A smad signaling network regulates islet cell proliferation

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Keywords: Pancreas; Regeneration; Smads; TGF-β
Running title: Smad2/3/7 Regulate Islet Proliferation
Word Count: 4350
ABSTRACT

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

INTRODUCTION

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.

TGFβ-superfamily signaling has been strongly implicated in pancreatic development and postnatal growth (7; 8). TGFβ-signaling seems to favor endocrine cell lineage-selection and maturation, and seems to be antiproliferative (9). TGFβs, GDF11 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 smads that are canonically downstream of BMP-signaling, i.e. smads 1,5,8 (16). Smad7 seems to be more globally active against all receptor-activated smads, i.e. smads 1,5,8, 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 TGFβ-receptor-II (TBRII), along with their inhibitor, smad7, in β-cell growth after a non-diabetogenic loss of β-cells (60% partial pancreatectomy, PPx) (18). The phosphorylated (active) forms of smad2 and smad3 (p-smad2/3) 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 PP-fold hormones, perhaps representing a specific “de-
differentiation” 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 de-differentiation of β-cells has been recently described as a possible mechanism leading to β-cell failure (19). Thus, TGFβ-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 IACUC. Smad3-exon2 null mutant mice were obtained from Jackson Lab. Transgenic mice expressing Smad2^{fx/fx} were gifts from Dr. Erwin Bottinger, Mt.Sinai School of Medicine. Transgenic mice expressing TGFβrI^{fx/fx} and TGFβrII^{fx/fx} were gifts from Prof.Stefan Karlsson, University of Lund, Sweden. Rosa LacZ mice (R26R^{lacZ}) have been described previously by Soriano (20) and were obtained from Jackson Lab. Insulin2-cre mouse have been previously described by Magnusson (21). Glucagon-cre (22) obtained from Mutant Mouse Regional Resource Centers. All transgenic mice were crossed with Pdxcre-ER^TM(23) (Mouse Models of Human Cancers Consortium, MMHCC), Ngn3cre(24) and PTF1a-cre (25) (MMHCC).

Tamoxifen Injection

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

**Partial-Pancreatectomy**

Described previously (18).

**Immunohistochemistry**

Insulin guinea-pig 1:500 (Dako), 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 all (Santa Cruz Biotech, CA), somatostatin rat-monoclonal 1:100 (LsBio), TGFβ1-ligand mouse-moniclonal antibody 1:100 (R&D systems), TGFβR-II rabbit-polyclonal antibody 1:100 (Millipore), p27Kip1 mouse-moniclonal antibody 1:100 (ThermoFisherScientific), Pancreatic polypeptide guinea-pig anti-rat 1:400 (Millipore), neuropeptideY (NPY) rabbit-polyclonal anti-body 1:500 (Millipore), peptideYY (PYY) rabbit-polyclonal antibody 1:500 (Abcam), PDX-1 goat-polyclonal 1:1000 (Abcam), Dolichos biflorus agglutinin FITC-conjugated (DBA) 1:100(Vector Laboratories, CA), pSmad3 (1:400), pSmad2 (1:50) and pSmad2/3 (1:500) all rabbit polyclonal (Santa Cruz Biotech, CA), Ki67 goat-polyclonal 1:100 (Santa Cruz Biotech, CA), anti-bromo-deoxyuridine (BrdU) rat-moniclonal antibody 1:400 (Abcam). Primary antibodies were incubated for 2 hrs at room temperature (RT) or at
4°C overnight. Biotinylated Vectastain ABC kit or AMCA/CY3/FITC fluorescent conjugated donkey secondary antibodies were used for 1.5hr at RT. Immunoperoxidase was detected by DAB kit (Dako, Carpintaria, CA) or AEC (Sigma, St. Louis, MO) and fluorescently labeled samples were imaged using a fluorescent microscope. Tissue sections were viewed on an upright AxioImager Z1 microscope or with an inverted Olympus-Fluoview-1000 confocal microscope to confocally image the tissue sections. Images were captured with the AxioCamMRc5 and processed using AxioVs40V4.8.2.0 software.

**Bromo-deoxyuridine (BrdU) incorporation and cell counting**

Adult mice were injected with BrdU (Sigma, St. Louis, MO) 200mg/Kg intraperitoneally after performing PPx and subsequently once a day for 7 consecutive days. The pancreas was harvested 1 week post surgery, fixed in 4%PFA overnight in 4°C then placed in 30%sucrose overnight in 4°C. Antigen-retrieval was done on the slides by treating it with 2MHC1 for 35min, followed by overnight incubation with primary antibodies. To quantify number of BrdU positive cells per islet in wild-type and transgenic pancreases, the whole frozen pancreas was sectioned 6µm-thick at -23°C in a cryostat and mounted on gelatin-coated glass microscope slides (Superfrost Plus, Fisherbrand), 8 sections/slide. Two random slides were picked and the average number of BrdU positive cells per islet was determined over 20islets, using the image tracing software (Stereoinvestigator, Microbrightfield) to count and tag individual BrdU positive/insulin positive cells. Using the Stereoinvestigator program, individual BrdU positive cells were tagged and linked to Neurolucida software (MicroBrightField, Inc.) to quantify 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 p-smad2/3 (Fig. 1A). After PPx we gave daily injections of BrdU for one-week. Here, we saw that many islet cells had become negative for p-smad2/3, and many of those p-smad2/3 negative islet cells were BrdU+ (Fig. 1B-D). Only rarely were p-smad2/3+ cells also BrdU+. One week after PPx 82% ± 2.5 SEM of p-smad2/3-negative endocrine (as defined by a non-endothelial appearance) (arrows) or p-smad2/3 weak cells (arrowheads) in islets are BrdU+. This value is compared to 18.2% ± 2.6 of p-smad2/3-positive cells being BrdU+. In addition, we noted that there was down-regulation within 24hrs of PPx of the phosphorylated (active) form of the TGFβ-type-II-receptor (p-TBRII) in the islet such that many insulin+ cells that had been p-TBRII+ prior to PPx had become p-TBRII-negative (Fig. 1E,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. 1A,B), but little or no expression in the islets or acinar tissue (Fig.1G) (28). Interestingly, we found that smad7 expression appeared very early after PPx (within 24hours) in a subset of pancreatic islet cells (Fig. 1H). 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. 1C). The smad7\(^+\) cells also stained for Ki67, and thus had entered the cell cycle (Supplementary Fig. 1D). Since both the smad7 and p-smad2/3 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 p-smad2/3 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. 1J). The smad7\(^+\) cells were sometimes localized to the outer (“mantle”) region of the islet, but could also be seen throughout many islets (Fig. 1H-L).

In order to confirm that this smad7 expression acted as an inhibitor of TGFβ-signaling in these islets, we stained for the ligand TGFβ1 (Fig. 1M,O). Similar to previous reports (29), TGFβ1 was found to be distributed evenly throughout the islet at baseline (Fig. 1M). However, 24 hrs post-PPx, TGFβ1 appeared to accumulate extracellularly in the immediate region of smad7\(^+\) cells (Fig. 1O, compare with smad7 staining in the immediately adjacent histologic section in Fig. 1N). Such an extracellular accumulation of TGFβ-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 TGFβ-signaling systems (30).

**Characterization of smad7\(^+\) islet cells post-pancreatectomy.**
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 co-stained with glucagon, insulin, pdx-1, somatostatin, nor Glut2 (Fig. 2A-E). However, surprisingly, essentially all of the smad7+ cells stained positive for pancreatic polypeptide (PP)(Fig. 2F-H). In addition, the three members of the “PP-fold” group of peptides (PP, peptideYY [PYY], and neuropeptideY [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. 2I-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 with NPY (Fig. 2L-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 pre-incubations of the antibodies either with their cognate peptide, or with the other two peptides. For all three antibodies pre-incubation with its own cognate peptide blocked staining, whereas pre-incubation with the other two peptides had no effect on staining, thus implying that there was not significant cross-reactivity of these newer antibodies (Supplementary Fig. 2,3). We also have recently published successful staining for PP in embryonic and early post-natal pancreas (28).

Smad7+ cells represent de-differentiated β-cells

We hypothesized that these smad7+ cells may be β-cells that recently turned off insulin, even within 24hours post-PPx. This possibility would be consistent with lineage-tagging
studies that found that new β-cells during regeneration 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 “de-differentiate”. 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-Isl-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. 3A-D). In the Ins2-Cre;R26R-Isl-tomato red mice these insulin-negative cells would be lineage-tagged, and still be tomato-red+. We found one-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-Isl-tomato mice (Fig. 3A-D). These results strongly suggest that the smad7+ cells had recently been positive for insulin (Fig. 3A-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-Isl-tomato red mice and harvested one 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. 3E-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 (36). To test whether smad7+ cells may have derived from α-cells that recently turned-off glucagon, we crossed a glucagon-cre mouse (22) with a R26R-Isl-tomato-red reporter mouse strain (22; 37). 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. 3I).

Enhanced islet cell proliferation after PPx in smad2 and/or smad3 null mice

In order to determine whether suppression of p-smad2 and p-smad3 plays a key role in islet cell proliferation post-PPx, we obtained smad2-conditional (Smad2$^{fx/fx}$) (38) and smad3 global mutant (exon2 deletion) mice (39). Smad3$^{−/−}$ (exon2) global null mutant mice are viable and fertile (39), 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 one week after tamoxifen treatment in pdx1-cre-ERT;smad2$^{fx/fx}$ mice, in the smad3$^{−/−}$ global null mutant mice, and in double smad2/3 mutant mice. For the pdx1-cre-ERT;smad2$^{fx/fx}$ mice, one-week post-PPx there was a 50% increase in the number of BrdU-labeled islet cells (Fig. 4A-D, quantification in Fig. 5G). In smad3$^{−/−}$ mutants there was a tripling of the number of BrdU$^+$ islet cells (Fig. 4E,F, 5G). At baseline in the smad2 mutants, there was a slightly increased proliferation rate compared with littermate controls (compare Fig. 4A,D, and Pdx-cre-ERT; smad2$^{fx/fx}$ sham in Fig. 5G). Interestingly, in the smad3$^{−/−}$ null mutant mice, although not quantified, we noticed that there was near absent proliferation of non-islet pancreatic tissue (Fig. 4F). 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 (Fig. 4G, 5G).

**Smad7 is necessary for islet cell proliferation after PPx**

While smad7 appeared to be a potential marker of proliferative, de-differentiated, 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<sup>fx/fx</sup> knock-in mouse. These mice were created with loxP-sites flanking exon5 (the TGFβ receptor interacting domain) of the smad7 locus (Supplementary Fig. 4A,B). We then conditionally deleted smad7 in the pancreas by crossing the smad7<sup>fx/fx</sup> 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 five days, starting one-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<sup>+</sup> islet cells between any of these three strains post-PPx, compared with sham-operated cre-negative littermates (Fig. 5A-C,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<sup>fx/fx</sup> animals, there was very little proliferation seen throughout the entire pancreas, including the exocrine tissue (see Fig. 5A, with a heavily BrdU<sup>+</sup> lymph node as an internal positive control). However, we noticed that some islets in the PTF1a-cre;smad7<sup>fx/fx</sup> mice did have several proliferative
cells. We suspected that there may be some mosaicism of cre recombination for PTF1α-cre mice, especially in the islets, so we crossed these mice with the R26R-Is1-lacZ conditional reporter mice (20) (Fig. 5D-F). We chose this reporter here rather than the tomato-red reporter used earlier because the floxed sequence of the R26R-Is1-LacZ mouse is closer in size to the floxed smad7 segment. One-week post-PPx in these PTF1α-cre;smad7<sup>fx/fx</sup>;R26R-Is1-lacZ mice we again saw a few islets that had several BrdU<sup>+</sup> cells, but essentially all of the BrdU<sup>+</sup> cells in the islet were β-gal negative, and therefore were either not of the PTF1α lineage (e.g. endothelial cells), or were not cre lineage-tagged due to mosaicism (Fig. 5D-F, quantified in 5H). 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.

*TGFβ receptor II mediates suppression of pancreatic islet proliferation*

Although smad2/3 were found to be important potential regulators of islet proliferation post-PPx (Fig. 4), multiple TGFβ-superfamily ligands and receptors can work through smad2/3. As described above, we noted that the TGFβ1-ligand and activated TBR-II-receptor staining pattern was rapidly altered in wild-type islets post-PPx, suggesting that down-regulation of signaling specifically through TβR-II may permit islet proliferation (Fig. 1E,F, L-N). In order to test this possibility, we performed PPx in mice with conditional inactivation of the TGFβ-receptor-I (TBR-I<sup>fx/fx</sup>, also called Alk-5, the canonical binding partner with TGFβrII) and/or TGFβ-receptor-II (TBR-II<sup>fx/fx</sup>) (40; 41). These receptor floxed mice were crossed with either PTF1α-cre mice or pdx1-cre-ERT mice. One-week after PPx we found that mice with TGFβ-receptor-II ablation (PTF1α-
cre;TBR-II^fx/fx or pdx1-cre-ERT;TBR-II^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. 4I,J, quantification in 5G). Mice with ablation of only TGFβ-receptor-I (PTF1a-cre;TBR-I^fx/fx or pdx1-cre-ERT2;TBR-I^fx/fx) had no difference in proliferation compared with controls (not shown), and there was no additive increased proliferation in the TGFβ-receptor-II ablated mice when additionally crossed to make a double TGFβ-receptor-I/TGFβ-receptor-II mutant mouse (PTF1a-cre;TBR-I^fx/fx;TBR-II^fx/fx or pdx1-cre-ERT2;TBR-I^fx/fx;TBR-II^fx/fx)(Fig. 4H). These results suggest that TGFβ-receptor-II is a key suppressor of islet cell proliferation after PPx, likely through smad2 and smad3 phosphorylation. Interestingly, here we found that increased proliferation in the pdx-cre-ERT2;TGFβr-II^fx/fx mice required that the tamoxifen be given at least one-month prior to PPx. If the tamoxifen was given just one-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 TGFβ-receptor-II 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 (40; 42-44). While mature β-cells have been shown to directly undergo cell division in certain systems (45), it remains unclear whether such an event is part of a normal physiologic program. In addition, a de-differentiation program in β-cells, resulting in an “empty β-cell”, has been recently
shown to be associated with β-cell failure (19). Here we found that a rapid and transient de-differentiation 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. TGFβ-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 TGFβ-superfamily signaling in the developing pancreas (7; 8; 11; 12). We previously found that TGFβ-receptor-II-mediated signals to the embryonic ductal structures suppress the recruitment of endocrine progenitors and suppress their proliferation (12). The canonical downstream effectors of TGFβ-receptor-II 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; 46), though these smad-mediated effects were thought to be representative of activin-ligand activity rather than TGFβ’s. 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; 47). Bonner-Weir showed that TGFβ-ligand localizes to the periductal region, and they proposed that TGFβ may act as a break on ductal proliferation post-PPx (48). In contrast with our findings of a 4-fold increase in baseline islet cell proliferation in pdx1-cre-ERT;smad2fx/fx islets one-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 (49). These differences may well reflect the ablation of smad2 beginning during the embryonic period versus acutely before the PPx. The 1.53-fold increase in islet cell proliferation that we saw post-PPx in the smad2 and/or smad3 mutant mice supports a role for either TGFβ or activin-signaling in suppressing β-cell proliferation, since both TGFβ and activin work through smads 2 and 3. Collectively, our data showing the rapid suppression of phosphorylated TBRII (Fig. 1E,F), the rapid accumulation of TGFβ-ligand in the vicinity of smad7 expression (Fig. 1M-O), and enhanced islet proliferation in the TBRII mutant mice (Fig. 4H-J,5G) all suggest that it is specifically TGFβ-signaling, rather 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 “de-differentiation” of β-cells in the islet within 24hrs 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 de-differentiation 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 de-differentiation 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 (50), which could explain why in our PPx model we did not observe β-cell de-differentiation to α-cells. In our PPx model, however, the de-differentiation appears to be part of a β-cell self-duplication process, with a de-differentiation and then re-differentiation. 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 de-differentiated smad7+ cells then “re-differentiate” after proliferation, logic would suggest that such a re-differentiation must occur. Specifically, since we saw multiple BrdU+/insulin+ cells one-week post-PPx, but little or no BrdU+ cells in the smad7-ablated islets one-week post-PPx (Fig. 5A-C) it would strongly suggest that the source of these insulin+/BrdU+ cells is β-cells that became insulin-negative/smad7+, and 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;smad7fl/fl;R26R-Isl-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. 5D-F,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 nearly 95% of islets post-
PPx, there was a large inter-islet variability within the same pancreas in terms of their smad7 expression pattern (Fig. 1H-K,N). A similar variability was observed by Talchai et al. in the different patterns of insulin immunoreactivity or insulin negative cells in different islets (19).

We found that smad7⁺ “de-differentiated” islet cells were derived from insulin⁺ cells. In addition, these “de-differentiated” β-cells had become positive for the PP-fold family of molecules (PP, NPY, PYY). The expression of these PP-fold molecules in the smad7⁺ cells suggests that the β-cells have de-differentiated along a developmental cell lineage pathway in which PP-fold expressing cells represent a more immature cell prior to “re-differentiation” 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 co-expressed 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 “de-differentiated” to form smad7⁺, PP-fold expressing cells (33; 51). 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 “de-differentiation” that we see, apparently guided in part by
smad7 expression, seems to represent a recapitulation of an embryonic phenotype with PP-fold expression. TGFβ-signaling may represent a target pathway for regulating β-cell proliferation in humans.

**Author Contribution:** YE and ST performed the experiments and wrote the manuscript. PG, CW and JW performed experiments. KP, JP, CS and XX shared in discussion of the paper. YW provided reagents and helped with the discussion of the paper. MD generated the Smad7fx/fx mouse. GG designed the overall experiment and wrote the manuscript.

**ACKNOWLEDGEMENT**

Financial support was from NIH (G.K.G., RO1 DK064952, R01 DK083541-01) and the Children's Hospital of Pittsburgh. M.D. was supported by Project Z01 ES101603 from the Division of Intramural Research of the NIH (NIEHS).

No potential conflicts of interest relevant to this article were reported.

The authors give special thanks to Lauren Brink, Jessica Thomas, and Sean-Paul Williams for technical assistance and mouse breeding. Thanks to Farzad Esni for helpful discussions. Thanks to Christine Kalinyak, Anne Meinert and Tamara Daviston for administrative assistance.

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**Figure Legends:**

**Fig. 1:** Phospho-smad (p-smad)2/3, smad7 and TGFβ1 ligand and phosphorylated TGFβ receptor II expression pattern in an adult pancreas. (A) p-Smad2/3 expression in a normal adult islet showing that most islet cells have nuclear staining. (B) p-Smad2/3 expression post-partial pancreatectomy where some cells appear to have become p-smad2/3 negative. (C,D) After partial pancreatectomy many of the cells that have switched off (arrows) or only weakly express (arrowheads) p-smad2/3 are BrdU+. (E) The phosphorylated TGFβ receptor type II (pTGFβrII, the active form of the receptor) is present throughout the adult islet at baseline (islet outlined by dotted line). (F) 24hrs post partial pancreatectomy, pTGFβrII is absent in many of the islet cells. (G-I) Smad7 expression pattern and time course, where Smad7 is not expressed in islets baseline, but becomes rapidly upregulated in islets within 24hours of performing a partial pancreatectomy, and expression persists in islets even 4 weeks after surgery. (J,K) Many islet cells one week after partial pancreatectomy express smad7, and these cells are insulin-negative. (L) Baseline expression of TGFβ1 ligand in an unperturbed adult islet. (M) Smad7 turns on in some cells within 24hrs of partial pancreatectomy; this mantle distribution of smad7 is frequently seen, but many islets have a more diffuse distribution of smad7 expression, as in (H,I,J). (N) Smad7 expression correlates with extracellular TGFβ1 ligand accumulation in the area of the islet where smad7 is acutely upregulated. (M & N) are consecutive histologic sections. Scale bar, A,B: 50µm, C-F, J-N: 10µm. G-I: 20µm.
**Fig. 2:** Characterization of smad7 positive cells. Smad7 does not co-localize with (A) glucagon, (B) insulin or (C) pdx1 (D) somatostatin or (E) Glut2. (F-H) However, smad7 does co-localize uniformly with pancreatic polypeptide (PP). (G-L) These PP⁺ cells also stain for NPY and PYY, although rare cells are PYY⁺/PP⁻ (arrowhead in G-I). Scale bar, A-E: 10µm, F-N: 20µm.

**Fig. 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 partial pancreatectomy, but remain tomato red positive indicating their derivation from insulin⁺ cells (D). (E-H) PP⁺ positive cells (arrowhead) co-staining with tomato-red cells in Ins2-cre;R26R-lsl-tomato red mouse islet harvested 1 week after partial pancreatectomy. (G) Smad7⁺ cells do not originate from glucagon positive cells as determined by using a glucagon-cre;R26R-lsl-tomato reporter mouse after partial pancreatectomy. Scale bar A-D: 2µm, E-I: 20µm.

**Fig. 4:** Islet proliferation analysis in wild-type, smad2, smad3 and TGFβ receptor mutant mice (see Figure 5G 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 partial pancreatectomy (PPx). (C) Further enhanced islet proliferation in pdx1-cre-ERT; smad2fx/fx pancreas 1 week after partial pancreatectomy. (D) At baseline (without pancreatectomy) pdx1-cre-ERT;smad2fx/fx sham islets have slightly higher proliferation compared to either wild-type sham islets (A) or cre- littermates (not shown). (E) Minimal proliferation in a smad3-exon2⁻/⁻ islet without pancreatectomy compared to
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**Fig. 5:** Islet proliferation in smad7 conditional mutant mice. Minimal islet proliferation is seen 1 week after partial pancreatectomy in (A) PTF1a-cre; smad7\(^{fx/fx}\), (B) pdx1-cre-ERT; smad7\(^{fx/fx}\), and (C) ngn3-cre; smad7\(^{fx/fx}\) islets. Lymph node in (A) demonstrating a positive internal control for proliferation, and peri-ductal proliferation was observed in (B) and (C). (D-F) PTF1a-cre; smad7\(^{fx/fx}\); R26R-Isl-lacZ pancreas, harvested 1 week after partial pancreatectomy, revealed mosaicism of cre lineage labeling in some islets. Here, 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 post-natally (personal communication, C. Murtaugh). (G) Islet proliferation quantification for pancreases harvested from the indicated transgenic and wild type mice 1 week after they had undergone 60% partial pancreatectomy (or sham-operation as indicated for some, at least 3 different mice in each group \(\pm\) SEM). Compared to proliferation in C57B6 islets 1 week post PPx, there was a significant increase in proliferation in all the different transgenic
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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-Isl-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 bar A: 50µm, B,C: 20µm, D-F: 10µm.
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Supplementary Fig. 1: (A,B) Smad7 is expressed in pancreatic ducts at baseline, with co-localization with the duct marker DBA. (C) Smad7+ cells are negative or only weakly positive for p27kip (see inset) and (D) positive for Ki67. Scale bar, A,B: 10µm, C: 20µm and D: 5µm.
Supplementary Fig. 2: PP, NPY & PYY staining in E16 pancreas. (A-C) PP staining in control E16 pancreas. (D-F) PP staining after PP neutralization. (G-I) control PYY staining. (J-L) PYY staining after PYY neutralization. (M-O) control NPY staining. (P-R) NPY staining after NPY neutralization.
Supplementary Fig. 3: PP, NPY & PYY staining in E16 pancreas. (A-C) PP staining after PYY neutralization. (D-F) PYY staining after PP neutralization. (G-I) PP staining after NPY neutralization. (J-L) NPY staining after PP neutralization. (M-O) co-staining PP with PYY. (P-R) co-staining NPY with PP.
Supplementary Fig. 4: (A) Genetic scheme for placement of loxP sites flanking exon5 (TGFβ receptor interacting domain) of the endogenous smad7 locus in mice. (B) PCR demonstrating wild-type (+), floxed, and deleted (del) gene amplifications.