells have the ability to differentiate into β-cells after parturition (36). We further demonstrated that the Alde$^+$ cells in the adult pancreas can be divided into four distinct subpopulations on the basis of E-cadherin and CD90 expression (36). Under normal conditions, the Alde population consists mainly of CD90$^-$ cells; however, the percentage of mice having CD90$^+$ cells increases concomitantly with gestation during pregnancy and peaks toward the end of gestation when Alde$^+$/CD90$^+$ cells can be found in 75–80% of dams (36). We isolated the four Alde$^+$ subgroups from PP1 pancreas and found robust *Lgr5* expression within the Alde$^+$/CD90$^+$

population (Fig. 4*A*). To visualize these Lgr5-expressing cells, we next studied $Lgr5^{EGFP\text{-}IRES\text{-}CreERT2}$ mice ($Lgr5^{CreERT}$). In these mice, EGFP expression at any given time point will mark cells actively expressing *lgr5*. Because stem cells typically reside within a niche, in order to determine a potential niche for these Lgr5-expressing cells we analyzed sections obtained from the pancreas of NP, pregnant, or postpartum $Lgr5^{CreERT}$ dams. While no EGFP$^+$ cells could be found in the NP pancreas (Fig. 4*B*), cells expressing EGFP were detected sporadically at GD14.5 (Fig. 4*B* and *C*). These EGFP$^+$ cells were localized either in the duct lining or within the ductal network (Fig. 4*F* and *G*). Though still few in number, the EGFP$^+$ cells were relatively more abundant in the pancreas of GD17.5 dams compared with those of the GD14.5 (Fig. 4*B* and *D*). Here, the presence of cells expressing EGFP
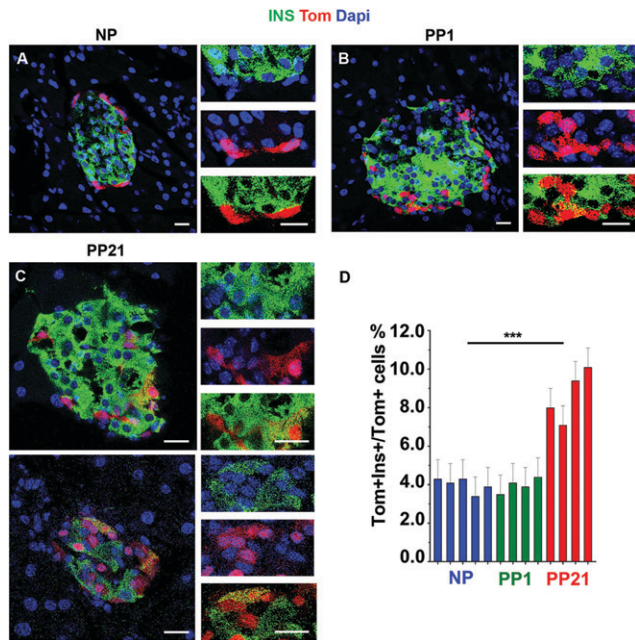
Figure 3—Conversion of α- to β-cells in the postpartum pancreas. A–C: Pancreatic tissues obtained from NP, PP1, and PP21 Gcg^{I-Cre};R26^{Tomato} dams for confocal detection of tomato (Tom) (red), insulin (INS) (green), and DAPI (blue). D: Quantification of the percentage of Tom$^+$/Ins$^+$ cells among Tom$^+$ cells in the indicated conditions. Data are mean ± SD of at least four biological replicates. Scale bars = 20 μm. ***$P < 0.0001$ by one-way ANOVA.

was more noticeable in the duct linings. Ultimately, the EGFP$^+$ cells decreased in number in the pancreas of PP1 $Lgr5^{CreERT}$ dams (Fig. 4B and E).

Lgr5 Progeny Differentiate Into Acinar and Endocrine Cells in the Postpartum Pancreas

In an attempt to visualize the progeny of Lgr5-expressing cells in situ, $Lgr5^{CreERT}$ mice were crossed with R26^{Tomato} reporter mice. In the $Lgr5^{CreERT}$;R26^{Tomato} mice, EGFP expression labels cells actively expressing $Lgr5$, whereas tomato expression as the result of tamoxifen treatment is a permanent lineage label of Lgr5$^+$ cells and their progeny. Cre-recombinase activity in $Lgr5^{CreERT}$;R26^{Tomato} females was induced before breeding, and the pancreata were harvested on PP21 (Supplementary Fig. 3A). As expected, tomato-labeled cells could not be detected in these mice or in NP tamoxifen-treated controls (data not shown). As demonstrated above, a subpopulation of cells in the pancreas of pregnant or postpartum dams express $Lgr5$. Therefore, pregnant $Lgr5^{CreERT}$;R26^{Tomato} dams were treated with tamoxifen at GD12, and the pancreata harvested at GD15 or PP21 (Supplementary Fig. 3B and C). Similar to the females treated with tamoxifen before pregnancy, we were not able to detect any tomato-labeled Lgr5 progeny (data not shown). Next, we induced Cre-recombinase activity on PP1 to study the fate of LGR5$^+$

cells as potential stem cells. In two of five mice euthanized 1 week after tamoxifen treatment, tomato-red–labeled cells were detected scattered outside the pancreatic parenchyma and throughout the pancreas (Fig. 5A). Upon closer examination, labeled cells localized within the parenchyma appeared to be either in proximity to ducts, but not cytokeratin positive (Fig. 5B), or within the acinar region, but not amylase positive (Fig. 5C and D). Both these results suggest that these labeled cells are nonductal nonacinar cells. Furthermore, the tomato-positive cells on PP7 (6 days after tamoxifen treatment) coexpressed EGFP (Fig. 5C and D), indicating that these cells were actively expressing $lgr5$ at the time of harvest and may still be in their stem-cell state. However, 2 weeks after tamoxifen injection at PP1, in two of five mice, cells expressing tomato only, along with persistent cells still coexpressing tomato and EGFP, were detected, implying that a progenitor-progeny relationship (similar to the intestine) may exist between early postpartum EGFP$^+$/tomato-positive cells and EGFP$^-$/tomato-positive cells 2 weeks postpartum (Supplementary Fig. 4A and B). Additionally, tomato-expressing cells in proximity to the islets were detected (Fig. 6A). In the pancreas of three out of five mice harvested 20 days after tamoxifen treatment (PP21), we could for the first time find tomato-labeled cells that expressed pancreatic terminally differentiated markers (Fig. 6B–G and Supplementary Fig. 4C and D), suggesting that the original EGFP$^+$/tomato-positive stem cells had given rise to these terminally differentiated pancreatic cells. Quantification analysis showed that on average, 48 ± 11% of islets in three of five mice that had tomato-positive cells 3 weeks after tamoxifen treatment also had tomato-labeled endocrine hormone-positive cells (Fig. 6H). Of note, we could not detect any insulin-positive/glucagon-positive bihormonal cells (Supplementary Fig. 4E). It should be emphasized that regardless of the time point for harvest, neither EGFP-expressing acinar cells nor EGFP$^+$ islet cells were found.
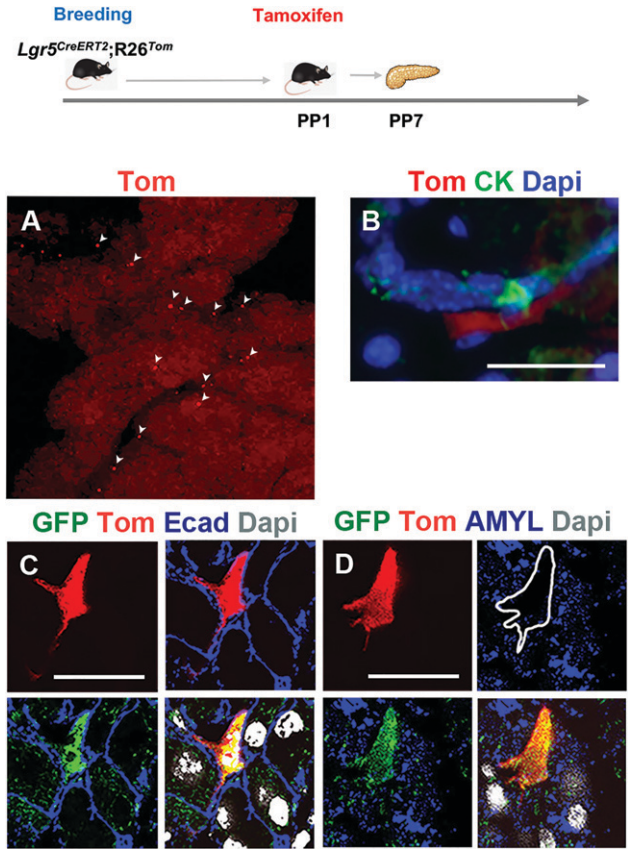
The periphery of the islets has been suggested to be a neogenic niche, as evidenced by the presence of a subset of immature insulin-producing β-cells that lack $Ucn3$ expression (19). Of note, these cells are at an intermediate stage in the transdifferentiation from α- to β-cells (19). Given the mainly peripheral localization of the tomato-labeled β-cells in $Lgr5^{CreERT}$;R26^{Tomato} islets (Fig. 5F), we suspected that one mechanism for β-cell formation from LGR5$^+$ stem cells could be via an α-cell stage. To address this question, we looked for UCN3 in the PP21 $Lgr5^{CreERT}$;R26^{Tomato} pancreas and found that UCN3 was not detected in the tomato-labeled β-cells (Fig. 7A–C). In line with this finding, a similar significant increase in the number of UCN3$^-$/INS$^+$ immature β-cells in wild-type postpartum pancreas was also detected (Fig. 7B–D).
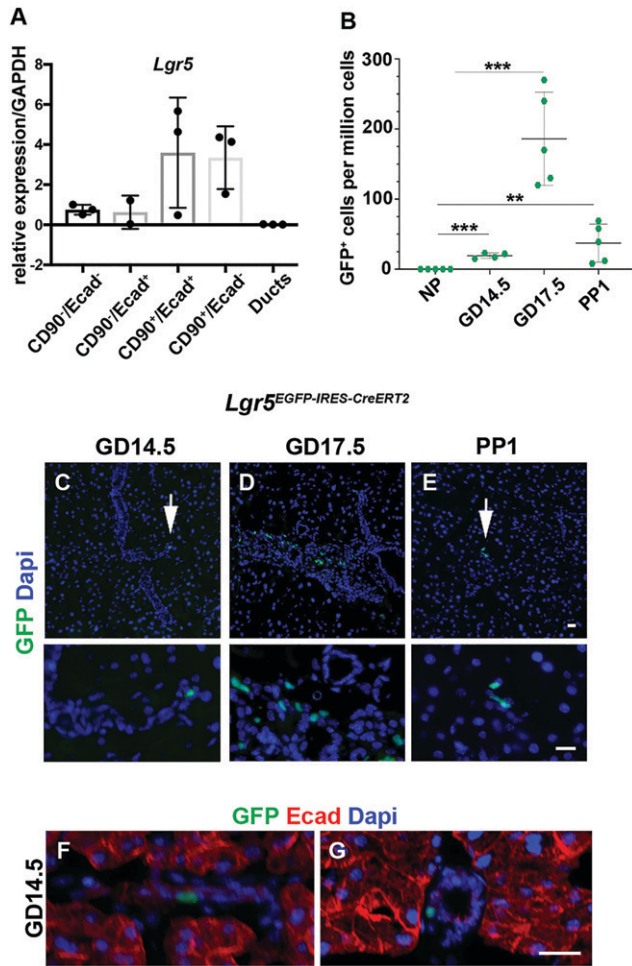
Figure 4—Identification of Lgr5-expressing cells residing in the adult pancreas. *A*: Quantitative RT-PCR for detection of *Lgr5* expression in the ducts or indicated four Alde$^+$ subpopulations. *B*: Quantification of the number of GFP$^+$ cells at the indicated time points. Data are mean ± SD of at least four biological replicates. *C–E*: Immunostaining for detection of GFP (green) and DAPI (blue) in sections obtained from the pancreas of GD14.5, GD17.5, and PP1 *Lgr5^{CreERT}* dams. Arrows highlight the GFP-expressing cells in the parenchyma. *F* and *G*: Immunostaining of pancreatic tissues obtained from GD14.5 *Lgr5^{CreERT}* dams for detection of GFP (green), E-cadherin (Ecad) (red), and DAPI (blue) showing GFP-expressing cells within (*D*) or lining (*E*) the ductal structures. Scale bars = 20 μm. **$P < 0.001$, ***$P < 0.0001$ by one-way ANOVA.

Figure 5—Detection of Lgr5 progeny in the adult pancreas. *A–D*: Detection of tomato-red (Tom) on whole-mount tissues or sections from the pancreas of *Lgr5^{CreERT}*;R26^{Tomato} mice treated with tamoxifen on PP1 and euthanized on PP7. *B–D*: Pancreatic tissues from *Lgr5^{CreERT}*;R26^{Tomato} mice for detection of Tom (red) along with cytokeratin (CK) (green) and DAPI (blue) (*B*), GFP (green), E-cadherin (Ecad) (blue) and DAPI (gray) (*C*), and GFP (green), amylase (AMYL) (blue), and DAPI (gray) (*D*). Note the absence of AMYL in the Tom$^+$/EGFP$^+$ cell (right panel). Scale bars = 20 μm.

DISCUSSION

There is an ongoing interconversion between α- and β-cells in healthy islets (19). The aim of the current study was to determine whether pregnancy would enhance this spontaneous conversion of α- to β-cells. A previous study has shown that there is no detectable β-cell neogenesis during pregnancy (38). However, the latest gestational time point studied in that report was at about GD17, suggesting that a potential conversion of α- to β-cells could therefore occur between GD17 and parturition. Analyzing the dynamics of α-cells during and after pregnancy using

timed pregnant wild-type or *Gcg^{iCre}*;R26^{Tomato} mice yielded surprising outcomes. We observed augmented conversion of α- to β-cells in the postpartum islets rather than during pregnancy. Additionally, we observed a significant expansion of α-cells at the end of gestation. A recent report has shown increased α-cell proliferation and α-cell mass during late gestational stages in mice (39). Intriguingly, we were not able to detect a higher proliferation rate among α-cells during pregnancy. This discrepancy is likely due to the observed variation in α-cell proliferation rate within each cohort. This exclusion of proliferation as the source for α-cell expansion encouraged us to identify the cells contributing to α-cell neogenesis.

The Wnt target gene Lgr5 marks adult stem cells in multiple organs (35,40,41). LGR5 is normally not detected in the adult mouse pancreas; however, its expression is strongly activated upon injury in regenerating pancreatic duct-like structures (11). Accordingly, cells actively
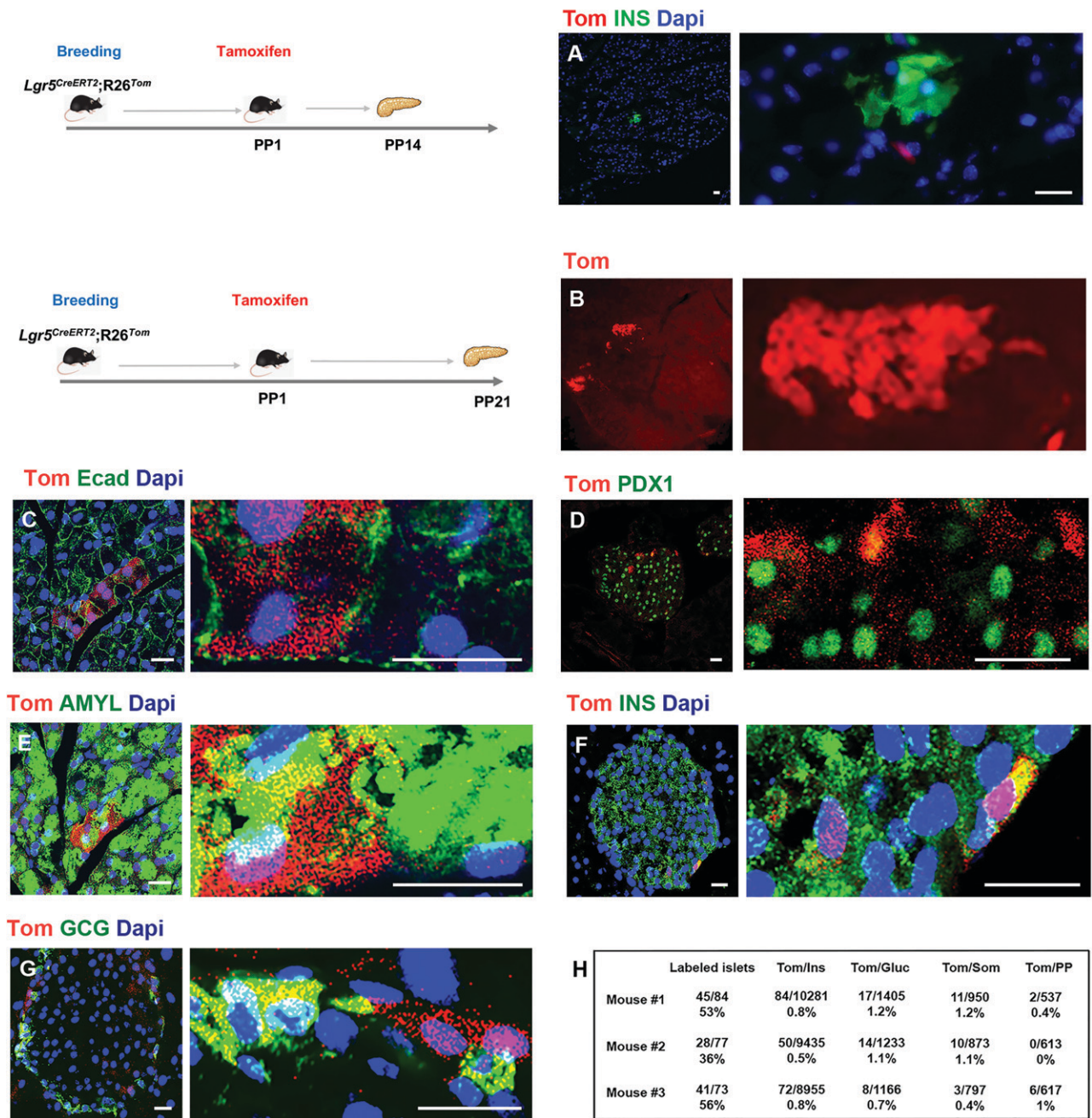
Figure 6—Lgr5-expressing cells can give rise to terminally differentiated cell types in vivo. *A*: Pancreatic tissues of *Lgr5*[CreERT];R26[Tomato] dams treated with tamoxifen on PP1 and euthanized on PP14 for detection of tomato-red (Tom) along with insulin (INS) (green) and DAPI (blue). Note the single Tom-expressing cell in proximity to an islet. *B–G*: Detection of Tom on whole-mount tissues or sections from the pancreas of *Lgr5*[CreERT];R26[Tomato] mice treated with tamoxifen on PP1 and euthanized on PP21. *C–G*: Confocal detection of Tom and E-cadherin (Ecad) (green) and DAPI (blue) (*C*), PDX1 (green) (*D*), amylase (AMYL) (green) and DAPI (blue) (*E*), insulin (INS) (green) and DAPI (blue) (*F*) , and glucagon (GCG) (green) and DAPI (blue). *H*: Quantification of the Tom-labeled endocrine cells in three *Lgr5*[CreERT];R26[Tomato] mice treated with tamoxifen on PP1 and euthanized on PP21. Labeled islets indicate the number and percentage of islets containing any numbers and any type of Tom-labeled endocrine cells. The remaining four columns display the number and percentages of Tom-labeled cells within each endocrine cell type. Scale bars = 20 μm. Tom/PP, tomato-pancreatic polypeptide; Tom/Som, tomato-somatostatin.

expressing *lgr5* in the pancreas of NP adult mice were not found. However, we were able to detect EGFP^{+} cells in the pancreas of late gestational as well as postpartum *Lgr5*[CreERT] dams. These EGFP^{+} cells were found mainly in proximity of the ducts and occasionally within the pancreatic ductal network in pregnant dams. Together, these results suggest that signals associated with pregnancy and those following injury may induce *lgr5* expression in the same subset of cells residing within or in the proximity of ducts. Future studies should reveal the exact nature of the signals required for triggering *lgr5* expression in the adult pancreas.
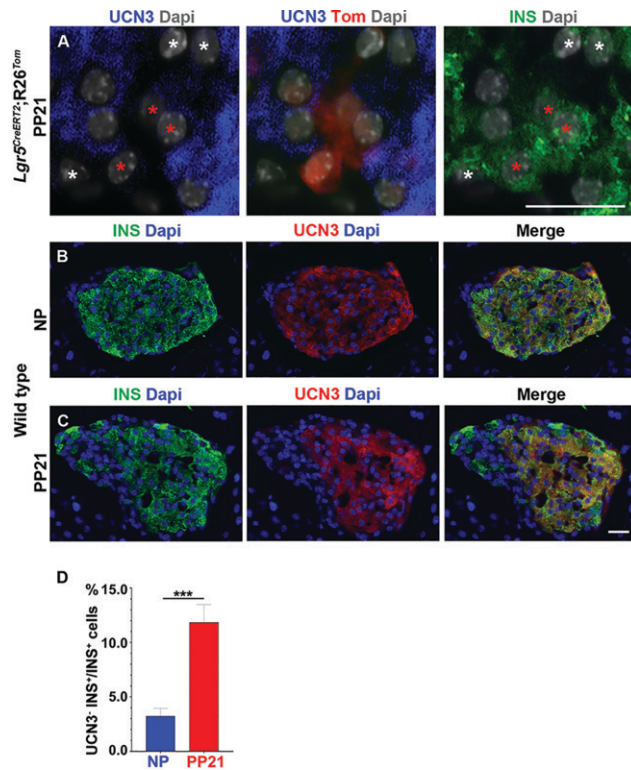
Figure 7—Lgr5 progeny differentiate into β-cells through intermediary α-cells. *A*: Detection of tomato-red (Tom), UCN3 (blue), insulin (INS) (green), and DAPI (gray) in pancreatic tissues of *Lgr5^{CreERT}*;R26^{Tomato} dams treated with tamoxifen on PP1 and euthanized on PP21. White asterisks highlight a UCN3$^-$/Tom$^-$/INS$^+$ cell, whereas red asterisks mark three UCN3$^-$/Tom$^+$/INS$^+$ cells. *B* and *C*: Detection of INS (green), UCN3 (red), and DAPI (blue) in tissues from NP and PP21 wild-type dams. *D*: Quantification of the number of UCN3$^-$/INS$^+$ among INS$^+$ cells in NP or PP21 wild-type islets. Data are mean ± SD of five biological replicates. Scale bars = 20 μm. ***$P < 0.0001$ by unpaired *t* test. Tom, tomato-red.

During pregnancy, the number of α-cells surges between GD17.5 and PP1. Consistent with the appearance of Lgr5$^+$ cells around GD15, administering tamoxifen before that stage did not lead to tomato labeling of any cells in the *Lgr5^{CreERT}*;R26^{Tomato} pancreas. Tamoxifen treatment at late gestation to lineage trace Lgr5 progeny during α-cell expansion led to abortion, presumably because of hormone imbalance caused by tamoxifen and, in most cases, death of the pregnant dams.

Thus, the PP1 time point was chosen because of the high likelihood of finding cells expressing *lgr5* in the pancreas. Although, we were not able to lineage trace Lgr5 progeny during its optimal window toward the end of gestation, careful timing of tamoxifen treatment in *Lgr5^{CreERT}*;R26^{Tomato} mice yielded confirmation that in the adult mouse pancreas, Lgr5-expressing progenitor cells can differentiate into acinar or endocrine cells after parturition. The observed replication-independent expansion of α-cells, which peaks around parturition, along with the presented progenitor-progeny relationship between Lgr5$^+$ and endocrine cells imply that Lgr5 progeny should have

differentiated into α-cells toward the end of gestation. This process declines significantly in the postpartum pancreas, as evidenced by the scarcity of the reporter-labeled endocrine cells at PP21 when tamoxifen was administered on PP1. However, the number of α-cells returns to NP levels by PP21. Immunostaining for cleaved caspase-3 and using *Gcg^{iCre}*;R26^{Tomato} mice to label α-cells and their progeny, we demonstrated that this reduction was not due to α-cell apoptosis but, rather, to the result of conversion of α- to β-cells. Given that the majority of tomato-positive/insulin-positive cells in the PP21 *Lgr5^{CreERT}*; R26^{Tomato} pancreas were found in the periphery of the islets and were UCN3$^-$, we propose that in the late gestational stages, *lgr5* expression is induced in dormant progenitor-like cells residing in the adult pancreas. These cells then commit to the α-cell lineage toward the end of gestation, which leads to an expansion of α-cells by PP1. Following parturition, α-cells further transform into β-cells, which reduces the number of α-cells back to NP levels.

As shown in Figs. 5 and 6, the progeny of Lgr5$^+$ cells can differentiate into endocrine cells between PP14 and PP21. Thus, while differentiation of LGR5$^+$ cells into α-cells during pregnancy takes ?5 days (GD15 to parturition), the same process in the postpartum pancreas seems to require at least 2 weeks. The higher pace of differentiation during pregnancy should not be surprising because several studies have shown an increased regenerative capacity associated with pregnancy (42–46), as seen when pregnancy protects against cardiac ischemic injury by initiating upregulation of cardiac progenitor cells in the affected site (46). Moreover, liver volume gain, liver function, and survival after partial hepatectomy are all markedly improved by pregnancy (43). Other studies have demonstrated an enhanced capacity to remyelinate white matter lesions in the maternal central nervous system (44,45) and beneficial effects on muscle regeneration through an increase in satellite cell number during pregnancy (42).

Pregnancy is among the few, if not the only, known condition where a relatively higher β-cell proliferation rate is observed in the healthy adult mouse pancreas. The expansion of β-cell mass during pregnancy is followed by β-cell apoptosis after parturition, which results in the return of β-cell numbers to baseline prepregnancy levels. The mechanism and signals that would initiate and subsequently terminate apoptosis among β-cells remain unknown. However, given that the proliferation rate among β-cells in the postpartum pancreas is low (29,31), maintaining this important dynamic equilibrium may not only rely on a balance between β-cell proliferation and β-cell death but also could entail neogenesis to recruit new β-cells from non–β-cells to protect from excessive β-cell loss. We therefore postulate that the postpartum transformation of α- to β-cells is unlikely a response to the

higher insulin demands but, instead, acts as a buffer to counteract excessive β-cell involution.

β-Cell turnover declines with age in both mice and humans (47,48); thus, pregnancy-associated β-cell proliferation in mice may reflect the fact that mice in these studies are generally bred at a relatively young age (8 weeks, which is ?6% of their natural life span). Humans are sexually mature later (?17% of the natural life span), and indirect evidence from the human pancreas during pregnancy and postpartum indicates that the increased number of β-cells is the result of neogenesis rather than replication of preexisting β-cells (49,50). Therefore, while the contribution of Lgr5 progeny to the endocrine lineage during and after pregnancy may appear redundant in mice, it may have a significant biological impact in humans.

In conclusion, we have identified a population of Lgr5^{+} cells that can differentiate into acinar or endocrine cells. Under normal conditions, these multipotent cells are primarily involved in maintaining β-cell homeostasis during postpartum pancreatic involution, but a better understanding of these cells' existence and biology may lead to directed therapeutic intervention to enhance β-cell mass for people with diabetes.

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