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A Smad Signaling Network Regulates Islet Cell Proliferation

Pancreatic β-cell loss and dysfunction are critical components of all types of diabetes. Human and rodent β-cells are able to proliferate, and this proliferation is an important defense against the evolution and progression of diabetes. Transforming growth factor-β (TGF-β) signaling has been shown to affect β-cell development, proliferation, and function, but β-cell proliferation is thought to be the only source of new β-cells in the adult. Recently, β-cell dedifferentiation has been shown to be an important contributory mechanism to β-cell failure. In this study, we tie together these two pathways by showing that a network of intracellular TGF-β regulators, smads 7, 2, and 3, control β-cell proliferation after β-cell loss, and specifically, smad7 is necessary for that β-cell proliferation. Importantly, this smad7-mediated proliferation appears to entail passing through a transient, nonpathologic dedifferentiation of β-cells to a pancreatic polypeptide–fold hormone-positive state. TGF-β receptor II appears to be a receptor important for controlling the status of the smad network in β-cells. These studies should help our understanding of properly regulated β-cell replication.

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ISLET STUDIES

New pancreatic β-cells form in response to a loss of β-cell mass, and this formation of new β-cells is thought to be an important defense against the evolution and progression of diabetes (1). β-cell replication (1,2) and neogenesis (3–5) are the two main mechanisms that have been proposed for the formation of new β-cells. β-cell replication is generally accepted as the predominant mechanism for the generation of new β-cells in the adult islet (1), but it remains unclear how such a terminally differentiated cell can undergo proliferation (6). Specifically, although much is known about β-cell cycle regulators and responses to growth factors, very little is known about the molecular mechanics of β-cell proliferation.

Transforming growth factor-β (TGF-β) superfamily signaling has been strongly implicated in pancreatic development and postnatal growth (7,8). TGF-β signaling seems to favor endocrine cell lineage selection and maturation and seems to be antiproliferative (9). TGF-βs, growth differentiation factor 11, and activins have all been closely tied to pancreatic endocrine development (10–12) and are all thought to work through the intracellular mediators smads2 and 3 (13). Specifically, smad2/3 have been associated with pancreatic differentiation toward an endocrine phenotype (14,15). Smad activity can be blocked by inhibitory smad6 or smad7 (16,17). Smad6 is thought to specifically inhibit those

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smads that are canonically downstream of bone morphogenetic protein signaling (i.e., smads 1, 5, and 8) (16). Smad7 seems to be more globally active against all receptor-activated smads (i.e., smads 1, 5, and 8 plus smad2/3) (16,17). Thus, effects specifically attributable to smad7, but not to smad6, are likely due to inhibition of smad2 and/or -3 (15).

In the current study, we examined the role of smad2/3 signaling, downstream of the TGF-β receptor II (TGFβrII), along with their inhibitor, smad7, in β-cell growth after a nondiabetogenic loss of β-cells (60% partial pancreatectomy [PPx]) (18). The phosphorylated (active) forms of smad2 and smad3 (psmad2/3) are strongly present specifically in the nucleus of islet cells, but become rapidly downregulated after PPx, specifically in proliferating cells. Mice with islet-specific smad2 and -3 gene ablation had a more robust proliferative islet cell response after pancreatectomy. Interestingly, many proliferating β-cells rapidly became smad7-positive, but also became insulin-negative, and appeared to become positive for the pancreatic polypeptide (PP)-fold hormones, perhaps representing a specific dedifferentiation of β-cells as part of the proliferative process. Mice with pancreatic or β-cell–specific ablation of smad7 had little β-cell proliferation after pancreatectomy. Such dedifferentiation of β-cells has been recently described as a possible mechanism leading to β-cell failure (19). Thus, TGF-β signaling, and smad7 in particular, may represent a key mechanistic link between normal β-cell proliferation and eventual β-cell failure.

RESEARCH DESIGN AND METHODS

Transgenic Animals

All animal experiments were performed in accordance with guidelines established by the International Animal Care and Use Committee. Smad3-exon2–null mutant mice were obtained from The Jackson Laboratory. Transgenic mice expressing Smad2$^{fx/fx}$ were gifts from Dr. Erwin Bottinger (Mt. Sinai School of Medicine). Transgenic mice expressing TGF-β receptor I (TGFβrI)$^{fx/fx}$ and TGFβrII$^{fx/fx}$ were gifts from Prof. Stefan Karlsson (University of Lund, Lund, Sweden). Rosa LacZ mice (R26R^{lacZ}) have been described previously by Soriano (20) and were obtained from The Jackson Laboratory. Insulin2-cre mouse have been previously described by Postic et al. (21). Glucagon-cre (22) was obtained from the Mutant Mouse Regional Resource Centers. All transgenic mice were crossed with Pdxcre-ER (23) (Mouse Models of Human Cancers Consortium), Ngn3cre (24), and PTF1a-cre (25) (Mouse Models of Human Cancers Consortium).

Tamoxifen Injection

In experiments with the cre-ER/LoxP system, tamoxifen (Sigma-Aldrich, St. Louis, MO) was dissolved at 20 mg/ml in corn oil (Sigma-Aldrich) and administered into adult mice intraperitoneally, 2 mg/40 g/day for 5 days, in order to induce cre recombination, and we then performed PPx 7 days after the first tamoxifen injection. When Pdxcre-ER was crossed with TGFβrI$^{fx/fx}$ or TGFβrII$^{fx/fx}$, PPx was performed 4 weeks after the first tamoxifen injection due to the long half-life of TGFβrI$^{fx/fx}$ and TGFβrII$^{fx/fx}$, with the same 5-day tamoxifen regimen being followed.

PPx

PPx was conducted as described previously (18).

Immunohistochemistry

Insulin guinea pig 1:500 (DakoCytomation, Carpinteria, CA), glucagon rabbit monoclonal 1:2000 (Linco), Glut2 goat-polyclonal 1:50, smad7 rabbit polyclonal IgG 1:50, TGFβrI (ALK5) rabbit polyclonal anti-human 1:75, ALK1 rabbit polyclonal anti-human 1:40 (Santa Cruz Biotechnology, Santa Cruz, CA), somatostatin rat monoclonal 1:100 (LSBio), TGF-β1 ligand mouse monoclonal antibody 1:100 (R&D Systems), TGFβrII rabbit polyclonal antibody 1:100 (Millipore), p27Kip1 mouse monoclonal antibody 1:100 (Thermo Fisher Scientific), PP guinea pig antirat 1:400 (Millipore), neuropeptide Y (NPY) rabbit polyclonal antibody 1:500 (Millipore), peptide YY (PYY) rabbit polyclonal antibody 1:500 (Abcam), PDX-1 goat polyclonal 1:1000 (Abcam), *Dolichos biflorus* agglutinin fluorescein isothiocyanate–conjugated 1:100 (Vector Laboratories, Burlingame, CA), pSmad3 (1:400), pSmad2 (1:50), and pSmad2/3 (1:500) all rabbit polyclonal (Santa Cruz Biotechnology), Ki67 goat polyclonal 1:100 (Santa Cruz Biotechnology), and anti–BrdU rat monoclonal antibody 1:400 (Abcam) were used. Primary antibodies were incubated for 2 h at room temperature or at 4°C overnight. Biotinylated Vectastain ABC kit or AMCA/CY3/FITC fluorescent-conjugated donkey secondary antibodies (Vector Laboratories) were used for 1.5 h at room temperature. Immunoperoxidase was detected by DAB kit (DakoCytomation) or AEC (Sigma-Aldrich), and fluorescently labeled samples were imaged using a fluorescent microscope. Tissue sections were viewed on an upright AxioImager Z1 microscope (Carl Zeiss) or with an inverted Olympus Fluoview-1000 confocal microscope (Olympus) to confocally image the tissue sections. Images were captured with the AxioCamMRc5 and processed using AxioVs40V4.8.2.0 software (Carl Zeiss).

BrdU Incorporation and Cell Counting

Adult mice were injected with BrdU (Sigma-Aldrich) 200 mg/kg intraperitoneally after performing PPx and subsequently once a day for 7 consecutive days. The pancreas was harvested 1 week postsurgery, fixed in 4% paraformaldehyde overnight in 4°C, and then placed in 30% sucrose overnight in 4°C. Antigen retrieval was done on the slides by treating it with 2 M HCl for 35 min followed by overnight incubation with primary antibodies. To quantify the number of BrdU-positive cells per islet in wild-type and transgenic pancreases, the whole

frozen pancreas was sectioned 6-μm thick at −23°C in a cryostat and mounted on gelatin-coated glass microscope slides (Superfrost Plus; Fisherbrand), eight sections per slide. Two random slides were picked, and the average number of BrdU-positive cells per islet was determined over 20 islets using the image tracing software (Stereo Investigator; MBF Bioscience) to count and tag individual BrdU-positive/insulin-positive cells. Using the Stereo Investigator program, individual BrdU-positive cells were tagged and linked to Neurolucida software (MBF Bioscience) to quantify the number of cells.

RESULTS

Smad Expression After 60% PPx

Based on the fact that the smad2/3/7 network seemed to be a potentially important regulator of embryonic pancreatic endocrine development (7,8,12,26,27), we hypothesized that this network may also play a role in islet cell growth post-PPx. We chose a 60% PPx to avoid the hyperglycemia that might have secondary affects on islet cell physiology and behavior (18). Most unperturbed wild-type pancreatic islet cells stained strongly in the nucleus for psmad2/3 (Fig. 1*A*). After PPx, we gave daily injections of BrdU for 1 week. In this study, we saw that many islet cells had become negative for psmad2/3, and many of those psmad2/3–negative islet cells were $BrdU^+$ (Fig. 1*B–D*). Only rarely were psmad2/3^+ cells also $BrdU^+$. One week after PPx, 82 ± 2.5% (± SEM) of psmad2/3–negative endocrine (as defined by a nonendothelial appearance) or psmad2/3 weak cells in islets are $BrdU^+$. This value is compared with 18.2 ± 2.6% of psmad2/3–positive cells being $BrdU^+$. In addition, we noted that there was downregulation within 24 h of PPx of the phosphorylated (active) form of TGFβrII (pTGFβrII) in the islet such that many $insulin^+$ cells that had been $pTGF\beta rII^+$ prior to PPx had become $pTGF\beta rII^-$ (Fig. 1*E* and *F*). To determine whether endogenous smad7 may be mediating the suppression of smad2/3 in these $BrdU^+$ islet cells, we performed immunostaining for smad7. At baseline in 10-week-old pancreas, there was only some ductal expression of smad7 (Supplementary Fig. 1*A* and *B*), but little or no expression in the islets or acinar tissue (Fig. 1*G*) (28). Interestingly, we found that smad7 expression appeared very early after PPx (within 24 h) in a subset of pancreatic islet cells (Fig. 1*H*). A spectrum of smad7 expression was seen in different islets, with some islets having over half of the cells $smad7^+$. Overall, 95.3 ± 0.11% (n = 3) of islets showed $smad7^+$ cells at 1 week post-PPx. In addition, we found that these $smad7^+$ islet cells stained only weakly for p27, a cell-cycle inhibitor (Supplementary Fig. 1*C*). The $smad7^+$ cells also stained for Ki67 and thus had entered the cell cycle (Supplementary Fig. 1*D*). Since both the smad7 and psmad2/3 antibodies were raised in rabbit, we were unable to confirm that the smad7 cells were smad2/3-negative. However, since smad2/3 cells were infrequently proliferative, whereas most $smad7^+$ cells were proliferative, it seems likely that for the most part, the $smad7^+$ cells were psmad2/3–negative. Furthermore, smad7 is a well-established inhibitor of smad2/3. Smad7 expression persisted in some islets for at least 4 weeks post-PPx (Fig. 1*J*). The $smad7^+$ cells were sometimes localized to the outer (mantle) region of the islet, but could also be seen throughout many islets (Fig. 1*H–L*). In order to confirm that this smad7 expression acted as an inhibitor of TGF-β signaling in these islets, we stained for the ligand TGF-β1 (Fig. 1*M* and *O*). Similar to previous reports (29), TGF-β1 was found to be distributed evenly throughout the islet at baseline (Fig. 1*M*). However, 24 h post-PPx, TGF-β1 appeared to accumulate extracellularly in the immediate region of $smad7^+$ cells (Fig. 1*O*, compare with smad7 staining in the immediately adjacent histologic section in Fig. 1*N*). Such an extracellular accumulation of TGF-β ligand in the vicinity of inhibited signal transduction, presumably in part due to smad7 expression in TGF-β target cells, has been described in other TGF-β signaling systems (30).

Characterization of $smad7^+$ Islet Cells Postpancreatectomy

In order to better characterize these $smad7^+$ cells in the islet post-PPx, we performed confocal triple immunofluorescence for smad7 and various islet cell–specific proteins. $Smad7^+$ cells never costained with glucagon, insulin, PDX-1, somatostatin, nor Glut2 (Fig. 2*A–E*). However, surprisingly, essentially all of the $smad7^+$ cells stained positive for PP (Fig. 2*F–H*). In addition, the three members of the PP-fold group of peptides (PP, PYY, and NPY) have together been implicated as markers of immature or, perhaps in this case, dedifferentiated pancreatic endocrine cells (22,31–33). For PYY, there were occasional PYY^+/PP^- cells (Fig. 2*I–K*). When we stained for these three peptides (PP, PYY, and NPY), along with smad7, we noted that there was essentially complete overlap of expression of smad7 with both PP and NPY (Fig. 2*L–N*). PP staining has caused some controversy in the past (32,34) because of the possible cross-reactivity with NPY and PYY, although the newer antibodies are purported to be more specific. Thus, we performed preincubations of the antibodies either with their cognate peptide or with the other two peptides. For all three antibodies, preincubation with its own cognate peptide blocked staining, whereas preincubation with the other two peptides had no effect on staining, thus implying that there was not significant cross-reactivity of these newer antibodies (Supplementary Figs. 2 and 3). We also have recently published successful staining for PP in embryonic and early postnatal pancreas (28).

$Smad7^+$ Cells Represent Dedifferentiated β-Cells

We hypothesized that these $smad7^+$ cells may be β-cells that recently turned off insulin, even within 24 h post-PPx. This possibility would be consistent with lineage-tagging studies that found that new β-cells during regeneration

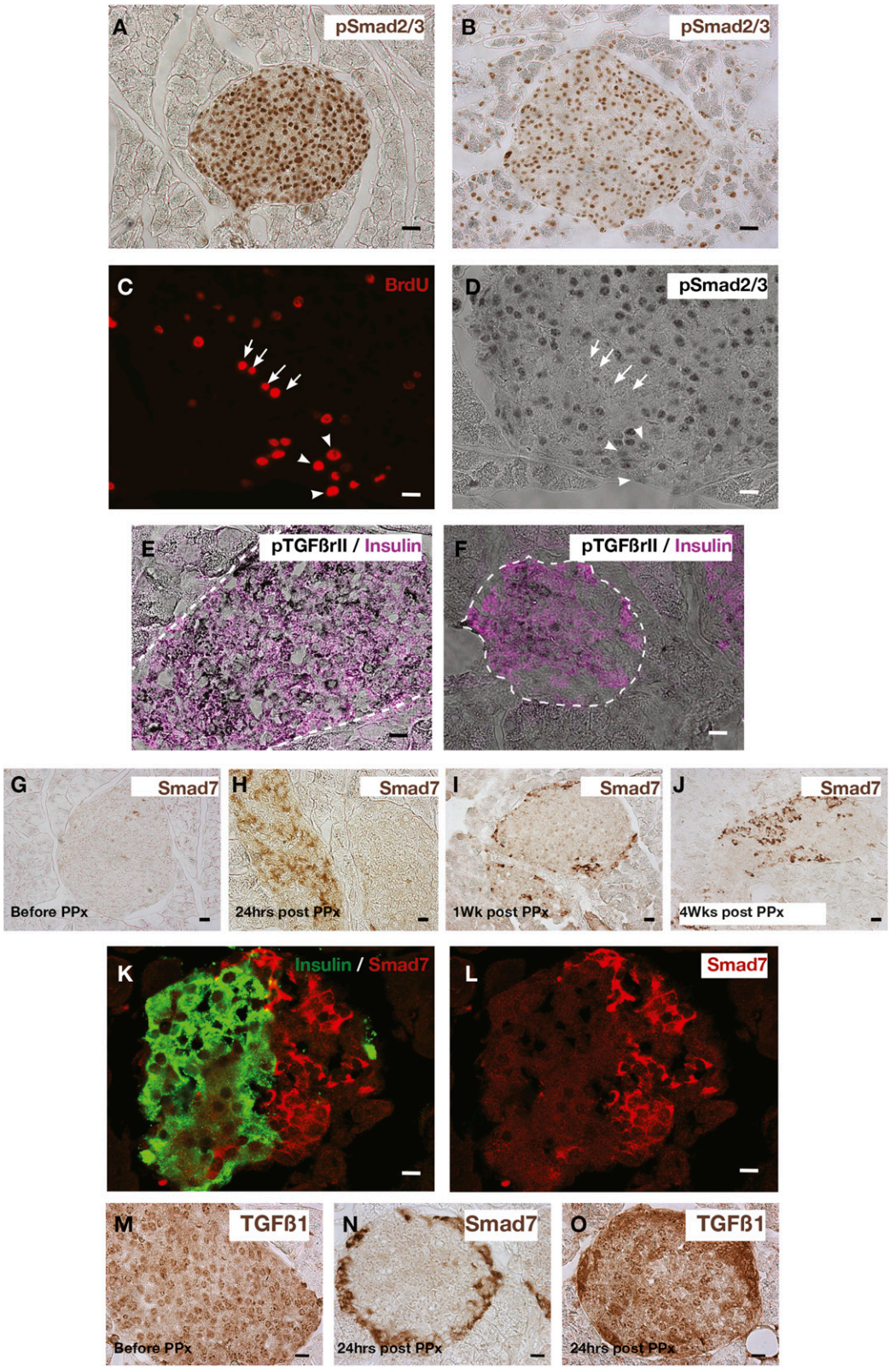


Figure 1—Expression pattern of psmad2/3, smad7, TGF-β1 ligand, and pTGFβrII in an adult pancreas. *A*: pSmad2/3 expression in a normal adult islet showing that most islet cells have nuclear staining. *B*: Expression of pSmad2/3 post-PPx, in which some cells appear to have become psmad2/3–negative. *C* and *D*: After PPx, many of the cells that have switched off (arrows) or only weakly express (arrowheads) psmad2/3 are $BrdU^{+}$. *E*: pTGFβrII (the active form of the receptor) is present throughout the adult islet at baseline (islet outlined by dotted line). *F*: At 24 h post-PPx, pTGFβrII is absent in many of the islet cells (islet outlined by dotted line). *G*–*J*: Smad7

derive only from pre-existing β-cells (1,2), but might suggest that in order for a highly differentiated mature cell such as a β-cell to undergo cell division, it may have to temporarily dedifferentiate. To test whether the smad7$^+$ cells represent recently insulin$^+$ cells that turned off insulin, we performed a lineage-tracing strategy in the PPx model to trace the origin of these smad7$^+$cells. We performed PPx on mice that were double-positive for Ins2-Cre and R26R-lsl-tomato-red reporter (21,35). The reporter activation frequency of Ins2-cre in β-cells has been reported to be 88% (19). Pancreatectomy was performed in 10-week-old mice and the pancreas harvested 1 week later (Fig. 3*A–D*). In the Ins2-Cre;R26R-lsl-tomato red mice, these insulin-negative cells would be lineage-tagged and still be tomato-red$^+$. We found 1 week post-PPx that the smad7$^+$ cells were again insulin-negative as seen earlier, but importantly, smad7$^+$ cells were tomato red–positive in Ins2-cre;R26R-lsl-tomato mice (Fig. 3*A–D*). These results strongly suggest that the smad7$^+$ cells had recently been positive for insulin (Fig. 3*A–D*). Thus, presumably as a result of PPx, some insulin$^+$ cells turned off insulin and became positive for smad7, and, based on the fact that all or most of the smad7$^+$ cells are PP$^+$, the former insulin-positive cells also turned on PP. To investigate this latter point, we stained for PP using Ins2-Cre;R26R-lsl-tomato red mice and harvested 1 week post-PPx. There we also found PP$^+$ staining with tomato red cells, indicating that insulin$^+$ β-cells appear to have turned into PP$^+$ cells (Fig. 3*E–H*).

Recently, glucagon$^+$ α-cells have been identified as a potential source of new β-cells in the islet in models of severe β-cell injury (5). To test whether smad7$^+$ cells may have derived from α-cells that recently turned off glucagon, we crossed a glucagon-cre mouse (22) with an R26R-lsl-tomato red reporter mouse strain (22,36). One week post-PPx in these glucagon-cre;R26R-lsl-tomato mice, we did not see any tomato-labeled smad7$^+$ cells, implying that the smad7$^+$ cells in the islet did not derive from α-cells that had turned off glucagon expression (Fig. 3*I*).

Enhanced Islet Cell Proliferation After PPx in smad2- and/or smad3-Null Mice

In order to determine whether suppression of psmad2 and psmad3 plays a key role in islet cell proliferation post-PPx, we obtained smad2-conditional (Smad2$^{fx/fx}$) (37) and smad3 global mutant (exon 2 deletion) mice (38). Smad3$^{-/-}$ (exon 2) global null mutant mice are viable and fertile (38), and to our knowledge, no smad3 conditional-mutant mice exist. Smad2$^{fx/fx}$ mice were crossed with a pdx1-cre-ERT mouse to create tamoxifen-inducible smad2 conditional mutants for the pancreas. We then performed PPx 1 week after tamoxifen treatment in pdx1-cre-ERT;smad2$^{fx/fx}$ mice, smad3$^{-/-}$ global null mutant mice, and double smad2/3 mutant mice. For the pdx1-cre-ERT;smad2$^{fx/fx}$ mice, 1 week post-PPx, there was a 50% increase in the number of BrdU-labeled islet cells (Fig. 4*A–D*, quantification in Fig. 5*G*). In smad3$^{-/-}$ mutants, there was a tripling of the number of BrdU$^+$ islet cells (Figs. 4*E* and *F* and 5*G*). At baseline in the smad2 mutants, there was a slightly increased proliferation rate compared with littermate controls (compare Fig. 4*A* and *D* and pdx-cre-ERT; smad2$^{fx/fx}$ sham in Fig. 5*G*). Interestingly, in the smad3$^{-/-}$ null mutant mice, although not quantified, we noticed that there was near-absent proliferation of non-islet pancreatic tissue (Fig. 4*F*). For double smad2/3 mutant mice, when crossed with a PTF1a-cre strain (25), it showed an enhanced islet proliferation rate similar to the smad3 mutants, but with no additive effect, thus suggesting that smad2 may somehow work via smad3, explaining the greater effect of smad3 mutation (28) on islet cell replication (Figs. 4*G* and 5*G*).

Smad7 Is Necessary for Islet Cell Proliferation After PPx

While smad7 appeared to be a potential marker of proliferative, dedifferentiated, insulin-negative β-cells, we hypothesized that smad7 expression may also be necessary for proliferation of these β-cells. To test this possibility, we generated a smad7$^{fx/fx}$ knockin mouse. These mice were created with loxP-sites flanking exon5 (the TGF-β receptor–interacting domain) of the smad7 locus (Supplementary Fig. 4). We then conditionally deleted smad7 in the pancreas by crossing the smad7$^{fx/fx}$ mice with three different cre strains: pdx1-cre-ERT, PTF1a-cre, and ngn3-cre (23–25). Again, PPx was performed in these mice, and in the case of the pdx1-cre-ERT mice, tamoxifen was given daily for 5 days, starting 1 week prior to pancreatectomy. In all three crosses, there was essentially complete blockage of pancreatectomy-induced islet proliferation, with no difference seen in the number of BrdU$^+$ islet cells among any of these three strains post-PPx compared with sham-operated cre-negative littermates (Fig. 5*A–C* and *G*), thus confirming that smad7 expression in islet cells is necessary for proliferation in response to a β-cell loss. The ngn3-cre cross showed the lowest overall proliferative rate. This result may reflect that ngn3-cre leads to a more effective deletion of both smad7 alleles in β-cell progenitors. Interestingly, in the PTF1a-cre;smad7$^{fx/fx}$ animals, there was very little proliferation seen throughout the entire pancreas, including the exocrine tissue (see Fig. 5*A*, with a heavily BrdU$^+$

expression pattern and time course, where Smad7 is not expressed in islets baseline, but becomes rapidly upregulated in islets within 24 h of performing a PPx, and expression persists in islets even 4 weeks after surgery. *K* and *L*: Many islet cells 1 week after PPx express smad7, and these cells are insulin-negative. *M*: Baseline expression of TGF-β1 ligand in an unperturbed adult islet. *N*: Smad7 turns on in some cells within 24 h of PPx; this mantle distribution of smad7 is frequently seen, but many islets have a more diffuse distribution of smad7 expression, as in *H–J*. *O*: Smad7 expression correlates with extracellular TGF-β1 ligand accumulation in the area of the islet where smad7 is acutely upregulated. *N* and *O* are consecutive histologic sections. Scale bars, *A* and *B*: 50 μm; *C–F* and *J–N*: 10 μm; *G–I*: 20 μm.

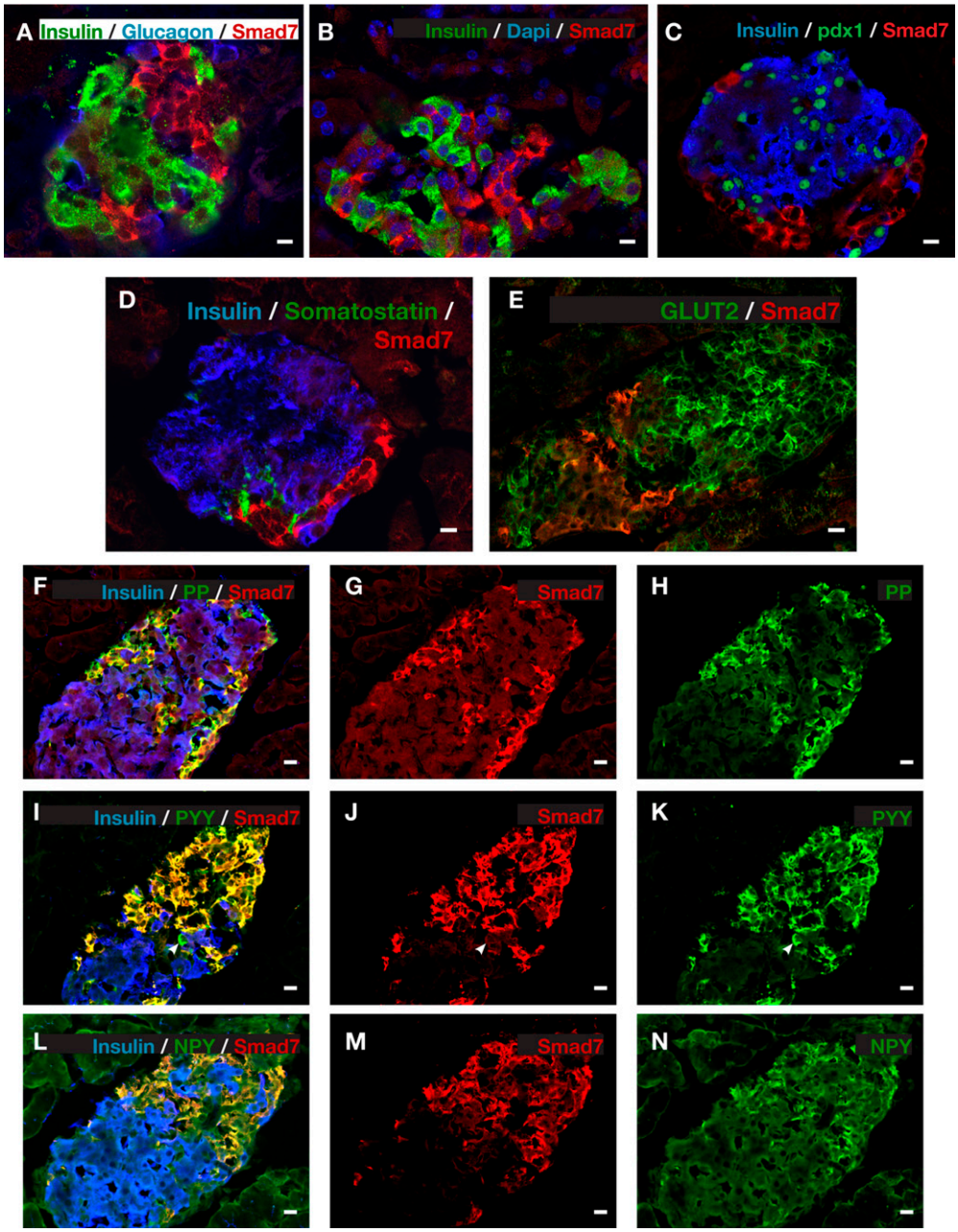


Figure 2—Characterization of smad7-positive cells. Smad7 does not colocalize with glucagon (*A*), insulin (*B*), pdx1 (*C*), somatostatin (*D*), or GLUT2 (*E*). *F–H*: However, smad7 does colocalize uniformly with PP. *I–N*: These PP^+ cells also stain for PYY and NPY, although rare cells are $PYY^+/Smad7^-$ (arrowheads in *I–K*). Scale bars, *A–E*: 10 μm; *F–N*: 20 μm.

lymph node as an internal positive control). However, we noticed that some islets in the PTF1a-cre;$smad7^{fx/fx}$ mice did have several proliferative cells. We suspected that there may be some mosaicism of cre recombination for PTF1a-cre mice, especially in the islets, so we crossed these mice with the R26R-lsl-lacZ conditional reporter mice (20) (Fig. 5*D–F*). We chose this reporter in this study rather than the tomato red reporter used earlier because the floxed sequence of the R26R-lsl-LacZ mouse is closer in size to the floxed smad7 segment. One week post-PPx in these PTF1a-cre;$smad7^{fx/fx}$;R26R-lsl-lacZ mice, we again saw a few islets that had several $BrdU^+$ cells, but essentially all of the $BrdU^+$ cells in the islet were β-gal–negative and therefore were either not of the PTF1a lineage (e.g., endothelial cells) or were not cre lineage-tagged due to mosaicism (Fig. 5*D–F*, quantified in Fig. 5*H*). These data suggest that the effect of smad7 deletion in inhibiting islet cell proliferation is cell autonomous (i.e., a given islet cell with smad7 deletion is less likely to divide than an adjacent islet cell with smad7 intact).

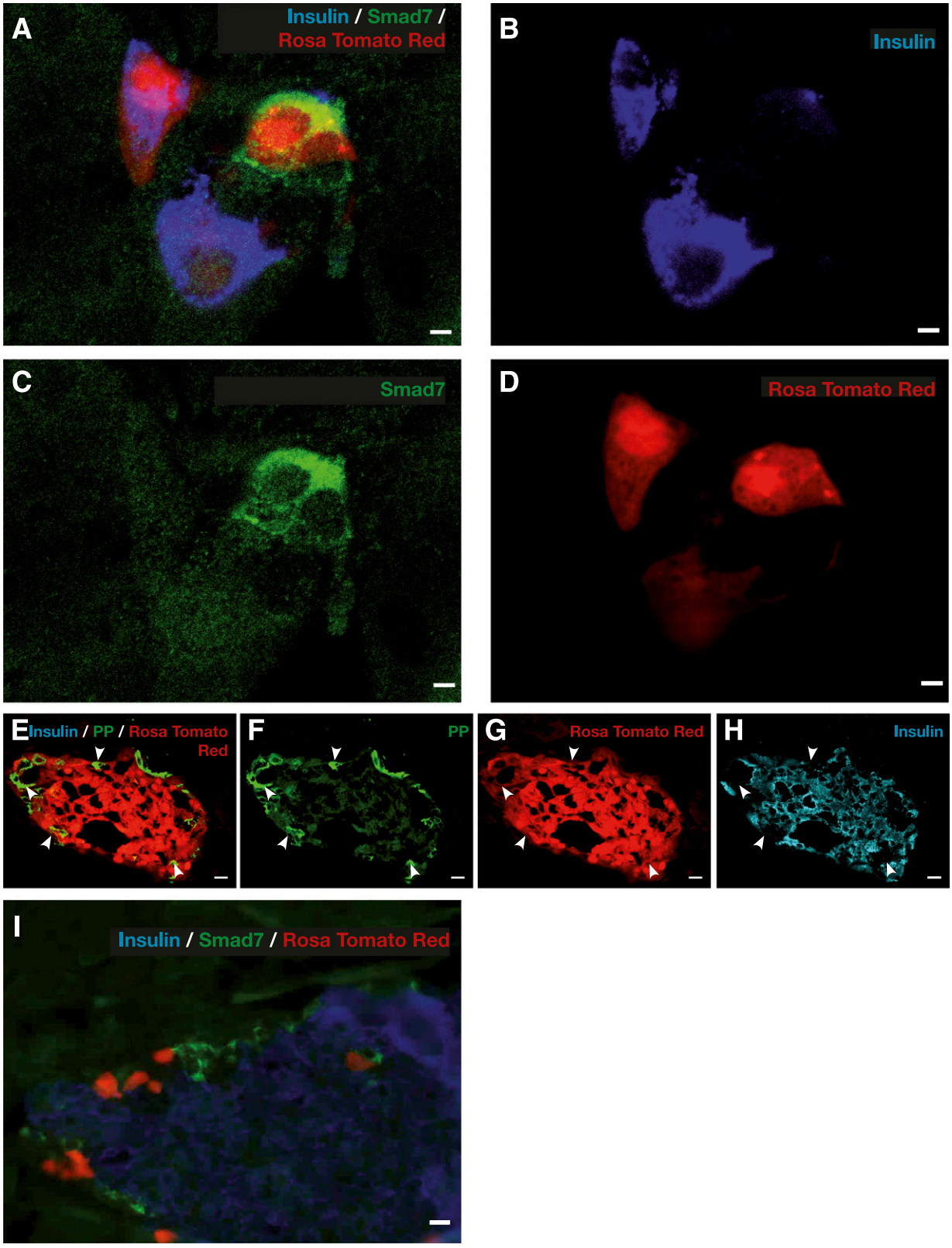


Figure 3—Origin of the smad7$^+$ cells. *A–D*: Confocal images showing lineage-tagging studies using Ins2-cre;R26R-lsl-tomato red mice reveal that these smad7$^+$ cells (*C*) derived from insulin$^+$ cells that are now insulin-negative after PPx, but remain tomato red–positive, indicating their derivation from insulin$^+$ cells (*D*). *E–H*: PP-positive cells (arrowheads) costaining with tomato red cells in Ins2-cre;R26R-lsl-tomato red mouse islet harvested 1 week after PPx. *I*: Smad7$^+$ cells do not originate from glucagon-positive cells as determined by using a glucagon-cre;R26R-lsl-tomato reporter mouse after PPx. Scale bars, *A–D*: 2 μm; *E–I*: 20 μm.

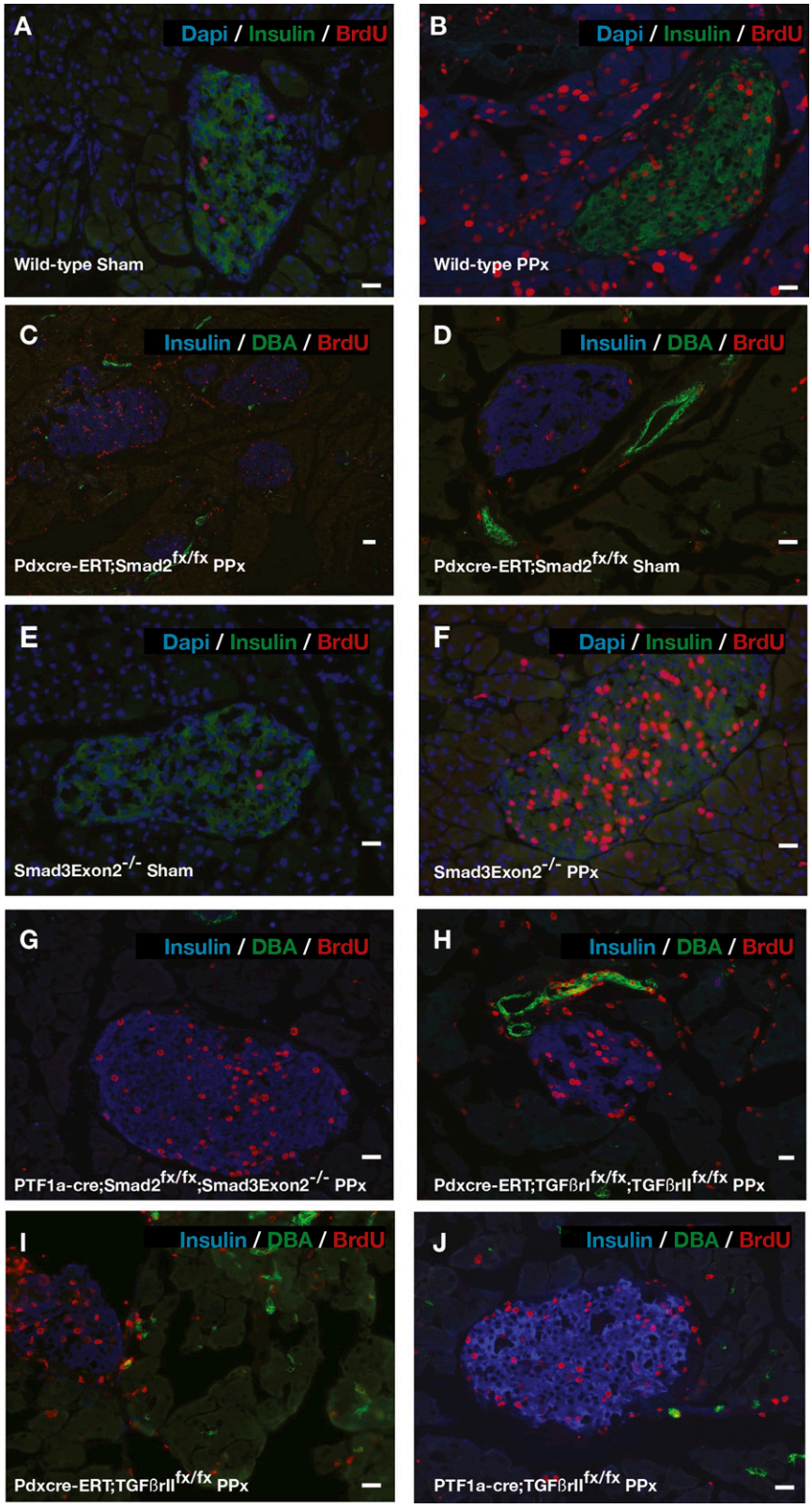


Figure 4—Islet proliferation analysis in wild-type, smad2, smad3, and TGF-β receptor mutant mice (see Fig. 5*G* for quantification). *A*: Minimal proliferation (BrdU uptake) in a wild-type islet without pancreatectomy. *B*: Enhanced proliferation in a wild-type islet 1 week after PPx. *C*: Further enhanced islet proliferation in pdx1-cre-ERT;smad2$^{fx/fx}$ pancreas 1 week after PPx. *D*: At baseline (without pancreatectomy), pdx1-cre-ERT;smad2$^{fx/fx}$ sham islets have slightly higher proliferation compared with either wild-type sham islets (*A*) or cre-littermates (not shown). Minimal proliferation in a smad3exon2$^{-/-}$ islet without pancreatectomy (*E*) compared with marked islet

TGFβrII Mediates Suppression of Pancreatic Islet Proliferation

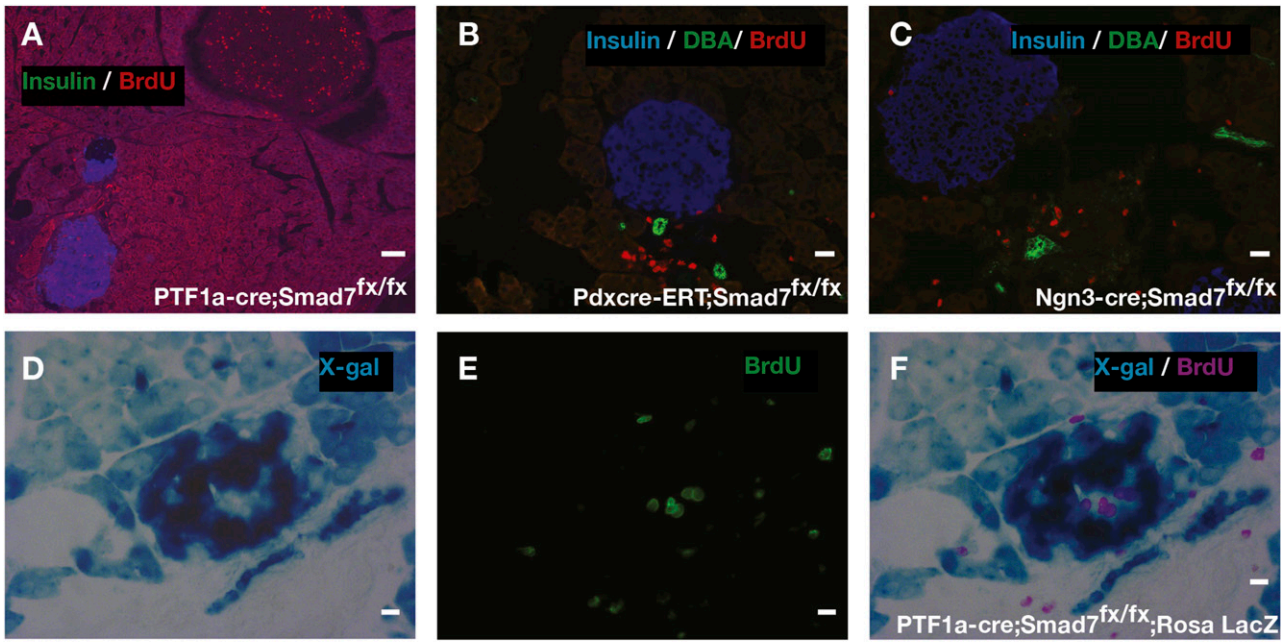
Although smad2/3 were found to be important potential regulators of islet proliferation post-PPx (Fig. 4), multiple TGF-β superfamily ligands and receptors can work through smad2/3. As described above, we noted that the TGF-β1 ligand and activated TGFβrII staining pattern was rapidly altered in wild-type islets post-PPx, suggesting that downregulation of signaling specifically through TGFβrII may permit islet proliferation (Fig. 1*E* and *F*, *M* and *O*). In order to test this possibility, we performed PPx in mice with conditional inactivation of the TGFβrI (TGFβrI$^{fx/fx}$, also called Alk-5, the canonical binding partner with TGFβrII) and/or (TGFβrII$^{fx/fx}$) (39,40). These receptor-floxed mice were crossed with either PTF1a-cre mice or pdx1-cre-ERT mice. One week after PPx, we found that mice with TGFβrII ablation (PTF1a-cre;TGFβrII$^{fx/fx}$ or pdx1-cre-ERT;TGFβrII$^{fx/fx}$) had elevated numbers of BrdU^{+} islet cells, in a range similar to islets after PPx in smad2 and/or smad3 mutant mice (Fig. 4*I* and *J*, quantification in Fig. 5*G*). Mice with ablation of only TGFβrI (PTF1a-cre;TGFβrI$^{fx/fx}$ or pdx1-cre-ERT2;TGFβrI$^{fx/fx}$) had no difference in proliferation compared with controls (not shown), and there was no additive increased proliferation in the TGFβrII-ablated mice when additionally crossed to make a double TGFβrI/TGFβrII mutant mouse (PTF1a-cre;TGFβrI$^{fx/fx}$;TGFβrII$^{fx/fx}$ or pdx1-cre-ERT2;TGFβrI$^{fx/fx}$;TGFβrII$^{fx/fx}$) (Fig. 4*H*). These results suggest that TGFβrII is a key suppressor of islet cell proliferation after PPx, likely through smad2 and smad3 phosphorylation. Interestingly, in this study, we found that increased proliferation in the pdx-cre-ERT2;TGFβrII$^{fx/fx}$ mice required that the tamoxifen be given at least 1 month prior to PPx. If the tamoxifen was given just 1 week ahead, as in the floxed smad mice, we saw no increase in proliferation. This requirement may reflect a relatively long half-life of the TGFβrII in the islet cells.

DISCUSSION

The evidence that new adult β-cells only originate from pre-existing β-cells is fairly strong (1,2), but a general tenet of cell biology is that mature, highly differentiated cells do not readily divide. Controversy exists as to whether β-cells are able to proliferate while in a fully differentiated, insulin-expressing state (39,41–43). While mature β-cells have been shown to directly undergo cell division in certain systems (44), it remains unclear whether such an event is part of a normal physiologic program. In addition, a dedifferentiation program in β-cells, resulting in an empty β-cell, has been recently shown to be associated with β-cell failure (19). In this study, we found that a rapid and transient dedifferentiation and proliferation of β-cells occurs after a loss of β-cell mass. These proliferative cells are marked by and dependent upon expression of smad7. These smad7^{+} proliferative islet cells may mirror the insulin-negative empty β-cells described by Talchai et al. (19).

Our findings implicate smads2/3/7 in regulating the proliferative state of β-cells after a loss of β-cells. TGF-β superfamily signaling has been shown to play an important role in regulating many developmental and physiologic processes in the pancreas and in the β-cell (8). Others have shown a specific role for TGF-β superfamily signaling in the developing pancreas (7,8,11,12). We previously found that TGFβrII-mediated signals to the embryonic ductal structures suppress the recruitment of endocrine progenitors and suppress their proliferation (12). The canonical downstream effectors of TGFβrII are smads 2 and 3 (30). Others have shown that smads 2 and 3 are potential regulators of transdifferentiation of a duct cell line (AR42J cells) into β-cells in vitro (26,45), though these smad-mediated effects were thought to be representative of activin-ligand activity rather than TGF-βs. In keeping with our results, a previous report showed that heterozygous smad2 global-null mutant embryos (smad2$^{+/-}$) have increased numbers of ngn3^{+} progenitor cells (10) and increased numbers of nkx2.2^{+} and nkx6.1^{+} progenitor cells (10,46). Bonner-Weir et al. (47) showed that TGF-β ligand localizes to the periductal region, and they proposed that TGF-β may act as a break on ductal proliferation post-PPx. In contrast with our findings of a fourfold increase in baseline islet cell proliferation in pdx1-cre-ERT;smad2$^{fx/fx}$ islets 1 week post–tamoxifen treatment, an adult mouse with pancreas-specific (pdx1-cre, not tamoxifen-inducible) ablation of smad2 was found to have poor insulin secretion and poor β-cell proliferation (48). These differences may well reflect the ablation of smad2 beginning during the embryonic period versus acutely before the PPx. The 1.5- to threefold increase in islet cell proliferation that we saw post-PPx in the smad2 and/or smad3 mutant mice supports a role for either TGF-β or activin signaling in suppressing β-cell proliferation, since both TGF-β and activin work through smads 2 and 3. Collectively, our data showing the rapid suppression of phosphorylated TGFβrII (Fig. 1*E* and *F*), the rapid accumulation of TGF-β ligand in the vicinity of smad7 expression (Fig. 1*M–O*), and enhanced islet proliferation in the TGFβrII mutant mice (Figs. 4*H–J* and 5*G*) all suggest that it is specifically TGF-β signaling, rather

hyperproliferation 1 week after PPx (*F*) in smad3exon2$^{-/-}$ mice. *G*: Enhanced proliferation in islets of PTF1a-cre;smad2$^{fx/fx}$;smad3exon2$^{-/-}$ mice (pancreatic smad2/3 double-knockout mice) 1 week after PPx. *H*: Enhanced proliferation in islets of double TGFβrI/TGFβrII receptor mutation (pdx1-cre-ERT;TGFβrI$^{fx/fx}$;TGFβrII$^{fx/fx}$) 1 week after pancreatectomy. *I*: Enhanced proliferation in islets of TGFβrII single-mutant mice (pdx1-cre-ERT;TGFβrII$^{fx/fx}$) 1 week after pancreatectomy. *J*: Enhanced proliferation in islets of TGFβrII single-mutant mouse with gene deletion in all pancreatic cells (PTF1a-cre;TGFβrII$^{fx/fx}$). Scale bars, *A*, *B*, and *D–J*: 20 μm; *C*: 50 μm.



G

Different Transgenic & Wild-type Mice	Avg. No. of BrdU$^+$ Cells / Islet (>100 μm in diameter)
C57B6	20.0 ± 4.1
Smad3 exon 2	60.0 ± 5.1 *
Pdx-cre-ERT ; Smad2$^{fx/fx}$	32.6 ± 2.9 **
PTF1a-cre ; Smad2$^{fx/fx}$	31.6 ± 2.0 **
PTF1a-cre ; Smad2$^{fx/fx}$; Smad3 exon 2	60.1 ± 2.7 *
Pdx-cre-ERT ; TGFβrII$^{fx/fx}$	49.9 ±4.3 ***
PTF1a-cre ; TGFβrII$^{fx/fx}$	56.0 ± 4.0 *
Pdx-cre-ERT ; TGFβrI$^{fx/fx}$; TGFβrII$^{fx/fx}$	51.0 ± 3.4 **
PTF1a-cre ; TGFβrI$^{fx/fx}$; TGFβrII$^{fx/fx}$	60.7 ± 6.6 ***
Ngn3-cre ; Smad7$^{fx/fx}$	0.8 ± 0.2 ***
Pdx-cre-ERT ; Smad7$^{fx/fx}$	1.4 ± 0.3 ****
PTF1a-cre ; Smad7$^{fx/fx}$	1.0 ± 0.1 ***
Pdx-cre-ERT ; Smad2$^{fx/fx}$ (Sham)	4.5 ± 0.6 ****
C57B6 (Sham)	1.2 ± 0.4

H

	No. of White Cells : BrdU$^+$ in 20 random Islets	No. of Blue Cells : BrdU$^+$ in 20 random Islets
PTF1a-cre ; Smad7$^{fx/fx}$; Rosa LacZ Islets	124 : 102	2657 : 18
Percentage with Cells that are BrdU$^+$	82.3%	0.68%

Figure 5—Islet proliferation in smad7 conditional-mutant mice. Minimal islet proliferation is seen 1 week after PPx in PTF1a-cre;smad7$^{fx/fx}$ (*A*), pdxcre-ERT;smad7$^{fx/fx}$ (*B*), and ngn3-cre;smad7$^{fx/fx}$ islets (*C*). Lymph node in *A* demonstrated a positive internal control for proliferation, and periductal proliferation was observed in *B* and *C*. *D–F*: PTF1a-cre;smad7$^{fx/fx}$;R26R-lsl-lacZ pancreas, harvested 1 week after PPx, revealed mosaicism of cre lineage labeling in some islets. In this study, smad7$^+$ cells that were not recombined with PTF1a-cre, seen as pale (lacZ$^-$) cells in the islet, were often proliferating (quantified in *H*). This mosaicism confirms the relatively poor penetrance of the PTF1a-cre in endocrine cells postnatally (C. Murtaugh, personal communication). *G*: Islet proliferation quantification for pancreases harvested from the indicated transgenic and wild-type mice 1 week after they had undergone 60% PPx (or sham operation as indicated for some, at least three different mice in each group ± SEM). Compared with proliferation in C57B6 islets 1 week post-PPx, there was a significant increase in proliferation in all of the different transgenic mice that have been harvested 1 week post-PPx (*P < 0.005, **P = 0.04, ***P < 0.01). Conversely, there was a significant decrease in proliferation in the different transgenic mice crossed with smad7$^{fx/fx}$ mice (****P = 0.01). *H*: Islet proliferation analysis of islets in PTF1a-cre;smad7$^{fx/fx}$;R26R-lsl-lacZ mice. BrdU$^+$ islet cells are predominantly cells that have escaped recombination with PTF1a-cre (i.e., white, lacZ$^-$ islet cells) and were likely still able to express smad7, whereas those islet cells that did undergo recombination with PTF1a-cre (i.e., blue cells) were predominantly BrdU-negative and unable to express smad7. Scale bars, *A*: 50 μm; *B* and *C*: 20 μm; *D–F*: 10 μm.

than activin signaling, that mitigates islet cell proliferation post-PPx.

Our results also show an important regulatory role for smad7, and not smad6, in endocrine differentiation and proliferation. Importantly, when smad6 was expressed under the pdx1 promoter, no pancreatic phenotype was seen (14). Smad7, however, when ectopically expressed under the pdx1 promoter, led to an increase in α-cells,

but a decrease in β-cells at the time of birth by 80–90% (15). The authors ascribed this phenotype to an inability of endocrine progenitor cells to mature.

We found that normal endogenous smad7 expression was associated with dedifferentiation of β-cells in the islet within 24 h of PPx and that this smad7 expression was necessary for β-cell proliferation. We feel that this phenomenon is not simply degranulation of β-cells, since the cells also begin to express PP-fold peptides. Along similar lines, β-cell dedifferentiation accompanied by the onset of expression of other peptide hormones, including PP, has recently been described in a model of β-cell failure (19). The authors attributed the dedifferentiation to β-cell exhaustion due to loss of FoxO1 in β-cells. There, some β-cells dedifferentiated into α-cells. We recently published that different injury models (pancreatic duct ligation vs. PPx) will result in different types of regenerative responses (49), which could explain why in our PPx model we did not observe β-cell dedifferentiation to α-cells. In our PPx model, however, the dedifferentiation appears to be part of a β-cell self-duplication process, with a dedifferentiation and then redifferentiation. The expression of PP-fold peptides in these dedifferentiated β-cells may reflect the established developmental lineage relationship between β-cells and PP cells (31–33).

Although we have not shown directly that these dedifferentiated smad7$^+$ cells then redifferentiate after proliferation, logic would suggest that such a redifferentiation must occur. Specifically, since we saw multiple BrdU$^+$/insulin$^+$ cells 1 week post-PPx, but little or no BrdU$^+$ cells in the smad7-ablated islets 1 week post-PPx (Fig. 5*A–C*), it would strongly suggest that the source of these insulin$^+$/BrdU$^+$ cells is β-cells that became insulin-negative/smad7$^+$, then divided (became BrdU$^+$), and then turned off smad7 expression and re-expressed insulin. An alternative explanation could be that the smad7$^+$ cells instead influence other cells in the islet to divide through a paracrine, non–cell-autonomous mechanism. However, we feel that such a non–cell-autonomous mechanism is less likely since in the PTF1a-cre;smad7$^{fx/fx}$;R26R-lsl-lacZ islets that showed a mosaic penetration of cre recombination in islet cells, non–cre-recombined cells were found to be the predominant cells that were BrdU$^+$ (Fig. 5*D–F* and *H*). Thus, the absence of smad7 in the majority of the islet cells did not prevent proliferation of those few cells that did not cre-recombine. Although we found smad7$^+$ cells to be present in ~95% of islets post-PPx, there was a large interislet variability within the same pancreas in terms of their smad7 expression pattern (Fig. 1*H–K* and *N*). A similar variability was observed by Talchai et al. (19) in the different patterns of insulin immunoreactivity or insulin-negative cells in different islets.

We found that smad7$^+$ dedifferentiated islet cells were derived from insulin$^+$ cells. In addition, these dedifferentiated β-cells had become positive for the PP-fold family of molecules (PP, NPY, and PYY). The expression of these PP-fold molecules in the smad7$^+$ cells suggests that the β-cells have dedifferentiated along a developmental cell lineage pathway in which PP-fold–expressing cells represent a more immature cell prior to redifferentiation to form β-cells. PP-fold peptides have been shown to be expressed in early embryonic endocrine cells and have been found to be frequently coexpressed with other islet hormones in the early embryo, but not late in gestation (31,32,34). Using cell ablation strategies, PP-fold–expressing cells were found to be uniquely necessary for the formation of β-cells and δ-cells, again consistent with our findings that β-cells, and possibly δ-cells, but not α-cells, appeared to have dedifferentiated to form smad7$^+$, PP-fold–expressing cells (33,50). Furthermore, using conditional cell lineage-tagging strategies, β-cells, but not α-cells, were shown to derive from a PP-expressing lineage (22), again consistent with our findings.

Thus, we have identified an important and necessary regulatory point for β-cell replication after β-cell loss, in the form of a smad2/3/7 network. In addition, our results begin to explain a concept of how a highly differentiated cell such as a β-cell could undergo cell division. The dedifferentiation that we see, apparently guided in part by smad7 expression, seems to represent a recapitulation of an embryonic phenotype with PP-fold expression. TGF-β signaling may represent a target pathway for regulating β-cell proliferation in humans.

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Author Contributions. Y.E. and S.T. performed the experiments and wrote the manuscript. J.W., P.G., and C.W. performed the experiments. K.P., J.P., C.S., and X.X. shared in discussion of the manuscript. Y.W. provided reagents and helped with the discussion of the manuscript. M.D. generated the Smad7$^{fx/fx}$ mouse. G.G. designed the overall experiment and wrote the manuscript. G.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.

References

1. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41–46
2. Georgia S, Bhushan A. Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. J Clin Invest 2004;114:963–968

3. El-Gohary Y, Tulachan S, Branca M, et al. Whole-mount imaging demonstrates hypervascularity of the pancreatic ducts and other pancreatic structures. Anat Rec (Hoboken) 2012;295:465–473
4. Inada A, Nienaber C, Katsuta H, et al. Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci USA 2008;105:19915–19919
5. Thorel F, Népote V, Avril I, et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010;464:1149–1154
6. Cozar-Castellano I, Fiaschi-Taesch N, Bigatel TA, et al. Molecular control of cell cycle progression in the pancreatic beta-cell. Endocr Rev 2006;27:356–370
7. Goto Y, Nomura M, Tanaka K, et al. Genetic interactions between activin type IIB receptor and Smad2 genes in asymmetrical patterning of the thoracic organs and the development of pancreas islets. Dev Dyn 2007;236:2865–2874
8. Rane SG, Lee JH, Lin HM. Transforming growth factor-beta pathway: role in pancreas development and pancreatic disease. Cytokine Growth Factor Rev 2006;17:107–119
9. Ungefroren H, Groth S, Sebens S, Lehnert H, Gieseler F, Fändrich F. Differential roles of Smad2 and Smad3 in the regulation of TGF-β1-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: control by Rac1. Mol Cancer 2011;10:67
10. Harmon EB, Apelqvist AA, Smart NG, Gu X, Osborne DH, Kim SK. GDF11 modulates NGN3+ islet progenitor cell number and promotes beta-cell differentiation in pancreas development. Development 2004;131:6163–6174
11. Miralles F, Czernichow P, Scharfmann R. Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. Development 1998;125:1017–1024
12. Tulachan SS, Tei E, Hembree M, et al. TGF-beta isoform signaling regulates secondary transition and mesenchymal-induced endocrine development in the embryonic mouse pancreas. Dev Biol 2007;305:508–521
13. Brown KA, Pietenpol JA, Moses HL. A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling. J Cell Biochem 2007;101:9–33
14. Goulley J, Dahl U, Baeza N, Mishina Y, Edlund H. BMP4-BMPR1A signaling in beta cells is required for and augments glucose-stimulated insulin secretion. Cell Metab 2007;5:207–219
15. Smart NG, Apelqvist AA, Gu X, et al. Conditional expression of Smad7 in pancreatic beta cells disrupts TGF-beta signaling and induces reversible diabetes mellitus. PLoS Biol 2006;4:e39
16. Park SH. Fine tuning and cross-talking of TGF-beta signal by inhibitory Smads. J Biochem Mol Biol 2005;38:9–16
17. Yan X, Liu Z, Chen Y. Regulation of TGF-beta signaling by Smad7. Acta Biochim Biophys Sin (Shanghai) 2009;41:263–272
18. Peshavaria M, Larmie BL, Lausier J, et al. Regulation of pancreatic beta-cell regeneration in the normoglycemic 60% partial-pancreatectomy mouse. Diabetes 2006;55:3289–3298
19. Talchai C, Xuan S, Lin HV, Sussel L, Accili D. Pancreatic β cell dedifferentiation as a mechanism of diabetic β cell failure. Cell 2012;150:1223–1234
20. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 1999;21:70–71
21. Postic C, Shiota M, Niswender KD, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem 1999;274:305–315
22. Herrera PL. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 2000;127:2317–2322
23. Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002;129:2447–2457
24. Schonhoff SE, Giel-Moloney M, Leiter AB. Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. Dev Biol 2004;270:443–454
25. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 2002;32:128–134
26. Zhang YQ, Kanzaki M, Furukawa M, Shibata H, Ozeki M, Kojima I. Involvement of Smad proteins in the differentiation of pancreatic AR42J cells induced by activin A. Diabetologia 1999;42:719–727
27. El-Gohary Y, Tulachan S, Guo P, et al. Smad signaling pathways regulate pancreatic endocrine development. Dev Biol 2013;378:83–93
28. Kiehne K, Otte JM, Fölsch UR, Herzig KH. Growth factors in development and diseases of the exocrine pancreas. Pancreatology 2001;1:15–23
29. Yamanaka Y, Friess H, Büchler M, Beger HG, Gold LI, Korc M. Synthesis and expression of transforming growth factor beta-1, beta-2, and beta-3 in the endocrine and exocrine pancreas. Diabetes 1993;42:746–756
30. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 2003;113:685–700
31. Liu G, Arnaud-Dabernat S, Kritzik MR, Kayali AG, Zhang YQ, Sarvetnick N. PYY in the expanding pancreatic epithelium. Endocrine 2006;30:103–112
32. Teitelman G, Alpert S, Polak JM, Martinez A, Hanahan D. Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development 1993;118:1031–1039
33. Herrera PL. Defining the cell lineages of the islets of Langerhans using transgenic mice. Int J Dev Biol 2002;46:97–103
34. Herrera PL, Huarte J, Sanvito F, Meda P, Orci L, Vassalli JD. Embryogenesis of the murine endocrine pancreas; early expression of pancreatic polypeptide gene. Development 1991;113:1257–1265
35. Gannon M, Shiota C, Postic C, Wright CV, Magnuson M. Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. Genesis 2000;26:139–142
36. Madisen L, Zwingman TA, Sunkin SM, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 2010;13:133–140
37. Ju W, Ogawa A, Heyer J, et al. Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. Mol Cell Biol 2006;26:654–667
38. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. Mol Cell Biol 1999;19:2495–2504
39. Russ HA, Bar Y, Ravassard P, Efrat S. In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing. Diabetes 2008;57:1575–1583
40. Billestrup N, Otonkoski T. Dedifferentiation for replication of human beta-cells: a division between mice and men? Diabetes 2008;57:1457–1458

41. Russ HA, Sintov E, Anker-Kitai L, et al. Insulin-producing cells generated from dedifferentiated human pancreatic beta cells expanded in vitro. PLoS ONE 2011;6:e25566

42. Russ HA, Ravassard P, Kerr-Conte J, Pattou F, Efrat S. Epithelial-mesenchymal transition in cells expanded in vitro from lineage-traced adult human pancreatic beta cells. PLoS ONE 2009;4:e6417

43. Morton RA, Geras-Raaka E, Wilson LM, Raaka BM, Gershengorn MC. Endocrine precursor cells from mouse islets are not generated by epithelial-to-mesenchymal transition of mature beta cells. Mol Cell Endocrinol 2007;270:87–93

44. Like AA, Chick WL. Mitotic division in pancreatic beta cells. Science 1969; 163:941–943

45. Yew KH, Prasadan KL, Preuett BL, et al. Interplay of glucagon-like peptide-1 and transforming growth factor-beta signaling in insulin-positive differentiation of AR42J cells. Diabetes 2004;53:2824–2835

46. Nomura M, Li E. Smad2 role in mesoderm formation, left-right patterning and craniofacial development. Nature 1998;393:786–790

47. Bonner-Weir S, Stubbs M, Reits P, Taneja M, Smith F. Partial pancreatectomy as a model of pancreatic regeneration. In *Pancreatic Growth and Regeneration*. Sarvetnick N, Ed. Austin, Landes Biosciences, 1997

48. Nomura M, Teramoto N, Morinaga H, Takayanagi R. Crucial roles of TGF-β signaling for pancreatic islet development and glucose-responsive insulin secretion. Presented at the Keystone Symposium "Islet and Beta Cell Biology (Z3)," Snowbird, UT, 6–11 April 2008

49. Xiao X, Wiersch J, El-Gohary Y, et al. TGFβ receptor signaling is essential for inflammation-induced but not β-cell workload-induced β-cell proliferation. Diabetes 2013;62:1217–1226

50. Herrera PL, Orci L, Vassalli JD. Two transgenic approaches to define the cell lineages in endocrine pancreas development. Mol Cell Endocrinol 1998;140:45–50