SMAD7 enhances adult β-cell proliferation without significantly affecting β-cell function in mice

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The interplay between the transforming growth factor β (TGF-β) signaling proteins, SMAD family member 2 (SMAD2) and 3 (SMAD3), and the TGF-β-inhibiting SMAD, SMAD7, seems to play a vital role in proper pancreatic endocrine development and also in normal β-cell function in adult pancreatic islets. Here, we generated conditional SMAD7 knockout mice by crossing insulinCre mice with SMAD7fx/fx mice. We also created a β cell–specific SMAD7-overexpressing mouse line by crossing insulinI1Cre mice with HPRT-SMAD7/RosaGFP mice. We analyzed β-cell function in adult islets when SMAD7 was either absent or overexpressed in β cells. Loss of SMAD7 in β cells inhibited proliferation, and SMAD7 overexpression enhanced cell proliferation. However, alterations in basic glucose homeostasis were not detectable following either SMAD7 deletion or overexpression in β cells. Our results show that both the absence and overexpression of SMAD7 affect TGF-β signaling and modulates β-cell proliferation but does not appear to alter β-cell function. Reversible SMAD7 overexpression may represent an attractive therapeutic option to enhance β-cell proliferation without negative effects on β-cell function.

Transforming growth factor β (TGF-β) family proteins regulate a wide range of cellular functions. They can either induce or inhibit cell proliferation and can regulate cell and organ development (1–3). In the developing pancreas, activated components of TGF-β signaling play a role throughout gestation. Several TGF-β receptors (RI or Alk5, RII, and Alk1) were localized to both the embryonic pancreatic epithelium and mesenchyme but then localized to the pancreatic islets and ducts at older ages (4). There is differential expression of various TGF-β signaling components across the embryonic pancreas. The mesenchyme of the embryonic pancreas expresses the TGF-β receptor antagonist follistatin, whereas the epithelium of the pancreas predominantly expresses receptor ligands (5–7). Either exogenous activin or the BMP inhibitor noggin both induced endocrine differentiation in the embryonic epithelium in vitro (8), whereas follistatin inhibited endocrine differentiation (6). TGF-β2 and TGF-β3 homozygous mutants are embryonically lethal (9, 10); however, the use of a dominant negative form of TGF-β receptor 2, the DNTBRII transgene, helped identify a possible role for TGF-β signaling in regulating pancreatic endocrine expansion and maturation during development (11–13). Using this mouse overexpressing the dominant negative form of the TGF-β type II receptor, Tulachan et al. (13) inhibited TGF-β signaling at the receptor level and found an increase in the number of endocrine precursors as well as a proliferation of endocrine cells that was progressively exaggerated with age.

In the developing pancreas and in mature β cells, key downstream transcription factors mediate TGF-β signaling, including the cytoplasmic SMAD proteins, SMAD2 and SMAD3 (14). Phosphorylation of cytoplasmic SMAD proteins leads to translocation of phospho-SMADs (p-SMADs) into the nucleus, where they regulate transcription of downstream targets (15, 16). Phosphorylated SMADs typically bind to SMAD-binding elements. In the pancreas, p-SMADs are localized to the nuclei of endocrine-committed, hormone-positive, nonproliferative embryonic pancreatic cells, suggesting a role for activated p-SMADs in endocrine terminal differentiation (17). Inhibiting SMAD2 or SMAD3 during pancreas development led to increased proliferation of immature hormone-positive cells severalfold, with reduced endocrine maturation (17). Inhibiting SMAD2 or SMAD3 in adult human β cells also led to enhanced proliferation (18). In vitro, blocking either SMAD2 or SMAD3 in embryonic mouse pancreas explants with antisense treatment significantly enhanced the insulin- and glucagon-positive cell numbers 2-fold (17), with a 2-fold increase in proliferation as well.

SMAD7 is a general negative regulator of TGF-β signaling; among many of its functions, it inhibits phosphorylation of SMAD2/3 and prevents their nuclear translocation (19–21). During development, SMAD7 is expressed in the hormone-negative cells in the developing pancreas and then later seems to shut off in hormone-positive endocrine cells (17). A previous study showed that overexpressing SMAD7 in pdx1-positive cells led to severe pancreatic hypoplasia and a >85% reduction in the number of β cells and an associated increase in the num-

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3. The abbreviations used are: TGF-β, transforming growth factor β; p-SMAD, phospho-SMAD; GSIS, glucose-stimulated insulin secretion; IPGTT, intraperitoneal glucose tolerance test; BrdU, bromodeoxyuridine; BAC, bacterial artificial chromosome; IPITT, intraperitoneal insulin tolerance test.

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This article contains Fig. S1.

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number of α cells at birth (21). On the other hand, SMAD7 knock-
down or deletion in endocrine progenitor cells resulted in sig-
nificant suppression of endocrine development (17). These
findings from overexpression and inhibitory studies suggest
that SMAD7 function may strike a delicate balance in the reg-
ulation of SMAD2/3 signaling to preserve normal pancreatic
development and function, especially β-cell function.
Our studies here of overexpression differ substantially
from the previous study by Smart et al. (21) in that here the cells
are not dependent on active pdx1 expression for SMAD7 over-
expression, and the entire insulin lineage will be SMAD7
overexpressors upon doxycycline treatment. In addition, the
novel ins1-dre knock-in that we use, instead of the pdx1-tTA
used previously, does not cause a baseline diabetic phenotype.
Last, we use a Tet-On system that allows for tighter initiation of
SMAD7 overexpression, without the need for waiting for the
pharmacologic clearance of doxycycline that is needed in the
tet-off system.

In the adult islet, β-cell renewal through proliferation is
thought to be important for proper maintenance of β-cell mass
and function. Our previous study showed that TGF-β receptor
signaling was necessary for inflammation-induced β-cell pro-
liferation, but not for workload-induced β-cell proliferation (22).
However, another of our studies used a global pancreatic
deletion of SMAD7 to demonstrate that, unlike TGF-β recep-
tors, SMAD7 is a crucial mediator of both workload and inflam-
mation-induced β-cell proliferation (23, 24). Specifically, lack of
SMAD7 expression prevented normal β-cell proliferation and
recovery either during regeneration after partial pancrea-
tectomy (increased workload) or after pancreatic duct ligation
(inflammation-induced), confirming an important role for
SMAD7 in promoting β-cell proliferation in both conditions
(22–24). Thus, a better understanding of how SMAD7 regulates
both β-cell proliferation and β-cell function seems warranted.
Here, we compare the effects of a seemingly more elegant β-
cell–specific SMAD7 deletion or overexpression system on
β-cell mass and function in an adult mouse islet.

Results

Generation of β cell–specific conditional SMAD7-
overexpressing mice

Our laboratory has previously developed a pancreas-specific
novel dual-lineage tracing technology, using a combination of
two recombinase systems, Dre/RoxP and Cre/LoxP, to inde-
dependently trace GFP1-expressing green fluorescent Pdx1-lineage
cells and tomato red fluorescent Ptf1a-lineage cells in the develop-
oping and adult mouse pancreas (25). We also previously
reported an important role for SMAD7 in promoting β-cell rep-
lication during pancreas development and during regeneration
after partial pancreatectomy or after pancreatic duct ligation
(17, 22–24). However, a baseline role for SMAD7 in adult
β-cell replication and function is less clear. To explore such a
role for SMAD7 in adult β cells, we developed a novel tetryc-
cline-regulatable Dre recombinase–dependent SMAD7-over-
expressing knock-in mouse (Fig. 1A). This mouse model resem-
bles the SMAD7 overexpressor published previously by the
Kim group (21), but with several important differences. First,
Dre recombinase–dependent SMAD7 overexpression in mice
allows for the use of Cre recombinase for other purposes, with-
out affecting SMAD7 overexpression. Thus, our model can
allow for a tissue-specific, knock-back-in strategy following
Cre-mediated SMAD7 knockout. Second, in our mice, the
expression of SMAD7 is up-regulated by doxycycline, rather
than down-regulated, and is independent of ongoing pdx1 or
insulin promoter activity, which may be unpredictable during
altered β-cell states. Third, SMAD7 overexpression is in the
HPRT locus, which is linked to the X chromosome, and thus
allows us to obtain roughly 100% of cells overexpressing
SMAD7 in males or homozygous females, but only 50% of cells
in heterozygous females, thus potentially facilitating cell-au-
tonomous analyses. Fourth, by crossing SMAD7Hprt mice with
our newly created insulin1Dre and Rosa26GFP reporter mice, we
were able to label SMAD7-overexpressing β cells with GFP pro-
tein for sorting (hereafter called Ins1DreSMAD7Hprt/Rosa26GFP
mice). In conclusion, our model may represent a greatly
improved approach for studying non-cell-autonomous and
cell-autonomous effects of SMAD7 overexpression on β cells.

β cell–specific overexpression of SMAD7 in
Ins1DreSMAD7Hprt/Rosa26GFP mouse islets

Ins1DreSMAD7Hprt/Rosa26GFP and MIP-GFP control mice were
randomized into groups receiving either doxycycline (625 mg)
or normal control diet for 3 weeks. We examined SMAD7 expres-
sion in flow-sorted GFP-positive β cells from Ins1DreSMAD7Hprt/Rosa26GFP
mice by quantitative RT-PCR and found a significant increase in SMAD7 gene transcripts (∼100-
fold over MIP-GFP mice) in Ins1DreSMAD7Hprt/Rosa26GFP
β cells when continuously exposed to doxycycline diet for 3
weeks (Fig. 1B). We did not see any significant difference
in SMAD7/mRNA levels between MIP-GFP and Ins1Dre
SMAD7Hprt/Rosa26GFP mice not exposed to doxycycline. Ins1Dre
SMAD7Hprt/Rosa26GFP mice on a regular diet did not
appear to show any “leaky” increase in the expression of
SMAD7 in β cells (Fig. 1B). The doxycycline exposure also
resulted in increased expression of SMAD7 protein in adult
islets confirmed by Western blotting (Fig. 1, C and D). Collec-
atively, the data confirm that SMAD7 overexpression is doxycy-
cline-dependent and appears to be β cell–specific, driven by
Ins1Dre in Ins1DreSMAD7Hprt/Rosa26GFP
mice.

Conditional SMAD7 overexpression is sufficient to disrupt TGF-
β signaling in β cells

TGF-β signaling pathways play an important regulatory role
in β-cell proliferation and homeostasis (21, 22). SMAD2 and
SMAD3 are specific SMADs that act downstream of TGF-βs
and seem to prevent β-cell proliferation and to maintain the
β-cell phenotype, whereas SMAD7, an inhibitory SMAD,
serves as an antagonist of TGF-β receptor signaling pathways
(17, 22). Normal adult β cells express both SMAD2 and
SMAD3, whereas SMAD7 is barely detectable (23). The
Ins1DreSMAD7Hprt/Rosa26GFP
mice on a regular diet had normal
islet levels of phosphorylated SMAD2 (Ser-465/467) (Fig. 2, A,
C, and D) and phosphorylated SMAD3 expression (Ser-423/
425) (Fig. 2, B–D), as shown by immunohistochemical staining
and Western blotting, suggesting normal TGF-β signaling in
the absence of doxycycline. Total SMAD2 and SMAD3 mRNA (Fig. 2, E and F) and protein levels (Fig. 2, C and D) were also measured in purified β cells and islets, respectively. With doxycycline-induced SMAD7 overexpression, we observed a significant reduction in phosphorylated SMAD2 (Ser-465/467) (Fig. 2, A, C, and D) and SMAD3 (Ser-423/425) (Fig. 2, B–D) as well as total islet SMAD2 and SMAD3 expression (Fig. 2, C–F). Collectively, the data confirm that β cell–specific doxycycline-dependent SMAD7 overexpression disrupted TGF-β signaling in adult pancreatic islet/β cells. This observation is in line with previous work by Smart et al. (21), who observed reduced phospho-SMAD2 and impaired TGF-β signaling following conditional transgenic expression of SMAD7 in pdx1-positive adult islet cells.

**SMAD7 overexpression increases β-cell proliferation**

SMAD7 is thought to be a regulator of the size of the pancreatic endocrine compartment during development (17). In adult islets, SMAD7 expression is barely detectable, if at all (23). However, SMAD7 expression re-emerged in regenerating islet cells after pancreatic injury in both partial pancreatectomy (a model for workload induced β-cell proliferation) and pancreatic duct ligation (a model of inflammation-induced β-cell proliferation) (22–24). Also, inhibition of SMAD7 expression in

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**Figure 1. Dre recombinase–dependent and doxycycline-inducible β cell–specific expression of SMAD7 in Ins1^DreSMAD7^Hprt/Rosa26GFP (Ins1^DreSMAD7^Hprt) mouse islets.** A, schematic for Dre-dependent tetracycline-inducible SMAD7 overexpression in an Hprt–TetOn–SMAD7 mouse. Dre activity permanently removes the stop cassette to activate TetOn3G expression (toward the right, green arrow). Upon doxycycline exposure, the TRE3G promoter is activated to initiate SMAD7 overexpression (toward the left, purple arrow). Pancreatic β cells in Hprt-SMAD7 mice are labeled with GFP by crossing with a mouse having a RoxP-flanked stop sequence preceding GFP, as a Dre reporter, inserted into the rosa locus. By crossing these two lines to make a quadruple-positive (homozygous female, Ins1^DreSMAD7^/SMAD7^Hprt/Rosa26GFP) or triple-positive mouse (male with X chromosome positive, Ins1^DreSMAD7^/yHprt/Rosa26GFP; or heterozygous female, Ins1^DreSMAD7/wHprt/Rosa26GFP), we here confirm that this dual expression system can function in one animal and that the SMAD7 and GFP reporter activity correlate well, with specific expression of both in the β cells of (Ins1^DreSMAD7^Hprt/Rosa26GFP) mouse islets. B, SMAD7 mRNA in FACS-sorted β cells from islets of 11-week-old Ins1^DreSMAD7^Hprt/Rosa26GFP and MIP-GFP mice, with and without doxycycline (3 weeks) treatment. Error bars, S.D. n = 5–8; *, p < 0.01. NS, no significance. C, SMAD7 protein expression was analyzed in isolated islets of 11-week-old Ins1^DreSMAD7^Hprt/Rosa26GFP and MIP-GFP mice, with and without doxycycline (3 weeks) treatment. Protein isolated from islets of three different mice from each group was loaded on separate gels, and a representative band from each gel is shown. D, Western blotting results were quantified by densitometry. β-Actin was used as a protein loading control for Western blot analysis. n = 3. *, p < 0.01. Error bars, S.D.
cells led to a loss of proliferation (23, 24). Therefore, we tested whether conditional and regulatable overexpression of SMAD7 in \( \text{H}9252 \) cells alone is sufficient to enhance proliferation. Ins1\text{Dre}SMAD7\text{Hprt}/Rosa26GFP mice were doxycycline-treated for 3 weeks and then fed with BrdU in the drinking water for 1 week to label replicating \( \text{H}9252 \) cells. In addition, the proliferation marker Ki-67 was quantified in \( \text{H}9252 \) cells from these mice. Quantification of BrdU/insulin cells in control-fed mice showed the typical low rate of proliferation, at \( \approx 0.5\% \) of insulin+ cells over the 7 days. However, the doxycycline-fed group showed a significant (3-fold) increase in \( \text{H}9252 \)-cell proliferation (Fig. 3A). We obtained similar results with Ki-67+/insulin+ staining (Fig. 3B). These data imply that doxycycline-regulated SMAD7 overexpression alone in \( \text{H}9252 \) cells can promote proliferation.

**Dedifferentiation of SMAD7-overexpressing \( \beta \) cells**

During dedifferentiation, \( \beta \) cells are thought to lose identity and function, with a decrease in the expression of crucial \( \beta \)-cell markers, such as maf-A, pdx1, and nkx6.1. These transcription factors are required for \( \beta \) cells to be fully functional. However, \( \beta \)-cell dedifferentiation is also thought to be a reversible process necessary for \( \beta \)-cell proliferation (26). Previous studies from

Figure 3. Conditional SMAD7 overexpression increases proliferation and dedifferentiation of \( \text{H}9252 \) cells. \( \beta \)-Cell proliferation in 11-week-old Ins1\text{Dre}SMAD7\text{Hprt}/Rosa26GFP mice with and without doxycycline (3-week) treatment, shown by representative images and quantification of BrdU (A) and Ki67 (B) staining. \( n = 5; *, p < 0.01 \). C, expression levels of endocrine-specific mRNAs were quantified by RT-PCR in purified \( \beta \) cells from islets of 11-week-old Ins1\text{Dre}SMAD7\text{Hprt}/Rosa26GFP with and without doxycycline (3-week) treatment. Values were normalized against \( \beta \)-actin. \( n = 6–8; *, p < 0.01 \). Error bars, S.D.

Figure 2. Conditional SMAD7 overexpression disrupts canonical TGF-\( \beta \) signaling. A and B, immunostaining and relative quantification for p-SMAD2 (Ser-465/467) (A) and p-SMAD3 (Ser-423/425) (B) in Ins1\text{Dre}SMAD7\text{Hprt}/Rosa26GFP islets at the age of 11 weeks, with and without doxycycline treatment. \( n = 5; *, p < 0.01 \). C and D, Western blotting (C) and quantification (D) of p-SMAD2 (Ser-465/467), p-SMAD3 (Ser-423/425), total SMAD2, and total SMAD3 in isolated islets of 11-week-old Ins1\text{Dre}SMAD7\text{Hprt}/Rosa26GFP with and without doxycycline (3-week) treatment. Protein isolated from islets of three different mice from each group was loaded on separate gels, and a representative band from each gel is shown (C). Western blotting results were analyzed by densitometry (D). \( \beta \)-Actin was used as a protein loading control. \( n = 3 \) mice. *, \( p < 0.01 \). SMAD2 (E) and SMAD3 (F) mRNA levels were analyzed in sorted \( \beta \) cells of 11-week-old Ins1\text{Dre}SMAD7\text{Hprt}/Rosa26GFP with and without doxycycline exposure. \( n = 6–8; *, p < 0.01 \). Error bars, S.D.
our laboratory indicated that SMAD7 is a critical marker of “dedifferentiated” β cells going through a proliferative program (17, 23). Therefore, here we examined the effect of conditional SMAD7 overexpression on key β-cell transcription factors, nkx6.1, pdx1, maf-A, and neuroD1, by quantitative RT-PCR (in sorted purified β cells via FACS) (Fig. 3C) and by immunohistochemistry (in islets) (Fig. S1) in normal and doxycycline-fed (SMAD7-overexpressing) mice. We detected a significant decrease in mRNA levels for pdx1, maf-A, and nkx6.1 in β cells after overexpression of SMAD7 with doxycycline in Ins1DreSMAD7Hprt/Rosa26GFP mice (Fig. 3C). Decreases in the number of pdx1, maf-A, or nkx6.1+ β cells by immunohistochemistry were also seen (Fig. S1). Maf-A is an essential regulator of the insulin gene and governs insulin production in β cells, whereas pdx1 expression is required for maintaining β-cell identity. Similarly, nkx6.1 is critical for maintaining β cells in their differentiated state (26). Following SMAD7 overexpression, we also observed a significant increase in neuroD1 mRNA expression (Fig. 3C). NeuroD1 is known to act as both a positive and negative regulator, activating β-cell–specific genes, including insulin, while repressing somatostatin (27). Thus, it is possible that increased expression of neuroD1 may help to maintain insulin levels and quasi-normal β-cell function despite SMAD7 overexpression–induced dedifferentiation and proliferation. However, further detailed studies are needed to confirm this possibility. Also, in contrast to the published study by Smart et al. (21), where SMAD7 induction impaired β-cell function, we observed that conditional overexpression of SMAD7 did not alter glucose physiology (explained below; Fig. 4). Here, it is possible that increased neuroD1 expression and altered expression of other unidentified factors might be contributing to the maintenance of normal pancreatic β-cell function. Our data provide strong additional in vivo evidence that SMAD7 may have a pivotal role during β-cell dedifferentiation and proliferation.

**β cell–specific SMAD7 overexpression does not impair β-cell function and glucose-stimulated insulin secretion (GSIS)**

We determined the effect of β-cell–specific SMAD7 overexpression on β-cell function and glucose tolerance. Intraportal glucose tolerance test (IPGTT) analysis of control and doxycycline-fed mice showed normal glucose tolerance (Fig. 4A). Likewise, insulin levels measured from serum samples during IPGTT showed normal *in vivo* glucose-stimulated insulin secretion (Fig. 4B). Furthermore, compared with Ins1Dre*SMAD7*Hprt/Rosa26GFP mice on a regular diet, we saw no difference in β-cell mass (Fig. 4C) in doxycycline-fed Ins1Dre*SMAD7*Hprt/Rosa26GFP mice. Our data thus show that mice with conditional overexpression of SMAD7 in β cells maintained a normal β-cell mass and a normal glycemic phenotype. Recently, Wang et al. (18) reported that glucose physiology and GSIS could remain intact despite the induction of proliferation in islets.

These *in vivo* findings prompted us to investigate the direct effects of glucose on insulin secretion in pancreatic β cells. Therefore, we isolated pancreatic islets from control and doxycycline-fed Ins1Dre*SMAD7*Hprt/Rosa26GFP mice, and the isolated islets were incubated in RPMI medium containing 11.1 mmol/liter glucose overnight, followed by examination of GSIS. As shown in Fig. 4D, similar to our *in vivo* observations, insulin secretion in response to both low (2.8 mM) and high (20 mM) glucose was not statistically significantly different between control and SMAD7 overexpressors. Thus, we postulate that despite some dedifferentiation and β-cell proliferation following SMAD7 overexpression, β cells as a whole maintained normal function in mice.

**Figure 4. SMAD7 overexpression does not impair β-cell function and GSIS.** A. IPGTT in normal and doxycycline-fed Ins1Dre*SMAD7*Hprt/Rosa26GFP mice (n = 20). Serum insulin levels after glucose challenge (n = 5) (B) and β-cell mass (n = 3) (C) were measured in Ins1Dre*SMAD7*Hprt/Rosa26GFP mice at age 11 weeks, with and without doxycycline treatment. D. SMAD7 overexpression failed to affect GSIS from isolated pancreatic islets. Isolated pancreatic islets from 11-week-old Ins1Dre*SMAD7*Hprt/Rosa26GFP mice with and without doxycycline (3 weeks) were incubated in RPMI1640 medium containing 11.1 mmol/liter glucose for 24 h, followed by examination of insulin secretion for 60 min in 2.8 or 20 mM glucose (n = 6). *p < 0.01; NS, no significance. Error bars, S.D.
cell–specific SMAD7 knockout mice have reduced β-cell proliferation

We next examined deletion of SMAD7 expression in islets from adult SMAD7 knockout and control mice by quantitative RT-PCR and found a significant decrease in SMAD7 gene transcripts in both homozygous (Ins1CreSMAD7fx/fx) and heterozygous (Ins1CreSMAD7fx/wt) mice (Fig. 5A).

To confirm whether there was a corresponding reduction in β-cell proliferation in these SMAD7 knockout mice, we analyzed BrdU labeling of β cells and found a significantly reduced number of BrdU+/insulin+ cells in the knockout islets compared with control mice (Fig. 5B and D). We obtained similar results with Ki67+/insulin+ staining (Fig. 5C and E).

β cell–specific SMAD7 knockout did not affect glucose physiology

We then measured the fasting blood glucose of adult SMAD7 knockout and control mice and found no significant differences (Fig. 6A). To further detect reduced insulin secretion in the SMAD7 knockout mice, we measured random serum insulin levels by ELISA and again found that the insulin levels were not significantly different from control mice (Fig. 6B).

SMAD7 knockout mice have normal insulin sensitivity and glucose clearance

We performed IPGTT to determine glucose tolerance in SMAD7 knockout mice. Homozygous SMAD7 knockout mice were able to clear the glucose normally within 2 h, similar to controls (Fig. 6C). Next, we performed insulin tolerance testing to determine insulin sensitivity in SMAD7 knockout mice. Again, we saw no difference in insulin tolerance between knockout and control mice (Fig. 6D).

Effect of SMAD7 knockout on GSIS ex vivo

At 2.8 mM low glucose concentrations, islets from SMAD7 knockout mice have a significantly increased insulin secretion compared with control mice islets (Fig. 6E). However, insulin secretion by islets from SMAD7 knockout and control mice at high glucose concentrations (20 mM) was not different (Fig. 6F). Because of the significant difference in the insulin secretion in response to low glucose, we measured the islet insulin content, which showed no difference between SMAD7 knockout and control islets (Fig. 6F).

Discussion

In the adult islet, β cells normally replicate at a very low rate (28, 29), but several studies have now shown that the primary
mechanism for β-cell regeneration following an increased demand for insulin or during repair after an injury is by increased replication of existing β cells (22, 23, 30, 31). Our previous work, and that of others, has shown that TGF-β signaling has an important role in β-cell proliferation, specifically mediated through SMAD7 expression in proliferating cells (22–24). When differentiated β cells enter a proliferative phase, the cells lose SMAD2 and SMAD3 expression and become SMAD7-positive. Early embryonic progenitor cells in the pancreas express SMAD7 but, later in gestation, instead then express only SMAD2 and SMAD3 in differentiated hormone-positive cells (17). Similarly, adult differentiated β cells do not express SMAD7 but are typically positive for SMAD2 and SMAD3 (23). During replication or regeneration, β cells undergo dedifferentiation and transiently express markers of proliferation and temporarily lose insulin expression (13) as well as other mature β-cell markers (32). Furthermore, in the absence of SMAD7, β-cell proliferation is inhibited, both dur-

Figure 6. Loss of SMAD7 in β cells does not impair β-cell function. A, fasting blood glucose levels were measured in WT, heterozygous, and homozygous SMAD7 knockout mice with no significant differences seen. n = 5–7; *, p < 0.01. B, random serum insulin levels trended lower but were not statistically significant between normal and SMAD7 knockout mice. n = 3; *, p < 0.01. C, IPGTT was done in control and SMAD7 knockout mice after overnight fasting, and the glucose tolerance was not different between the groups. n = 3–4; *, p < 0.01. D, IPITT was done in SMAD7 knockout mice along with control and heterozygous mutants after a 6-h fast. Blood was collected from the tail vein, and glucose was measured at specified intervals. We saw no difference between the groups, again suggesting that SMAD7 knockout mice have normal insulin sensitivity. n = 3; *, p < 0.01. Pancratic islets were isolated from 8-week-old WT and SMAD7 knockout mice and then incubated in RPMI1640 medium containing 11.1 mmol/liter glucose for 24 h, followed by examination of insulin secretion (E) for 60 min in 2.8 or 20 mm glucose (n = 4) and quantification of total insulin content (F) (n = 3). *, p < 0.01. Error bars, S.D.
TGF-β signaling in β-cell function

...ing development (17) and in regenerating adult islets (23, 24). Here, in this study, we wished to pursue whether SMAD7-dependent β-cell changes, which appear to be necessary for adult β-cell proliferation, carry a necessary concomitant loss of β-cell function in the process. To test the role of SMAD7 in β-cell function, in this study, we compare two separate models, one in which SMAD7 expression is specifically deleted in the β cells and another in which SMAD7 is conditionally overexpressed specifically in β cells, to identify the interplay between SMAD7 and other factors for regulating both β-cell mass and function.

Loss of SMAD7 in β cells did not affect glucose physiology

SMAD7 can inhibit all TGF-β ligand–mediated signaling, suggesting an important regulatory role for SMAD7 in endocrine development, differentiation, and maturation (33–35). Following the deletion of SMAD7 expression in β cells, we saw reduced β-cell proliferation. However, in these mice, although lacking SMAD7, β-cell physiology appeared normal. Rodents have been shown to retain normal glucose metabolism even after loss of a significant fraction of the pancreas (60–80% loss) (22, 23). In stark contrast to rodents, humans develop severely impaired glucose tolerance after 50–65% loss of their pancreas (36–38). The rodent islet seems to be able to compensate for such loss by the proliferation of existing β cells (39), whereas humans may not. The TGF-β signaling inhibitor, SMAD7, appears to be a key factor required for β-cell regeneration, because mice lacking SMAD7 in the entire pancreas failed to regenerate following partial pancreatectomy (23). Interestingly, these mice still maintained euglycemia (23), suggesting that the rodent pancreas has the ability to maintain normal glucose physiology despite a significant deficiency in β cells, which may explain our findings that, after β-cell-specific loss of SMAD7 expression, the resulting partial loss of β cells did not alter glucose physiology. Thus, SMAD7 may represent a therapeutic target in humans to allow enhanced β-cell proliferation without concomitant loss of β-cell function.

β-Cell SMAD7 overexpression does not affect baseline β-cell functions

Inhibiting TGF-β signaling by way of knocking out SMAD2 or -3 in the pancreas significantly increased endocrine cell numbers (17), further suggesting that inhibiting TGF-β signaling may be a normal, endogenous mechanism to allow for β-cell proliferation. Because SMAD7 is a key inhibitor of TGF-β signaling, and because we found that β-cell–specific loss of SMAD7 affected both β-cell proliferation and β-cell mass, we then developed a Dre recombinase method to overexpress SMAD7, specifically in β cells. Here, we saw a significant increase in β-cell proliferation following SMAD7 overexpression. Thus, SMAD7 alone is sufficient to drive β-cell proliferation. However, the increased β-cell proliferation did not translate into an increase in β-cell mass, perhaps because β-cell mass is a much less sensitive indicator of β-cell proliferation. Similar to an earlier finding (21), we saw an increase in dedifferentiation of β cells, with a decrease in the expression of mature β-cell markers, pdx1, maf-A, and nkx6.1, in SMAD7-overexpressing islets. Also, we saw reduced expression of phosphorylated SMAD2 and SMAD3 after SMAD7 overexpression in β cells, further indicating the dedifferentiation of β cells. However, in contrast to a previous study (21), we saw normal glucose physiology and insulin secretion in our model of SMAD7 overexpression. The difference between these two models may be in the manner of regulation of expression of SMAD7. We used insulin1 Dre to activate the SMAD7 overexpression allele, but then the actual overexpression is directly regulated by exogenous doxycycline. In the previous model (21), pdx1 promoter activity determined SMAD7 expression, and the heterozygous knock-in to the pdx locus created a baseline diabetic phenotype. In that study by Smart et al. (21), which used pdx1 Cre to drive SMAD7 overexpression, there was a significant increase in the number of glucagon-positive cells and a significant decrease in the number of insulin-positive cells (21). In our study, we did not see a similar switch in the islet cell population; nor did we see a significant difference in β-cell mass following SMAD7 overexpression with insulin Dre, which may explain the normal glucose physiology and normal insulin secretion. In summary, our study shows that β-cell–specific SMAD7 expression can drive β-cell proliferation, but without negative effects on β-cell function and glucose homeostasis.

Experimental procedures

Mouse manipulations

All mouse experiments were approved by the Animal Research and Care Committee at the Children’s Hospital of Pittsburgh and the University of Pittsburgh Institutional Animal Care and Use Committee. MIP-GFP mice (GFP reporter under the control of a mouse insulin promoter) have been described before (40). All mice had a C57BL/6 background (purchased from the Jackson Laboratory, Bar Harbor, ME).

SMAD7 knockout

Floxed SMAD7 knock-in (SMAD7fx/fx) and insulin1Cre knock-in mice were obtained from the Jackson Laboratory. Insulin1Cre mice were crossed with SMAD7fx/fx to generate β-cell–specific SMAD7 (Ins1Cre;SMAD7fx/fx) knockout mice. SMAD7 deletion is confirmed by real-time PCR (Fig. 5A).

SMAD7 overexpression

Insulin1Dre knock-in, SMAD7Hprt, and rosa26GFP (Dre reporter) mice were all recently generated in our laboratory. Previously, we developed a novel Dre/RoxP system in combination with a Cre/LoxP system to create a dual lineage-tracing system for both Pdx1 lineage and Ptf1a lineage cells in the developing and adult mouse pancreas (25). The SMAD7Hprt knock-in mouse has a Dre-dependent tetracycline-inducible SMAD7 expression cassette at 1.5 kb upstream of the HPRT gene exon 1. The expression cassette consists of two elements in head-to-head arrangement: the Tet-On 3G transactivator gene, whose expression occurs after removal of a transcriptional stop signal by Dre, and the SMAD7 gene, whose expression is controlled by a tetracycline response element (TRE3G). In these mice, SMAD7 overexpression is localized to the HPRT locus (SMAD7Hprt), on the X chromosome. SMAD7 expression is tissue-specific, based on Dre expression, and regulatable, based on tet-on (Fig. 1A). The insulin1Dre mice have an insu-
lin1-Dre knock-in allele, and thus express Dre under the control of the endogenous insulin1 gene promoter. The insulin1

**Generation of SMAD7<sup>Hprt</sup> mice**

pTetOne vector (Clontech, catalog no. 634303) was used to construct a Dre-dependent tetracycline-inducible SMAD7 expression cassette. The mouse SMAD7 coding sequence with a Kozak consensus sequence attached on its 5’ end was inserted into a multiple cloning site located downstream of TRE3Gs promoter in pTetOne. The PKG promoter that drives expression of Tet-On 3G transactivator in pTetOne was replaced with a CAG promoter followed by a Dre recognition site (Rox) flanked transcriptional stop signal (RSR). The resulting SMAD7 expression cassette was then transferred to the Hprt targeting vector, pEMS1307, to generate an Hprt-SMAD7 targeting vector. pEMS1307 was a gift from Elizabeth Simpson (Addgene plasmid 29149) and was designed for a single-copy knock-in at the Hprt1 locus using ES cells carrying a spontaneous deletion at the Hprt1 locus, Hprt<sup>1b-m3</sup> (41). Complementary Hprt1 sequence within pEMS1307 restores the Hprt1 gene when targeted to the Hprt<sup>1b-m3</sup> gene, which enables selection of correctly targeted cells using HAT medium. To take advantage of superior competence of G4 ES cells (a gift from Andras Nagy, MMRRC 011986-MU) for generation of aggregation chimeras (42), the Hprt1<sup>1b-m3</sup> mutation was introduced to G4 cells by traditional gene targeting. The Hprt-SMAD7 targeting vector was introduced into G4-Hprt<sup>1b-m3</sup> cells by electroporation, and targeted cells were selected by HAT medium. Chimeric mice from correctly targeted ES cells were generated by aggregation with CD1 E2.5 morula.

**Generation of insulin1<sup>Dre</sup> knock-in mice**

The BAC clone RPCI-23-175M3, which harbors the Ins1 gene locus of C57BL/6J mouse genomic DNA, was obtained from the BACPAC Resources Center at the Children’s Hospital Oakland Research Institute (Oakland, CA) and was modified by BAC recombining (43) to construct a targeting vector. Briefly, the entire coding region and 3’ UTR of the Ins1 gene, both located in exon 2, were replaced with the Dre coding (44) and SV40 poly(A) sequences, respectively. A neo cassette flanked by two FRT sites was inserted immediately downstream of the Dre-poly(A) sequence for positive selection of targeted ES cells. The modified Ins1 gene, including a 2.2-kb 5’-homologous arm and a 0.9-kb 3’-homologous arm, was retrieved to plasmid PGKdtabpA (a gift from Philippe Soriano, Addgene plasmid 13440). The resulting ins-Dre targeting vector was introduced into G4 ES cells, and correctly targeted cells were used to produce aggregation chimeras. The neo cassette was removed from F1 progeny by crossing the chimeric mice with FLP deleter mice (The Jackson Laboratory, catalog no. 009086).

**Generation of Dre reporter mice**

The Rosa26 targeting vector for Cre reporter knock-in (Ai6, a gift from Hongkui Zeng, Addgene plasmid 22798) was modified to a Dre reporter knock-in vector by replacing the LoxP sequences flanking a stop cassette with the Rox sequences. The ZsGreen gene was also replaced with the AcGFP1 gene. A gene targeting was performed in G4 ES cells, and aggregation chimeras were produced to establish the Rosa26<sup>GFP</sup> Dre reporter mouse line.

To generate insulin1<sup>Dre</sup>/SMAD7<sup>Hprt/Rosa26GFP</sup> (Ins1<sup>Dre</sup> SMAD7<sup>Hprt/Rosa26GFP</sup>) mice, we crossed Insulin1<sup>Dre</sup> mice with SMAD7<sup>Hprt</sup> and Rosa26<sup>GFP</sup> mice to assess β cell–specific autonomous effects of SMAD7 overexpression. For the current study, we used 8–11-week-old triple-positive males (Ins1<sup>Dre</sup>/SMAD7<sup>Hprt/Rosa26GFP</sup>) and/or homozygous quadruple-positive females (Ins1<sup>Dre</sup>/SMAD7<sup>Hprt/Rosa26GFP</sup>). To induce SMAD7 overexpression in Ins1<sup>Dre</sup>/SMAD7<sup>Hprt/Rosa26GFP</sup> mice, doxycycline (625 mg) was provided in the diet at 8 weeks of age, and mice were maintained on a doxycycline diet for 3 weeks. Glucose tolerance tests and serum were collected for measuring insulin concentrations before sacrificing mice for post-mortem analysis. For β-cell proliferation analysis, 0.1% BrdU (Sigma–Aldrich) in 1% sucrose was supplemented in the drinking water for 1 week before euthanasia. All mice were caged with free access to fresh water and fed a standard diet.

**Intraperitoneal glucose tolerance test and serum insulin measurement**

For the IPGTT, 16-h-fasted mice were injected with 2 g/kg glucose (Sigma–Aldrich). For the intraperitoneal insulin tolerance test (IPITT), 6-h-fasted mice were injected with 0.075 units/kg insulin (Humulin<sup>R</sup>, Lilly). Blood glucose levels were detected at 0, 15, 30, 60, 90, and 120 min after injection using a glucometer. During IPGTT, tail vein blood was collected at 0, 15, and 30 min after glucose injection for measuring serum insulin concentration. Insulin levels were measured using an insulin ELISA kit (Alpco, Salem, NH).

**Pancreatic digestion, islet isolation, and FACS for β cells**

The islet isolation was performed following a previously published protocol (45). The pancreatic duct was perfused and subsequently digested with Type V collagenase (1.95 mg/ml). Islets were separated from the exocrine tissue with a discontinuous Ficoll gradient and then washed with Hanks’ balanced salt solution (Gibco) containing 20 mM HEPES buffer (Gibco) and 0.2% BSA (Sigma–Aldrich). Islets were then handpicked to eliminate any contamination from exocrine tissue. β-Cell purification from islets of Ins1<sup>Dre</sup>/SMAD7<sup>Hprt/Rosa26GFP</sup> and MIP-GFP mice was done by taking advantage of the specific expression of the GFP in β cells following a protocol described previously (24).

**Glucose-stimulated insulin secretion (GSIS) assay (ex vivo)**

Insulin secretion ex vivo was assessed by static incubation using isolated islets. Briefly, after overnight culturing in RPMI containing 11.1 mM glucose, 30 islets from each mouse were transferred to new plates and treated with Krebs–Ringer medium containing low-glucose (2.8 mM) and high-glucose (20 mM) conditions. Assessment of insulin content in islets was performed by acid-ethanol extraction using 20 islets/mouse. Secreted insulin levels and islet insulin content were measured using an insulin ELISA kit (Alpco, Salem, NH). All insulin secretion data were normalized to the islet protein content. Results are reported as insulin secreted (ng)/μg of islet protein.
TGF-β signaling in β-cell function

Isolation of RNA and quantitative RT-PCR

Total RNA was extracted from purified β cells using the RNeasy Plus extraction kit (Qiagen, Hilden, Germany). RNAs were reverse-transcribed into cDNA using Superscript II reverse transcriptase (Qiagen, Germantown, MD). The following primers all were purchased from Qiagen (Valencia, CA): SMAD7 (PPM03073F), SMAD2 (PPM04430C), SMAD3 (PPM04461C), pdx1 (PPM04509D), neuroD1 (PPM05527D), mafA (PPM39984A), and nks6.1 (PPM32738G). Gene expression was normalized for the β-actin (PPM02945B), which proved to be stable across the samples. -Fold changes from the control are shown in the figures.

Western blotting

Western blotting was performed as described before (24), using total protein isolated from the islets, which was then separated on SDS-polyacrylamide gels and subsequently transferred to polyvinylidene difluoride membrane. Membranes were then incubated with one of the following antibodies: mouse monoclonal anti-SMAD7 (R&D Systems), rabbit monoclonal anti-total SMAD2/3 (Cell Signaling), rabbit monoclonal anti-phospho-SMAD2(Ser-465/467)/SMAD3(Ser-423/425), and rabbit monoclonal anti-β-actin (Cell Signaling) antibodies. The secondary antibody was either horseradish peroxidase–conjugated anti-rabbit (Bio-Rad) or anti-mouse (Cell Signaling). Band quantification was performed using the ImageJ software.

Immunohistochemistry

For immunohistochemistry, all pancreas samples were fixed with 4% paraformaldehyde and cryoprotected in 30% sucrose overnight before freezing and sectioning at 6 μm. GFP was detected by direct fluorescence. Primary antibodies for immunostaining were as follows: guinea pig polyclonal anti-insulin, guinea pig polyclonal anti-pdx1, rabbit monoclonal anti-nks6.1, and rabbit monoclonal anti-phospho-SMAD3 (Ser-423/425) all purchased from Abcam; rabbit monoclonal anti-mafA (Cell Signaling); rat monoclonal anti-Ki67 (Invitrogen); and rabbit polyclonal anti-phospho-SMAD-2 (Ser-465/467) (Thermo Fisher Scientific). BrdU staining has been described before (17, 22, 23). fluorescent conjugated (FITC, CY3, CY5) secondary antibodies against the primary antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclear staining and mounting were performed with Fluoroshield with 4′,6-diamidino-2-phenylindole (Sigma–Aldrich).

Quantifications and data analysis

For quantification of β-cell proliferation, the percentages of BrdU+/Ki-67+ per insulin+ cell were calculated. Pdx1+1, Maf-A+, Nkx6.1+, p-SMAD2 (Ser-465/467)+, and p-SMAD3 (Ser-423/425)+ β cells were manually quantified from at least six sections that were 100 micron apart for each mouse. At least 3,000 cells were counted for each experimental condition. Counting continued beyond 3,000 cells until 50 positive cells were tallied if the percentage of positive cells was low. All values are depicted as mean ± S.D. from five animals for each experimental condition. The β-cell mass/area was quantified as described previously (22). In brief, 10 sections at 100 micron intervals from the whole pancreas were immunostained for insulin and 4′,6-diamidino-2-phenylindole and imaged using a confocal microscope. Captured images of whole sections were analyzed using ImageJ software. Average β-cell mass was calculated by multiplying the islet (insulin-positive)/pancreas area ratio by pancreatic weight. The β-cell mass was estimated by examining pancreata from three animals for each group. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by the Fisher exact test or by unpaired Student’s t test. Data were considered significant when p < 0.05.

Data availability

All data presented in this paper are contained within the article.

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References

1. Moses, H. L., Arteaga, C. L., Alexandrow, M. G., Dagnino, L., Kawabata, M., Pierce, D. F., Jr., and Serra, R. (1994) TGF-β regulation of cell proliferation. *Princess Takamatsu Symp.* 24, 250–263 [Medline]
2. Moses, H. L., Coffey, R. J., Jr., Leof, E. B., Lyons, R. M., and Keski-Oja, J. (1987) Transforming growth factor β regulation of cell proliferation. *J. Cell. Physiol. Suppl.* 5, 1–7 [CrossRef Medline]
3. Moses, H. L., Yang, E. Y., and Pietenpol, J. A. (1990) TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* 63, 245–247 [CrossRef Medline]
4. Gittes, G. K. (2009) Developmental biology of the pancreas: a comprehensive review. *Dev. Biol.* 326, 4–35 [CrossRef Medline]
5. Maldonado, T. S., Kadison, A. S., Crisera, C. A., Grau, J. B., Alkasab, S. L., Longaker, M. T., and Gittes, G. K. (2000) Ontogeny of activin B and follistatin in developing embryonic mouse pancreas: implications for lineage selection. *J. Gastrointest. Surg.* 4, 269–275 [CrossRef Medline]
6. Miralles, F., Czerneckow, P., and Scharffman, R. (1998) Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. *Development* 125, 1017–1024 [PubMed Medline]
7. Szabat, M., Johnson, J. D., and Piret, J. M. (2010) Reciprocal modulation of adult β-cell maturity by activin A and follistatin. *Diabetologia* 53, 1680–1689 [CrossRef Medline]
8. Zhang, Y. Q., Cleary, M. M., Si, Y., Liu, G., Eto, Y., Kritzik, M., Dabernat, S., Kayali, A. G., and Sarvetnick, N. (2004) Inhibition of activin signaling induces pancreatic epithelial cell expansion and diminishes terminal differentiation of pancreatic β-cells. *Diabetes* 53, 2024–2033 [CrossRef Medline]
9. Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L., and Doetschman, T. (1997) TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development* 124, 2659–2670 [PubMed Medline]
10. Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., and Bu, D., Heisterkamp, N., Groffen, J. (1995) Abnormal lung development and cleft
pate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interaction. Nat. Genet. 11, 415–421 CrossRef Medline

11. Böttger, E. P., Jakubczak, J. L., Haines, D. C., Bagnall, K., and Wakefield, L. M. (1997) Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor β receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz[a]-anthracene. Cancer Res. 57, 5564–5570 Medline

12. Böttger, E. P., Jakubczak, J. L., Roberts, I. S., Mumy, M., Hemmati, P., Bagnall, K., Merlino, G., and Wakefield, L. M. (1997) Expression of a dominant-negative mutant TGF-β type II receptor in transgenic mice reveals essential roles for TGF-β in regulation of growth and differentiation in the exocrine pancreas. EMBO J. 16, 2621–2633 CrossRef Medline

13. Tulachan, S. S., Tei, E., Hembre, M., Crisera, C., Prasad, K., Koizumi, M., Shah, S., Guo, P., Böttger, E., and Gittes, G. K. (2007) TGF-β isoform signaling regulates secondary transition and mesenchymal-induced endocrine development in the embryonic mouse pancreas. Dev. Biol. 305, 508–521 CrossRef Medline

14. Brorsen, M., Hougaard, D. M., Nielsen, J. H., Tornehave, D., and Larsson, L. I. (2001) Expression of SMAD signal transduction molecules in the pancreas. Histochem. Cell Biol. 116, 263–267 CrossRef Medline

15. Matsuzaki, K. (2013) SMAD phospho-isoforms direct context-dependent TGF-β signaling. Cytokine Growth Factor Rev. 24, 385–399 CrossRef Medline

16. Wrighton, K. H., Lin, X., and Feng, X. H. (2009) Essential for inflammation-induced but not normal and streptozotocin-treated newborn rats: site, dynamics and capacity. Diabetologia 37, 1088–1096 CrossRef Medline

17. El-Gohary, Y., Tulachan, S., Guo, P., Wiersch, J., Prasad, K., Paredes, J., Shiota, C., Xiao, X., Wada, Y., Diaz, M., and Gittes, G. (2013) SMAD signaling pathways regulate pancreatic endocrine development. Dev. Biol. 378, 83–93 CrossRef Medline

18. Wang, P., Karakose, E., Liu, H., Swartz, E., Ackeifi, C., Zlatanic, V., Wilson, L. I. (2001) Expression of SMAD signal transduction molecules in the pancreas. Proc. Natl. Acad. Sci. U.S.A. 98, 103, 1858–1863 CrossRef Medline

19. Smart, N. G., Apelqvist, A. A., Gu, X., Harmon, E. B., Topper, J. N., Mac-Donald, R. J., and Kim, S. K. (2006) Conditional expression of SMAD7 in pancreatic β cells disrupts TGF-β signaling and induces reversible diabetes mellitus. PLoS Biol. 4, e39 CrossRef Medline

20. Kuang, C., Xiao, Y., Liu, X., Stringfield, T. M., Zhang, S., Wang, Z., and Chen, Y. (2006) In vivo disruption of TGF-β signaling by SMAD7 leads to premalignant ductal lesions in the pancreas. Proc. Natl. Acad. Sci. U.S.A. 103, 1858–1863 CrossRef Medline

21. Smaller, N. G., Apelqvist, A. A., Gu, X., Harmon, E. B., Topper, J. N., Mac-Donald, R. J., and Kim, S. K. (2006) Conditional expression of SMAD7 in pancreatic β cells disrupts TGF-β signaling and induces reversible diabetes mellitus. PLoS Biol. 4, e39 CrossRef Medline

22. Xia, P., Wiersch, J., El-Gohary, Y., Guo, P., Prasad, K., Paredes, J., Shiota, C., Xiao, X., Wada, Y., Diaz, M., and Gittes, G. K. (2013) TGF-β receptor signaling is essential for inflammation-induced but not β-cell workload-induced β-cell proliferation. Diabetes 62, 1217–1226 CrossRef Medline

23. El-Gohary, Y., Tulachan, S., Wiersch, J., Guo, P., Welsch, K., Prasad, K., Paredes, J., Shiota, C., Xiao, X., Wada, Y., Diaz, M., and Gittes, G. (2014) A SMAD signaling network regulates islet cell proliferation. Diabetes 63, 224–236 CrossRef Medline

24. Xiao, X., Gafar, I., Guo, P., Wiersch, J., Fischbach, S., Peiris, L., Song, Z., El-Gohary, Y., Prasad, K., Shiota, C., and Gittes, G. K. (2014) M2 macrophages promote β-cell proliferation by up-regulation of SMAD7. Proc. Natl. Acad. Sci. U.S.A. 111, E1211–E1220 CrossRef Medline

25. Chen, C., Shiota, C., Agostinelli, G., Ridley, D., Jiang, Y., Ma, J., Prasad, K., Xiao, X., and Gittes, G. K. (2019) Evidence of a developmental origin for β-cell heterogeneity using a dual lineage-tracing technology. Development 146, dev164913 CrossRef Medline

26. Prasad, K., Shiota, C., Xiangwei, X., Ricks, D., Fusco, J., and Gittes, G. (2016) A synopsis of factors regulating β cell development and β cell mass. Cell Mol. Life Sci. 73, 3623–3637 CrossRef Medline