TGFβ Receptor Signaling Is Essential for Inflammation-Induced but Not β-Cell Workload–Induced β-Cell Proliferation

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Protection and restoration of a functional β-cell mass are fundamental strategies for prevention and treatment of diabetes. Consequently, knowledge of signals that determine the functional β-cell mass is of immense clinical relevance. Transforming growth factor β (TGFβ) superfamily signaling pathways play a critical role in development and tissue specification. Nevertheless, the role of these pathways in adult β-cell homeostasis is not well defined. Here, we ablated TGFβ receptor I and II genes in mice undergoing two surgical β-cell replication models (partial pancreatectomy or partial duct ligation), representing two triggers for β-cell proliferation, increased β-cell workload and local inflammation, respectively. Our data suggest that TGFβ receptor signaling is necessary for baseline β-cell proliferation. By either provision of excess glucose or treatment with exogenous insulin, we further demonstrated that inflammation and increased β-cell workload are both stimulants for β-cell proliferation but are TGFβ receptor signaling dependent and independent, respectively. Collectively, by using a pancreas-specific TGFβ receptor–deleted mouse model, we have identified two distinct pathways that regulate adult β-cell proliferation. Our study thus provides important information for understanding β-cell proliferation during normal growth and in pancreatic diseases. ***Diabetes*** **62:1217–1226, 2013**

Preservation and restoration of a functional β-cell mass are fundamental objectives in diabetes therapy (1), which require an understanding of the regulation of β-cell mass in the adult pancreas. During embryogenesis, β-cell mass is generated by both the proliferation and differentiation of pancreatic progenitor cells—a process called neogenesis (2,3)—whereas β-cell replication was shown to be the predominant way to expand β-cell numbers to compensate for increased insulin demands after birth (4–7).

Transforming growth factor β (TGFβ) superfamily signaling has diverse roles in various cellular and developmental pathways, beginning with binding of ligands to type II receptors to catalyze phosphorylation of the type I receptors to activate either the transcription factors called Smads or alternative signaling pathways (2,8–10). The complex TGFβ signaling cascade entails overlapping, redundant, and diverse roles in different types of cells (11,12).

Many studies have demonstrated that TGFβ signaling plays a role in pancreas development (2) and pancreatic diseases like pancreatitis and pancreatic carcinoma (13,14). During embryogenesis, TGFβ signaling regulates the balance between endocrine and exocrine pancreas by favoring endocrine cell differentiation and maturation and inhibiting acinar cell growth (15–19). In the adult pancreas, TGFβ signaling in acinar cells seems to be crucial for maintenance of differentiation (15–17,19). In contrast to acinar cells, the effect of TGFβ on adult pancreatic β cells appears quite different (20–25). First, TGFβ was shown to increase insulin release from fetal rat islets when exposed to glucose at 200 mg/dL without affecting β-cell replication (25). In contrast, TGFβ counteracted the mitogenic effect on β cells by 300 mg/dL glucose and no longer induced insulin secretion (25). Furthermore, although TGFβ and epidermal growth factor are able to stimulate extracellular signal–related kinase 1/2 and phosphatidylinositol 3-kinase signaling pathways in β cells, the effect was not prolonged enough to commit β cells to a mitogenic state—unlike stimulation by glucose or insulin-like growth factor-1 (23,25). Collectively, these studies suggest that the net effect of TGFβ signaling on insulin release and β-cell proliferation is TGFβ dose and glucose concentration dependent.

Even though the importance of TGFβ signaling in regulating adult β-cell proliferation is suggested by the above-mentioned studies, most of the data are from in vitro experiments. In the current study, we used mice with both the type I TGFβ receptor (TBRI) and the type II TGFβ receptor (TBRII) deleted in the pancreas (26,27), with different β-cell proliferation models, including partial pancreatectomy (PPX) (28) or partial duct ligation (PDL) (29,30), with or without drinking water containing high glucose (31,32) and with or without exogenous insulin treatment. Comparison of β-cell proliferation under these various conditions enables us to dissect the different ways in which TGFβ signaling affects adult pancreatic β-cell proliferation.

RESEARCH DESIGN AND METHODS

Mouse manipulation. All mouse experiments were performed in accordance with the guidelines from the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and the University of Pittsburgh Institutional Animal Care and Use Committee. C57/6 mice were purchased from The Jackson Laboratory. Transgenic mice expressing TGFβ receptor I fx/fx (Alk5) and TGFβ receptor II fx/fx were generous gifts from Prof. Stefan Karlsson, University of Lund, Lund, Sweden (26,27). Pancreas transcription factor 1a (PTF1a) promoter cre reporter (PTF1a^{cre}) mice have previously been described (33). Besides the nearly exclusive expression of PTF1a in the

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pancreas, the use of the PTF1a promoter to drive CRE recombinase to delete TBRI and TBRII in the mouse pancreas avoids the need for injection of tamoxifen, which has been found to affect cell proliferation (not shown). PTF1a^{cre} mice were bred with TBRI(fx/fx)/TBRII(fx/fx) mice to generate PTF1a^{cre}/TBRI(fx/fx)/TBRII(fx/fx) (PTF1a^{cre}TBR12F) mice. The CRE-negative TBRI(fx/fx)/TBRII(fx/fx) (TBR12F) littermates were used as controls and showed no difference from wild-type C57/6 mice (not shown). Only 8-week-old male mice were used for experiments. Measurements of mouse blood glucose and intraperitoneal glucose tolerance test were performed as previously described (34). PPX and PDL were performed as previously described (28,30). Adequacy of the PDL was assured by examination of gross morphology, immunohistochemistry, and gene expression for potential differentiation factors like ngn3. Degree of inflammation was evaluated by quantitatively measuring inflammatory factors interleukin (IL)-6, interferon-γ (IFN-γ), and tumor necrosis factor (TNF) in addition to the pan-leukocyte marker CD45. For quantification of β-cell proliferation, 1 mg/mL BrdU (Sigma) was added into either 1 or 10% sucrose drinking water supplied to mice ever since surgery for 1 week. For insulin pellet experiments, each mouse received subcutaneous implantation of one mouse insulin pellet (LinBit) according to the manufacturer's instruction.

Pancreatic digestion and analysis. For islet isolation, pancreatic duct perfusion and subsequent digestion were performed with 0.2 mg/mL collagenase (Roche) for 15–20 min. Islets were handpicked three times to avoid contamination of nonislet cells. Nonislet fraction was prepared from islet-deprived pancreatic digests. The purity of the fractions was evaluated by the gene expressions of insulin and amylase as markers for islets and nonislet exocrine pancreas, respectively.

RNA isolation and quantitative RT-PCR. RNA was extracted from pancreas tissue, isolated islets, or nonislet pancreatic digests with Trizol (Invitrogen) and quantified with Nanodrop1000 (Thermo Scientific) according to the manufacturer's instructions, followed by cDNA synthesis (Qiagen). Conventional PCR was performed using gene-specific, exon-spanned primers (Supplementary Table 1). Quantitative PCR primers were purchased from Qiagen (Supplementary Table 2).

Immunohistochemistry. All of the mice were perfused through the heart with PBS to remove blood cells from the circulation and to reduce nonspecific immunostaining. Pancreata were subsequently fixed in 4% formalin for 4 h, followed by cryo-protection in 30% sucrose overnight before freezing in a longitudinal orientation (from tail to head of the pancreas) and sectioned at 6 μm. Primary antibodies for immunostaining were as follows: DBA (Vector Laboratories), rat polyclonal BrdU specific (Abcam), Ki-67 specific (DAKO), CD45 specific (BD), and guinea pig polyclonal insulin specific (Dako). Pretreatment with protease for 5 min followed by incubation with 2N HCl for half an hour was performed for the antigen retrieval for BrdU and Ki-67 staining. HCl was neutralized with Tris-borate-EDTA buffer (Sigma) before further steps. Indirect fluorescent staining was performed with Cy2- or Cy3-conjugated secondary antibodies generated from donkey (Jackson). Nuclear staining was performed with Hoechst (BD). Imaging of cryosections was performed using an AxioImager Z.1 microscope (Zeiss) with image analysis with AxioVision software (Zeiss). Gross images were obtained using an Olympus SZX12 stereomicroscope and captured with SPOT imaging software (SPOT Imaging Solutions).

Quantification and statistics. Percentage of BrdU$^+$ β cells, Ki-67$^+$ β cells, BrdU$^+$ duct cells, and β-cell mass/area was quantified on the basis of at least six sections that were 100 μm apart and determined as previously described (35). At least 5,000 cells were counted for each experimental condition. Counting continued beyond 5,000 cells until 50 positive cells were tallied if the percentage of positive cells were low. The quantitative values are depicted as

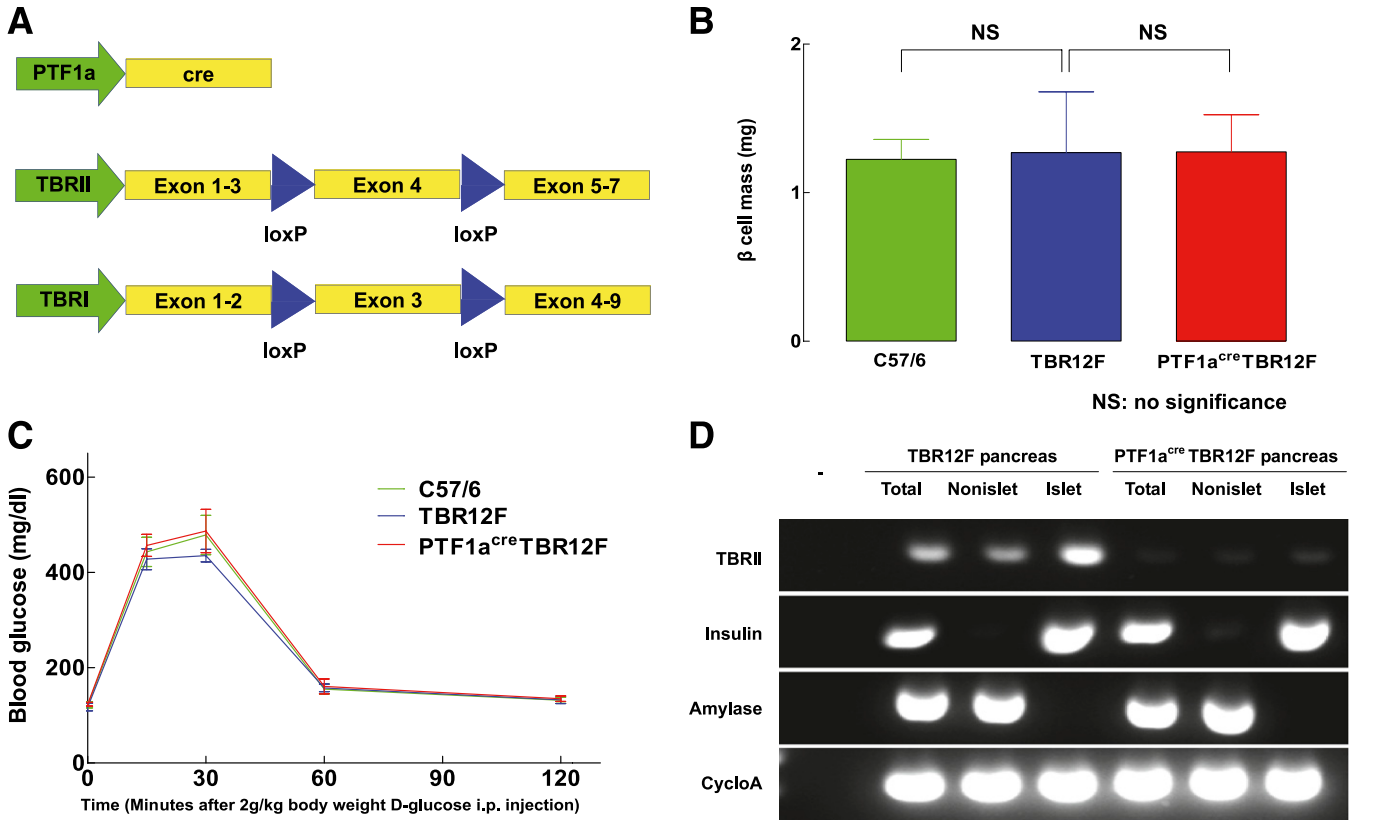


FIG. 1. Production of pancreas-specific TGFβ receptor–deleted mice. *A*: PTF1a^{cre} mice were bred with TBRI(fx/fx)/TBRII(fx/fx) mice to generate PTF1a^{cre}TBR12F mice. Activation of Cre recombinase in PTF1a$^+$ pancreatic cells can remove exon 4 and exon 3 in TBRII and TBRI, respectively, which are both required for normal receptor functions. *B*: At the age of 8 weeks, β-cell mass of PTF1a^{cre}TBR12F mice along with littermate TBR12F controls and wild-type C57/6 controls was analyzed and showed no differences among the three groups. *C*: Intraperitoneal (i.p.) glucose tolerance test was also performed in PTF1a^{cre}TBR12F mice and showed normal glucose response like that in controls. *D*: For validation of a TGFβ receptor deletion in PTF1a^{cre}TBR12F mice in which exon 4 of TBRII was removed during recombination, a primer pair was designed to amplify a 1,195 bp nucleotide encoding exon 4 to exon 8 of the *TBRII* gene product. RT-PCR was performed on whole pancreas samples (total) and samples of islets (islet) and nonislet pancreatic fractions (nonislet) from control TBR12F and PTF1a^{cre}TBR12F mice, showing efficient TBRII deletion in the pancreas of PTF1a^{cre}TBR12F mice. Insulin and amylase were used to evaluate the purity of the islet and nonislet cell populations, respectively. CyclophilinA (CycloA) is a housekeeping gene. -, water instead of cDNA as negative control.

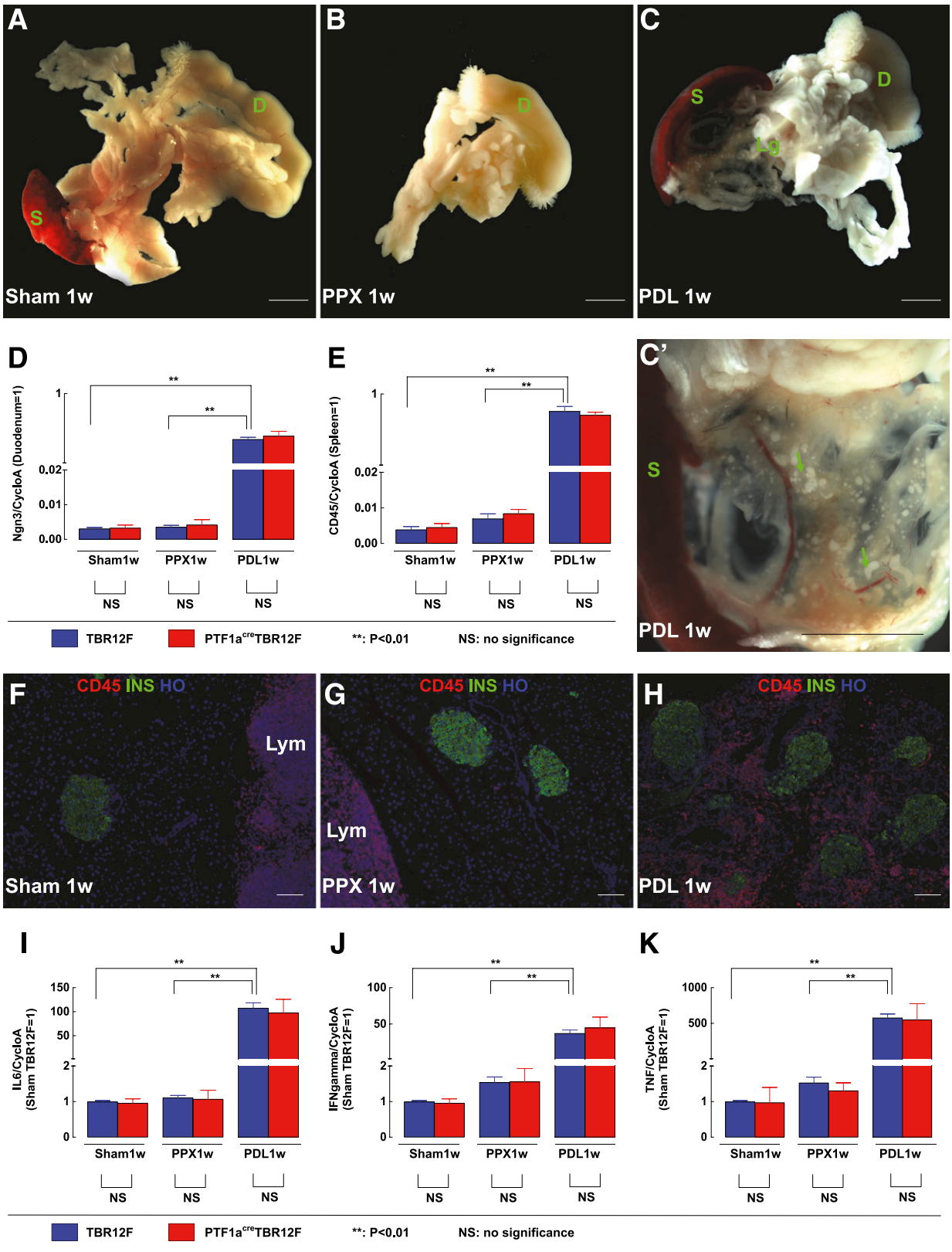


FIG. 2. Models for study of β-cell proliferation. *A–C*': Representative gross images of mouse pancreata 1 week (1w) after sham operation (*A*), 50% PPX (*B*), and PDL (*C*). (An enlarged view of the ligated tail is shown in *C*'.) Islets (arrows) in the ligated part of PDL pancreas can easily be visualized due to the atrophy of the acinar tissue (*C*'). *D*: Quantitative RT-PCR was performed to check Ngn3 transcription in control TBR12F and in PTF1a^{cre}TBR12F pancreas. Cyclophilin A (CycloA) was used as a housekeeping gene to normalize Ngn3 values. Relative enrichment of Ngn3 from samples was normalized to duodenum (equal to 1). No difference in Ngn3 expression was found between control and PTF1a^{cre}TBR12F pancreas in sham-treated, PPX, or PDL mice. Ngn3 levels in PDL pancreas were significantly higher (>100 times) than in sham and PPX pancreas. *E*: Quantitative RT-PCR was performed to check CD45 transcription in control TBR12F and the PTF1a^{cre}TBR12F pancreas. Relative enrichment of CD45 from samples was normalized to spleen (equal to 1). No difference in CD45 mRNA expression was found between control and PTF1a^{cre}TBR12F pancreas in sham-treated, PPX, or PDL mice. CD45 levels in PDL pancreas were significantly higher (>100 times) than those in sham and PPX

means ± SEM from five animals for each experimental condition. All data were statistically analyzed by unpaired Student *t* test. The significance was considered if $P < 0.05$.

RESULTS

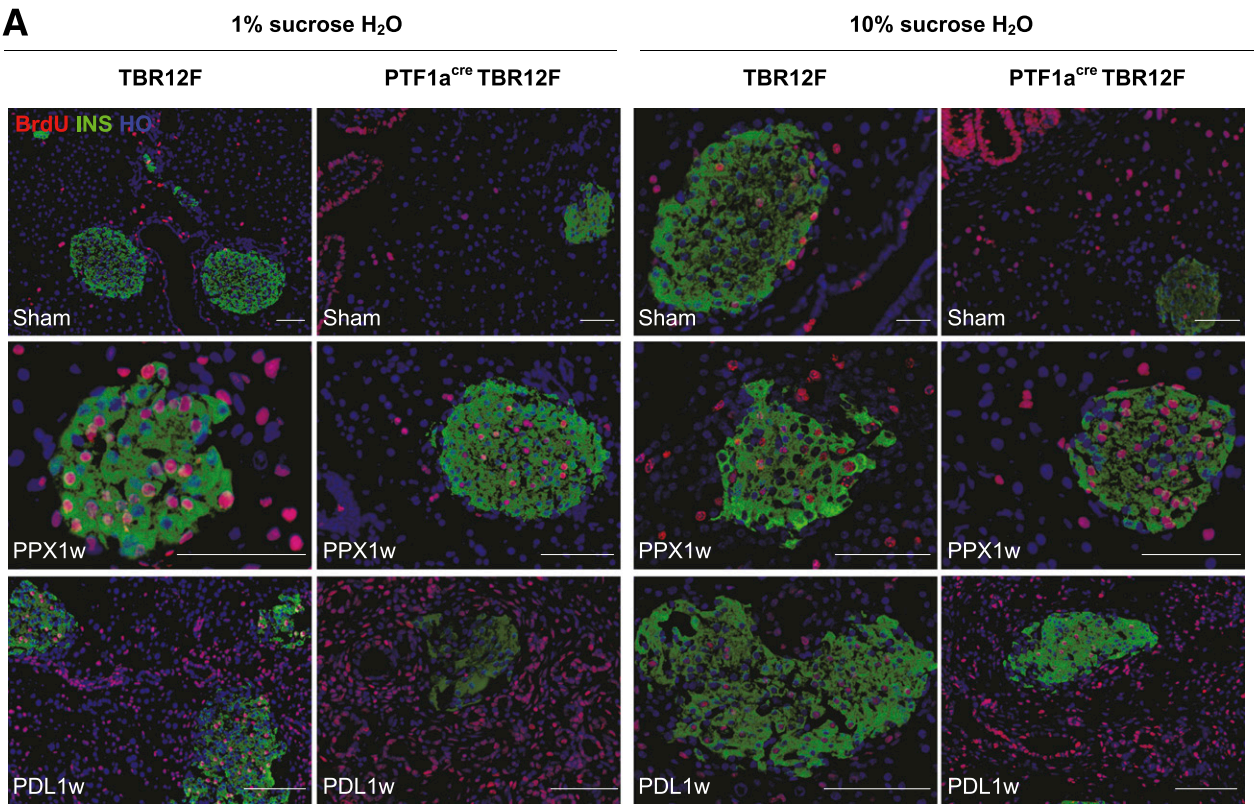
Models to study the effect of defective TGFβ receptor signaling on adult β-cell proliferation. To explore whether TGFβ signaling plays a role in adult pancreatic β-cell proliferation, we generated PTF1a^{cre}TBR12F mice with TBRI and TBRII specifically deleted in pancreas (26,27) (Fig. 1*A*). The CRE-negative TBR12F littermates were used as controls (see RESEARCH DESIGN AND METHODS). At the age of 8 weeks, male PTF1a^{cre}TBR12F mice were compared with littermate TBR12F and wild-type C57/6 mice and did not show differences in blood glucose levels [either fasting or nonfasting (not shown)], β-cell mass (Fig. 1*B*), or glucose response (Fig. 1*C*). Moreover, there were no observed developmental defects. Only 8-week-old male mice were used for the current study.

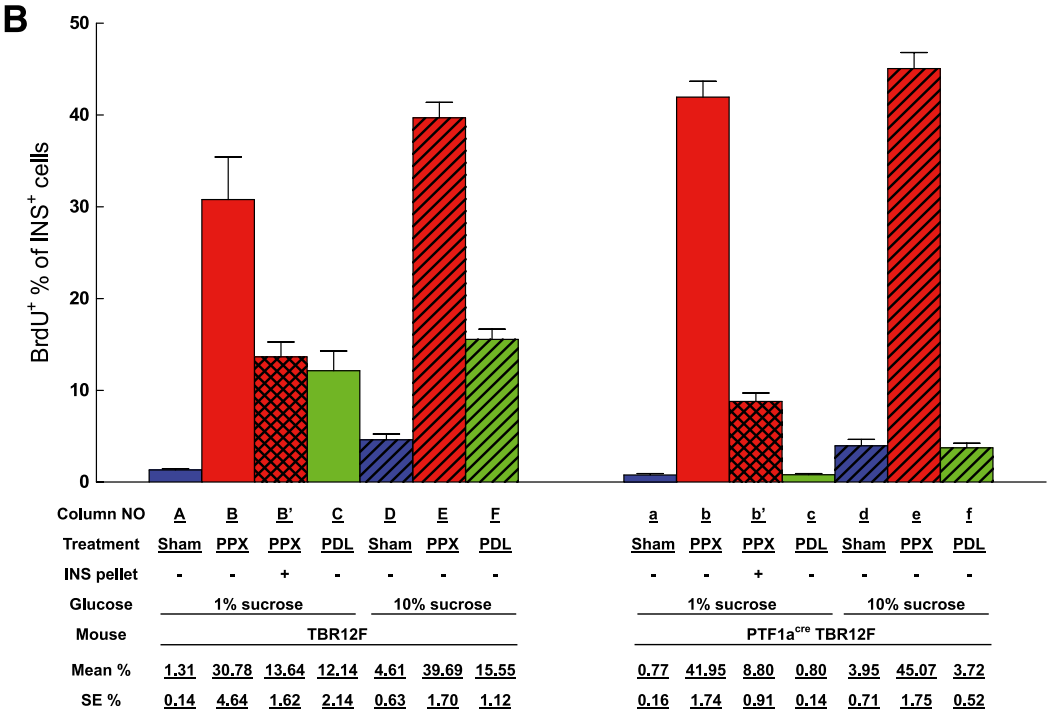
As both TBRI and TBRII are part of canonical TGFβ signaling, confirmation of only TBRII deletion was used to validate proper CRE function. The TBRII(fx/fx) mouse has LoxP sites flanking exon 4, the functional domain of TBRII. When CRE recombinase is activated, exon 4 is then removed, TBRII is inactivated, and TGFβ signaling is inhibited (27). However, the most commonly used TBRII antibody (Millipore) was generated against the first 28 NH_2-terminal residues of TBRII, which are encoded by exons 1 and 2 of the *TBRII* gene. Consequently, the truncated TBRII resulting from cre-mediated recombination may still permit antibody detection. Therefore, neither immunohistochemistry nor Western blot with this antibody is a proper method to confirm recombination. We thus used a primer pair that amplifies a 1,195 bp nucleotide from intact TBRII-mRNA–encoded cDNA but not from cDNA in which recombination had occurred, as the sense primer resides in exon 4. Conventional PCR was performed on either total pancreas or isolated islet and nonislet pancreatic fractions from both control and PTF1a^{cre}TBR12F mice. Amylase and insulin were used as markers for the purity of the islets and nonislet cell populations, respectively (Fig. 1*D*). Our data showed TBRII expression in the whole pancreas of control mice and at a higher level in islets. Only very weak TBRII signals were detected in the various cell populations from the pancreas of PTF1a^{cre}TBR12F mice, which could derive from the cells in the pancreas that never expressed PTF1a during development, e.g., endothelial and mesenchymal cells (Fig. 1*D*).

We performed three different treatments on the mice: 50% PPX, PDL, and sham operation. Sham operation did not have any effect on β-cell proliferation (not shown). Sham-operated mice (Fig. 2*A*) were used as a control. In 50% PPX, less than one-half of the β cells remain after surgery, since the removed tail pancreas contains more islets and a higher β-cell percentage per islet than the head (Fig. 2*B*). PDL, on the contrary, does not remove any β cells but leads to the destruction of acinar cells and the emergence of many duct-like tubular structures from the ligated part of pancreas, along with a severe local inflammation and β-cell proliferation (Fig. 2*C* and *C'*). We found a >100-fold increase in Ngn3 mRNA levels by quantitative PCR in the ligated tail compared with the unligated head (not shown) or compared with sham-operated or PPX pancreas (Fig. 2*D*), consistent with previous reports (30,36). Interestingly, differences in ngn3 mRNA levels between control and PTF1a^{cre}TBR12F mice were not detected in either total pancreatic samples (Fig. 2*D*) or separate islet or nonislet populations (not shown), suggesting that TGFβ receptor signaling is not necessary for the presumed β-cell neogenesis in PDL pancreas (30,36). Quantitative PCR and immunostaining were performed on pancreas samples for CD45, a specific marker for recruited inflammatory cells, to confirm that a properly performed PPX only causes a slight increase in CD45$^+$ cells compared with sham-treated mice (Fig. 2*E–G*). In contrast, a 100-fold increase in CD45 gene expression and a robust recruitment of CD45$^+$ cells were detected in PDL pancreas, suggesting severe local inflammation (Fig. 2*E–H*). Therefore, the proliferation of β cells seen in PDL should be mainly triggered by local inflammation (37), whereas the proliferation of β cells seen in PPX should mainly result from an increased β-cell workload. Leukocytes do not express PTF1a during development. Therefore, TGFβ receptor signaling should be intact in recruited inflammatory cells. For confirmation, the degree of inflammation was evaluated by quantitatively measuring inflammatory factors IL6 (Fig. 2*I*), IFN-γ (Fig. 2*J*), and TNF (Fig. 2*K*) in addition to the pan-leukocyte marker CD45 (Fig. 2*E*). No difference in the levels of these factors was found between the pancreas of control and PTF1a^{cre}TBR12F mice, suggesting little change in the degree of local inflammation due to pancreas-specific deletion of TGFβ receptor signaling.

TGFβ receptor signaling is required for baseline adult β-cell proliferation. To quantitatively analyze β-cell proliferation, we gave BrdU in the drinking water, with 1% sucrose to encourage adequate intake, starting immediately after surgery for 1 week before harvest (31,32). To ensure that variation in the water intake between the different experimental groups did not affect our results, we also diluted the BrdU from 1 mg/dL, which was used in our study, down to 0.2 mg/dL. We found no change in the labeling percentage of β cells (not shown), suggesting that the labeling efficiency of BrdU does not decrease even if the mice drink five times less water. Also, we did not find an obvious difference in the volume of consumed drinking water by mice from different experimental groups, suggesting that the measured difference in BrdU labeling results entirely from a difference in β-cell proliferation. We only assessed β-cell proliferation in the first week because of concerns that long-term changes in β-cell proliferation might induce compensatory or secondary regulation of β-cell homeostasis. Besides BrdU, we also analyzed Ki-67 expression in β cells at the time of

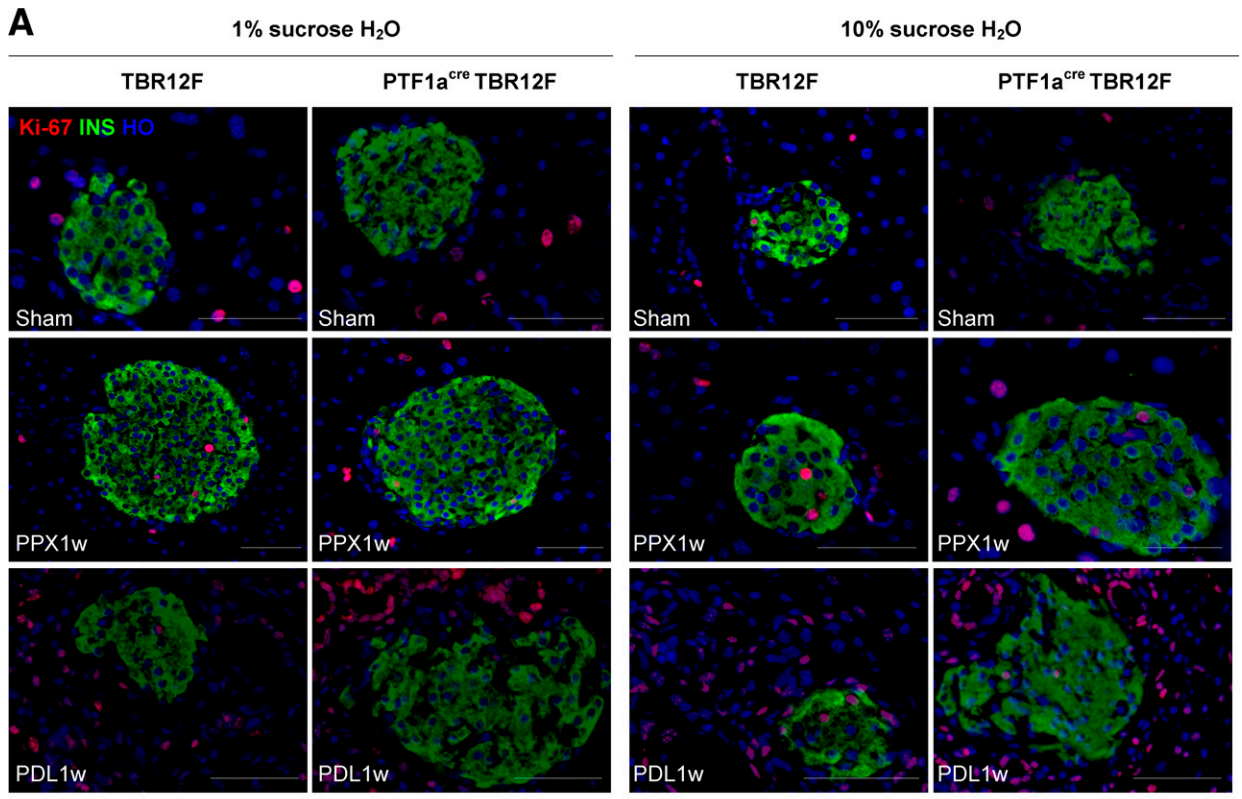
pancreas. *F–H*: Representative immunofluorescent images for insulin (INS) and CD45 were shown in mouse pancreata 1 week after sham operation (*F*), 50% PPX (*G*), and PDL (*H*). The PPX pancreas possessed few CD45 cells—similar to sham pancreas. PDL pancreas, on the contrary, possessed many more CD45 cells. HO, nuclear staining by Hoechst. *I–K*: Quantitative RT-PCR was performed to check transcription of IL-6 (*I*), IFN-γ (*J*), and TNF (*K*) in control TBR12F and the PTF1a^{cre}TBR12F pancreas. Relative enrichment of the genes from samples was normalized to sham-operated TBR12F mice (equal to 1). No differences in expressions of these three genes were found between control and PTF1a^{cre}TBR12F pancreas after sham treatment, PPX, or PDL. Levels of IL-6, IFN-γ, and TNF mRNA in PDL pancreas were significantly higher (>100-, >30-, and >400-fold, respectively) than in sham and PPX pancreas. D, duodenum; Lg, point of ligation; Lym, lymph node; S, spleen. Scale bars in *A–C'* are 2 mm. Scale bars in *D–F* are 50 μm.

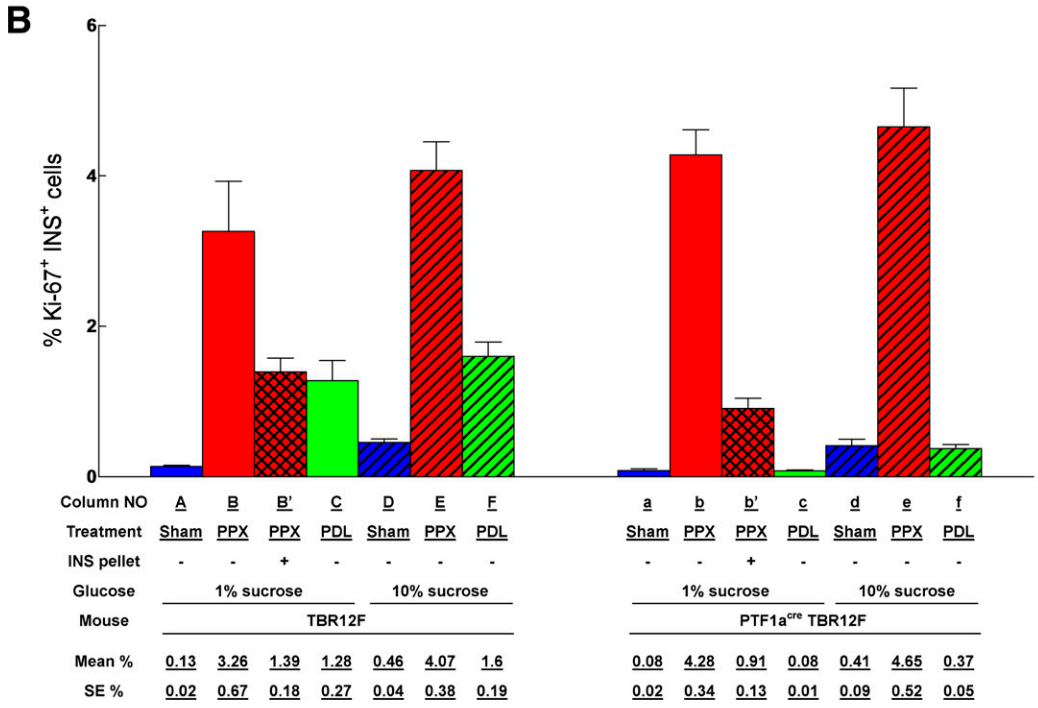




Column NO	A	B	B'	C	D	E	F	a	b	b'	c	d	e	f
Treatment	Sham	PPX	PPX	PDL	Sham	PPX	PDL	Sham	PPX	PPX	PDL	Sham	PPX	PDL
INS pellet	-	-	+	-	-	-	-	-	-	+	-	-	-	-
Glucose	1% sucrose				10% sucrose			1% sucrose				10% sucrose		
Mouse	TBR12F							PTF1a^cre TBR12F						
Mean %	1.31	30.78	13.64	12.14	4.61	39.69	15.55	0.77	41.95	8.80	0.80	3.95	45.07	3.72
SE %	0.14	4.64	1.62	2.14	0.63	1.70	1.12	0.16	1.74	0.91	0.14	0.71	1.75	0.52

FIG. 3. Quantification of β-cell proliferation by 7 days' BrdU incorporation. *A*: BrdU in either 1 or 10% sucrose drinking water was supplied to the control TBR12F and the PTF1a^{cre}TBR12F mice for 1 week (1w) immediately after surgery. Representative BrdU (red) and insulin (INS) (green) staining is shown in sham-operated (*top row*), 50% PPX (*middle row*), and ligated part of PDL (*bottom row*) pancreas. HO, nuclear staining by Hoechst (blue). Scale bars = 50 μm. *B*: BrdU$^+$ β-cell percentage was quantified in each experimental condition (1% sucrose: control sham solid blue, control PPX solid red, control PDL solid green; 10% sucrose, corresponding to the color in control from various treatments but diagonal lines instead of solid; insulin pellet treatment after PPX, cross-hatched red). Significance was considered when $P < 0.05$. $P < 0.05$: A and B, B and C, A and C, B and B', D and E, D and F, E and F, A and D, B and E, a and b, b and c, b and b', d and e, c and f, e and f, a and d, A and a, C and c, B and b, B' and b'. No significance: a and c, d and f.





Column NO	A	B	B'	C	D	E	F	a	b	b'	c	d	e	f
Treatment	Sham	PPX	PPX	PDL	Sham	PPX	PDL	Sham	PPX	PPX	PDL	Sham	PPX	PDL
INS pellet	-	-	+	-	-	-	-	-	-	+	-	-	-	-
Glucose	1% sucrose				10% sucrose			1% sucrose				10% sucrose		
Mouse	TBR12F							PTF1acre TBR12F						
Mean %	0.13	3.26	1.39	1.28	0.46	4.07	1.6	0.08	4.28	0.91	0.08	0.41	4.65	0.37
SE %	0.02	0.67	0.18	0.27	0.04	0.38	0.19	0.02	0.34	0.13	0.01	0.09	0.52	0.05

FIG. 4. Quantification of β-cell proliferation by Ki-67 staining. *A*: Ki-67 (red) and insulin (INS) (green) staining was performed on mice 7 days after surgery, and representative pictures are shown in sham-operated (*top row*), 50% PPX (*middle row*), and the ligated part of the PDL (*bottom row*) pancreas. HO, nuclear staining by Hoechst (blue). Scale bars = 50 μm. *B*: BrdU$^+$ β-cell percentage was quantified in each experimental condition (1% sucrose: control sham solid blue, control PPX solid red, control PDL solid green; 10% sucrose, corresponding to the color in control from various treatments but diagonal lines instead of solid; insulin pellet treatment after PPX, cross-hatched red). Significance was conditioned when $P < 0.05$. $P < 0.05$: A and B, B and C, A and C, B and B', D and E, D and F, E and F, A and D, a and b, b and c, b and b', d and e, c and f, e and f, a and d, A and a, C and c, B' and b'. No significance: a and c, d and f.

sacrifice as an additional confirmation. The difference between the quantification of BrdU$^+$ β cells and Ki-67$^+$ β cells is due to the fact that 7-day BrdU should label all of the β cells that have proliferated in the 7-day period, while Ki-67 should only label the β cells that are within the G1 to M phase of the cell cycle at the time the mice are killed. Because edema in the PDL pancreas can lead to significant overestimation of β-cell mass (36), at the time of death

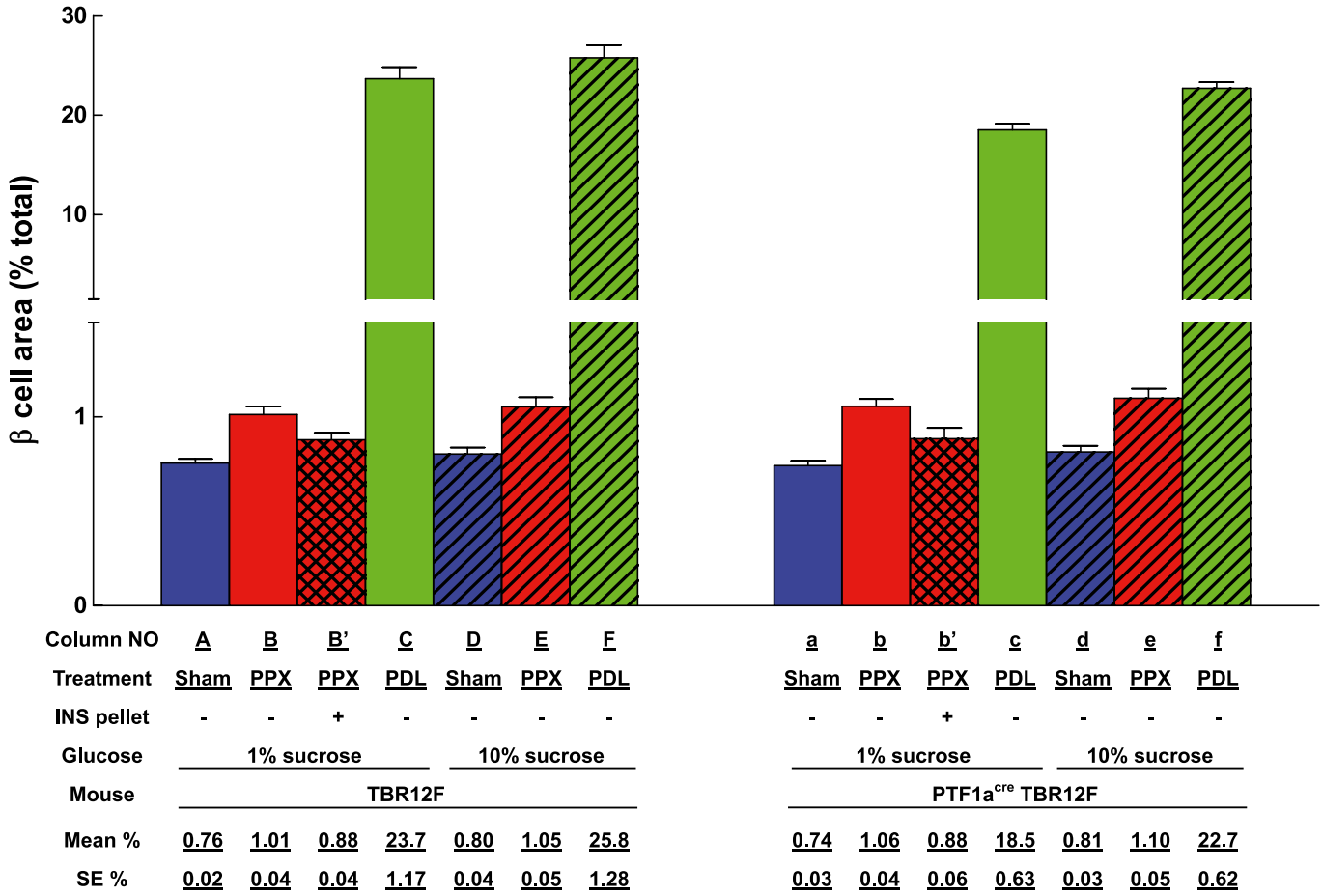


FIG. 5. Quantification of the change in β-cell area. Percentages of β-cell area in total pancreas area were quantified in each experimental condition (1% sucrose: control sham solid blue, control PPX solid red, control PDL solid green; 10% sucrose, corresponding to the color in control from various treatments but diagonal lines instead of solid; insulin pellet treatment after PPX, cross-hatched red) 7 days after surgery. High values in PDL pancreas are due to atrophy of acinar tissues. Comparisons can only be performed within sham and PPX groups or within PDL groups. Significance was conditioned when $P < 0.05$. $P < 0.05$: A and B, B and B', D and E, a and b, b and b', d and e, c and f, C and c. No significance: A and D, C and F, a and d, A and a.

β-cell area, rather than β-cell mass, was measured to detect the overall effect of any change in β-cell proliferation. All of the experiments were performed under normoglycemic conditions, as high or low glucose may have an important effect on β-cell homeostasis (Supplementary Fig. 1*A*). Our data showed that β-cell proliferation in sham-operated control animals was significantly greater than that of sham-operated PTF1a^{cre}TBR12F mice (BrdU: Fig. 3*A* and *B*, column A versus a, $P < 0.05$; Ki-67: Fig. 4*A* and *B*, column A versus a, $P < 0.05$). As the proliferation baseline is very low, this difference in β-cell proliferation did not result in a significant difference in β-cell area (Fig. 5, column A versus a, no significance). Therefore, TGFβ receptor signaling is important for normal, baseline β-cell replication, which is consistent with previous reports (20,25).

Inflammation-induced β-cell proliferation is TGFβ receptor signaling dependent. We then compared β-cell proliferation in animals after PDL (Figs. 3*A* and 4*A*). In control mice, our data showed a significant increase in β-cell proliferation after PDL compared with after sham operation (BrdU: Fig. 3*B*, column C versus A, $P < 0.05$; Ki-67: Fig. 4*B*, column C versus A, $P < 0.05$). However, PDL did not increase β-cell proliferation in PTF1a^{cre}TBR12F mice (BrdU: Fig. 3*B*, column c versus a, no significance; Ki-67: Fig. 4*B*, column c versus a, no significance), resulting in a striking difference in β-cell proliferation between control and PTF1a^{cre}TBR12F (BrdU: Fig. 3*B*, column C versus c, $P < 0.05$; Ki-67: Fig. 4*B*, column C versus c, $P < 0.05$) mice after PDL. Moreover, the significant difference in β-cell proliferation during 7 days resulted in a significant difference in β-cell area (Fig. 5, column C versus c, $P < 0.05$) between control and PTF1a^{cre}TBR12F mice. This result suggests that inflammation-induced β-cell proliferation is TGFβ receptor signaling dependent.

Workload-induced β-cell proliferation is TGFβ receptor signaling independent. Next, we examined β-cell proliferation in animals after 50% PPX (Fig. 3*A*). Here, we saw a dramatic increase in proliferation of β cells over sham-operated mice in both control (BrdU: Fig. 3*B*, column B versus A, $P < 0.05$; Ki-67: Fig. 4*B*, column B versus A, $P < 0.05$) and PTF1a^{cre}TBR12F (BrdU: Fig. 3*B*, column b versus a, $P < 0.05$; Ki-67: Fig. 4*B*, column b versus a, $P < 0.05$) mice, resulting in a significant difference in β-cell area after 7 days (Fig. 5, column B versus A, $P < 0.05$; column b versus a, $P < 0.05$) between sham and PPX in both control and PTF1a^{cre}TBR12F mice. Therefore, inhibition of TGFβ receptor signaling does not prevent the β-cell proliferation induced by increased β-cell workload, suggesting that workload-induced β-cell proliferation is TGFβ signaling independent. Moreover, increased

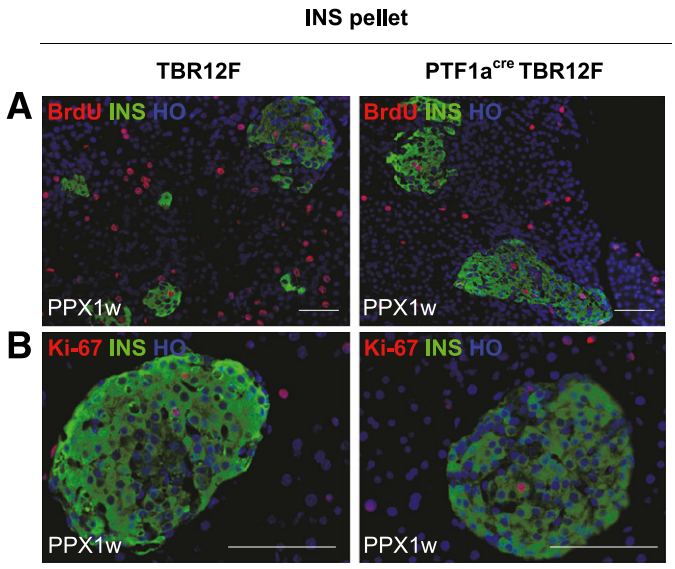


FIG. 6. Exogenous insulin (INS) significantly reduces β-cell proliferation after PPX. Mice received implantation of an insulin pellet at the time of PPX and were provided with 1% sucrose BrdU drinking water. *A*: Representative pictures for BrdU (red) and insulin (INS) (green) staining are shown for both control and PTF1a^{cre}TBR12F pancreas. *B*: Representative pictures for Ki-67 (red) and insulin (green) staining are shown for both control and PTF1a^{cre}TBR12F pancreas. HO, nuclear staining by Hoechst (blue). Scale bars = 50 μm.

workload appears to be a more potent activator of β-cell proliferation than PDL-induced inflammation (BrdU: Fig. 3*B*, column B versus C, $P < 0.05$; Ki-67: Fig. 4*B*, column B versus C, $P < 0.05$). In addition, proliferation after PPX was significantly greater in the PTF1a^{cre}TBR12F mice compared with after PPX in control mice (BrdU: Fig. 3*B*, column b versus B, $P < 0.05$), suggesting an actual suppressive role of TGFβ receptor signaling on workload-induced β-cell proliferation.

High glucose intake partially mimics the effect of 50% PPX. As an alternative to PPX to increase β-cell workload, we provided drinking water containing 10% sucrose to the mice and repeated sham, PPX, and PDL treatments. Because hyperglycemia itself may have secondary effects on pancreatic β cells, we used a dose of sucrose that would maintain normoglycemia. The blood glucose level from the mice receiving 10% sucrose did not exceed 200 mg/dL, which we regarded as a normoglycemic value for adult mice (Supplementary Fig. 1*A*).

We then compared β-cell proliferation in 10% sucrose-fed mice with 1% sucrose-fed mice (Figs. 3*A* and 4*A*). We found a significant increase in proliferation of β cells in both control (BrdU: Fig. 3*B*, column D versus A, $P < 0.05$; Ki-67: Fig. 4*B*, column D versus A, $P < 0.05$) and PTF1a^{cre}TBR12F (BrdU: Fig. 3*B*, column d versus a, $P < 0.05$; Ki-67: Fig. 4*B*, column d versus a, $P < 0.05$) mice with 10% sucrose-fed mice compared with 1% sucrose-fed mice, again suggesting that increased workload indeed induces β-cell proliferation and that this effect does not depend on TGFβ receptor signaling. This finding was further supported by the fact that 10% sucrose feeding enhanced proliferation despite the inhibition of inflammation-induced β-cell proliferation by TGFβ receptor deletion in the PDL mice. Specifically, the PTF1a^{cre}TBR12F mice showed an increase in β-cell proliferation after PDL if given 10% sucrose (BrdU: Fig. 3*B*, column f versus c, $P < 0.05$; Ki-67: Fig. 4*B*, column f versus c, $P < 0.05$), resulting in a significant difference in β-cell area after 7 days (Fig. 5, column f versus c, $P < 0.05$).

Exogenous insulin reduced the mitogenic effect of PPX on β cells. To further confirm whether the mitogenic effect of PPX is due to increased β-cell workload, we implanted a mouse insulin pellet subcutaneously at the time of PPX and supplied the mice with 1% sucrose plus BrdU drinking water. The insulin pellet alone did not cause hypoglycemia in mice (RESEARCH DESIGN AND METHODS and Supplementary Fig. 1*B*). Interestingly, the provision of the exogenous insulin, which presumably decreased β-cell workload, indeed decreased the mitogenic effect of PPX on β-cell proliferation in both control (BrdU: Figs. 6*A* and 3*B*, column B' versus B, $P < 0.05$; Ki-67: Figs. 6*B* and 4*B*, column B' versus B, $P < 0.05$) and PTF1a^{cre}TBR12F (BrdU: Figs. 6*A* and 3*B*, column b' versus b, $P < 0.05$; Ki-67: Figs. 6*B* and 4*B*, column b' versus b, $P < 0.05$) mice,

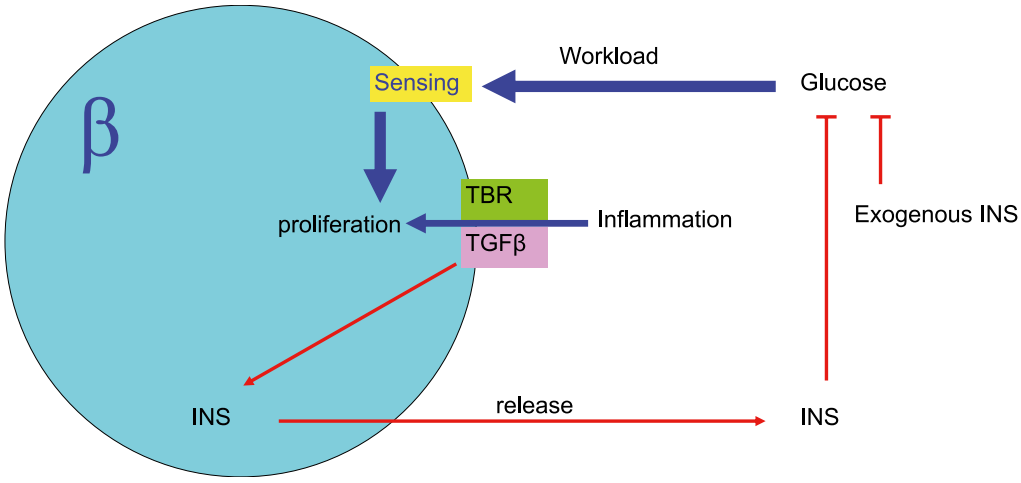


FIG. 7. TGFβ receptor signaling in adult β-cell proliferation. A model of how adult β-cell proliferation is regulated in baseline conditions by inflammation (PDL) and in states of increased workload is proposed. TGFβ receptor signaling is necessary for the normal low rate of β-cell turnover. Inflammation is a modest stimulant for β-cell proliferation and is dependent on TGFβ receptor signaling. As a more potent β-cell proliferation stimulant, β-cell workload is TGFβ receptor signaling independent. TGFβ can also counterbalance the mitogenic effect of increased workload on β cells by inducing insulin (INS) release.

resulting in a significant difference in β-cell area after 7 days (Fig. 5, column B' versus B, $P < 0.05$; column b' versus b, $P < 0.05$) between sham and PPX in both control and PTF1a^{cre}TBR12F mice. Interestingly, implanting an insulin pellet after PPX blunted β-cell proliferation more so in PTF1^{acre}TBR12F mice than in control mice (BrdU: Fig. 3*B*, column b' versus B', $P < 0.05$; Ki-67: Fig. 4*B*, column b' versus B', $P < 0.05$). These data support the conclusions from the previous experiments, suggesting that β-cell workload activates a TGFβ receptor signaling–independent pathway to stimulate β-cell proliferation.

DISCUSSION

Cell replacement therapy for diabetes requires knowledge for understanding how functional β-cell mass is maintained in the adult pancreas. As cell replication is the predominant way for adult β cells to increase their number (4,5), numerous growth factor pathways have been extensively studied for their involvement in β-cell replication. Even though TGFβ signaling was shown to play an important role in pancreas development (15–19,38), pancreatitis-induced fibrosis (14,15,39–41), and pancreatic cancer development (13), its involvement in adult β homeostasis is very poorly understood. In the current study, we generated PTF1a^{cre}TBR12F mice that were double mutant for TBRI and TBRII in the adult pancreas (26,27) and selected two surgical manipulations (PPX and PDL) to study the effect of TGFβ signaling on adult pancreatic β-cell proliferation. Although β-cell toxins like alloxan or streptozotocin (42) have been extensively studied for β-cell destruction and possible regeneration, we took them as much inferior models to study β-cell proliferation compared with PPX and PDL because of the concern that the regeneration of β cells in toxin models is much less robust, as the survived β cells were not healthy anymore after exposure to the toxin. Both PPX and PDL require certain technical skills, and we found only very limited local inflammation in the PPX pancreas and very little degeneration of islets in the ligated PDL pancreas (Fig. 2). Therefore, PPX and PDL represent rather clean models for β-cell proliferation stimulated by inflammation and β-cell proliferation, respectively. We further showed that deletion of TGFβ receptor in the pancreas does not affect the local inflammation level, suggesting that the effect of PDL on β-cell proliferation in PTF1a^{cre}TBR12F mice results from deletion of TGFβ receptor signaling in pancreatic β cells rather than from altered inflammation. Based on these quality controls, we are confident that alterations in β-cell proliferations are due to pancreas-specific TGFβ receptor deletion in PPX and PDL models.

In PTF1a^{cre}TBR12F mice, we found that loss of the TGFβ receptor signaling improved β-cell proliferation after PPX. This enhanced proliferation may be due to loss of a tonic inhibition by TGFβ receptor signaling on the β-cell proliferative response to an increased workload. Interestingly, implantation of an insulin pellet after PPX blunted β-cell proliferation more so in PTF1a^{cre}TBR12F mice than in control mice. Since TGFβ has been shown to stimulate insulin secretion (24,43), the loss of this TGFβ-stimulated insulin secretion can serve as a secondary stimulus for proliferation. Therefore, the blunted proliferation in TGFβ receptor–deleted mice owing to insulin pellets could be the result of both the loss of the increased β-cell workload stimulus and the loss of TGFβ-mediated insulin release, consistent with reports that TGFβ regulates β-cell proliferation in vitro both positively and negatively through different mechanisms (20,21,23–25,43).

PDL has been reported to possibly involve adult β-cell neogenesis from ducts (30). However, subsequent studies provided conflicting results (5,36,44–47). While not the primary focus of our study, we quantified proliferating duct cells across all experimental conditions. We found no significant difference between controls and PTF1a^{cre}TBR12F mice after PDL with or without high glucose intake (Supplementary Fig. 2), suggesting that although there may be neogenesis, it is unaffected by pancreas-specific deletion of TGFβ receptors or high glucose. Similarly, we also found a >100-fold increase in ngn3 mRNA levels after PDL but again found no effect of pancreas-specific deletion of TGFβ receptors or high glucose on this 100-fold increase (Fig. 2*D*). These data suggest that presumed β-cell neogenesis in the adult pancreas after PDL is not affected by pancreas-specific deletion of TGFβ receptor signaling.

Based on our findings in this study and the related literature, we propose a model of how adult β-cell proliferation is regulated by TGFβ receptor signaling at baseline, during inflammation, and under increased workload in a normoglycemic state (Fig. 7). TGFβ receptor signaling appears to be necessary for normal baseline β-cell proliferation. PDL-induced inflammation is a modest β-cell proliferation stimulant and is dependent on TGFβ receptor signaling. On the other hand, β-cell workload is a more potent β-cell proliferation stimulant but is apparently not dependent on TGFβ receptor signaling. In fact, TGFβ may suppress such workload-induced proliferation through promoting insulin release.

Collectively, by using a pancreas-specific TGFβ receptor–deleted mouse model, we have identified two distinct pathways that regulate adult β-cell proliferation. Our study thus provides important information for understanding β-cell proliferation during normal growth and in pancreatic diseases.

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X.X. contributed to the study concept and design, researched data, and wrote the manuscript. J.W., Y.E.-G., and P.G. researched data. K.P., J.P., C.W., and C.S. participated in critical discussions. G.K.G. contributed to the study concept and design, acquired funding, supervised the study, and edited the manuscript. G.K.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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