Transforming Growth Factor-β/Smad3 Signaling Regulates Insulin Gene Transcription and Pancreatic Islet β-Cell Function

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Huei-Min Lin, Ji-Hyeong Lee, Hariom Yadav, Anil K. Kamaraju, Eric Liu, Duan Zhigang, Anthony Vieira, Seong-Jin Kim, Heather Collins, Franz Matschinsky, David M. Harlan, Anita B. Roberts, and Sushil G. Rane

From the Diabetes Branch, NIDDK, the Laboratory of Cell Regulation and Carcinogenesis, NCI, National Institutes of Health, Bethesda, Maryland 20892, the Lee Gil Ya Cancer & Diabetes Institute, Gachon University of Medicine & Science, Songdo, Incheon 406-840, Korea, and the Department of Biochemistry & Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Pancreatic islet β-cell dysfunction is a signature feature of Type 2 diabetes pathogenesis. Consequently, knowledge of signals that regulate β-cell function is of immense clinical relevance. Transforming growth factor (TGF)-β signaling plays a critical role in pancreatic development although the role of this pathway in the adult pancreas is obscure. Here, we define an important role of the TGF-β pathway in regulation of insulin gene transcription and β-cell function. We identify insulin as a TGF-β target gene and show that the TGF-β signaling effector Smad3 occupies the insulin gene promoter and represses insulin gene transcription. In contrast, Smad3 small interfering RNAs relieve insulin transcriptional repression and enhance insulin levels. Transduction of adenoviral Smad3 into primary human and non-human primate islets suppresses insulin content, whereas, dominant-negative Smad3 enhances insulin levels. Consistent with this, Smad3-deficient mice exhibit moderate hyperinsulinemia and mild hypoglycemia. Moreover, Smad3 deficiency results in improved glucose tolerance and enhanced glucose-stimulated insulin secretion in vivo. In ex vivo perfusion assays, Smad3-deficient islets exhibit improved glucose-stimulated insulin release. Interestingly, Smad3-deficient islets harbor an activated insulin-receptor signaling pathway and TGF-β signaling regulates expression of genes involved in β-cell function. Together, these studies emphasize TGF-β/Smad3 signaling as an important regulator of insulin gene transcription and β-cell function and suggest that components of the TGF-β signaling pathway may be dysregulated in diabetes.

Incidence of the “metabolic syndrome,” a complex condition linked to insulin resistance, type 2 diabetes and obesity, is increasing worldwide (1). The pancreatic islet β-cell, due to its unique function of insulin synthesis and glucose-stimulated insulin secretion, is a prime target of affliction in diabetes (2). In addition, a majority of Type 2 diabetes patients develop insulin resistance in target organs of insulin action: liver, muscle, and adipose tissue (3). Improved mechanistic understanding of normal β-cell function and insulin action is needed to enable early diagnosis of β-cell dysfunction and insulin resistance and to facilitate development of new rational therapeutics for diabetes.

The transforming growth factor-β (TGF-β)³ superfamily, which includes the TGF-β isoforms, activins, and the bone morphogenetic proteins (BMPs), regulates gene expression in diverse cell types and is involved in a myriad of cellular processes including cell proliferation, differentiation, and apoptosis (4–6). Activated TGF-β family isoforms signal via dual Type I and Type II transmembrane serine/threonine kinase receptors and effector Smad transcription factors (4–6). Ligand binding and receptor activation leads to phosphorylation and activation of Smads, which translocate to the nucleus and regulate transcription of target genes.

Development of the endocrine and exocrine pancreas is controlled by factors that include members of the TGF-β superfamily (7, 8). In addition, TGF-β signaling has been implicated in pancreatic diseases (9). BMP signaling plays an instructive role during early pancreatic development (7–9) and regulates mature β-cell function (10, 11), whereas activin signaling regulates islet morphogenesis and β-cell mass (12, 13). TGF-β isoforms are expressed in the epithelium and mesenchyme of embryonic pancreas and in adult pancreas (14). Islet cells demonstrate diffuse cytoplasmic immunostaining for TGF-β isoforms with most of the positive islet cells co-expressing insulin. TGF-β receptors (TBR1 and TBRII) are present in the pancreatic epithelium and mesenchyme during early stages of development and postnatally in pancreatic islets and ducts. Furthermore, Smad proteins are expressed in the pancreas, which elucidates that components needed for activation of the canonical TGF-β signaling exist within the pancreas.

³ The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; CA-ALK, constitutively active activin-like kinase; Ad, adenoviral; GSSG, glucose-stimulated insulin secretion; GSR, glucose-stimulated insulin release; FBS, fetal bovine serum; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; SBE, Smad-binding element; ERK, extracellular signal-regulated kinase.

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We dedicate this article to the memory of Dr. Anita B. Roberts in appreciation of her insightful suggestions.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables A–C and Figs. S1–S7.

Both authors contributed equally to this work.

To whom correspondence should be addressed: CRC, 5-5940, 10 Center Dr., Bethesda, MD 20892. Tel.: 301-451-9834; E-mail: ranes@mail.nih.gov.
Disruption of TGF-β signaling at the receptor level using mice expressing the dominant-negative TGF-β type II receptor (DNTβRII) resulted in increased proliferation of pancreatic acinar cells and severely perturbed acinar differentiation (15). Additionally, DNTβRII mice exhibit increased endocrine precursors and proliferating endocrine cells, with an abnormal accumulation of endocrine cells around the developing ducts of mid-late stage embryonic pancreas (16). Transgenic mice expressing TGF-β1 in β-cells exhibit abnormal small islet cell clusters without formation of normal adult islets although the overall islet cell mass is not significantly diminished (17). Although these studies underscore the importance of TGF-β signaling in β-cell development, they do not address its role in postnatal β-cell growth and function. In this article, we examined the role of TGF-β signaling in β-cell function and uncover its importance in regulating insulin levels and glucose-stimulated insulin secretion.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The pancreatic β-cell line INS-1E (obtained from Dr. C. Wollheim) was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 50 μg/μl β-mercaptoethanol, 1 mM sodium pyruvate, and 10 mM HEPES. βHC9 and NIT (ATCC, VA) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Cells were cultured in medium with 10% FBS, which contains less than 0.5–1 ng/ml of total TGF-β (greater than 99% of TGF-β1 is in latent form, which is not immediately available to the cells). During TGF-β stimulation, cells were washed with phosphate-buffered saline and replaced with medium containing 0.2% FBS with 11 mM glucose after which they were treated with/without 2–5 ng/ml of TGF-β1 (R&D Systems, Indianapolis, IN).

**Islet Isolation**—Non-human primate and cadaver human pancreatic islets were isolated and cultured as described previously (18, 19) with minor modifications. Mouse and rat islets were isolated using similar methods. Animals were euthanized and their pancreases were inflated with cold collagenase type V (Sigma, C-9263) dissolved in Ca2+ and Mg2+ containing Hanks’ balanced salt solution. The pancreatic tissues were digested for 15–20 min at 37 °C. Islets were separated by Ficoll gradient (density: 1.077) by centrifuging at 1700 × g for 5 min. Middle islet-enriched fraction was collected and washed with Dulbecco’s modified Eagle’s media. The islets were handpicked using a glass pipette under a stereomicroscope for further assays.

**TGF-β Treatment of Primary Islets**—Islets were isolated from 6-week-old wild-type mice as described above and 70–80 islets were plated per well in a 24-well plate with 1 ml of RPMI 1640 medium (with 0.2% FBS). After 1 h, the medium was replaced with fresh RPMI 1640 medium containing 5 ng/ml TGF-β1 and 0.2% FBS. Control islets were incubated in RPMI 1640 medium without TGF-β1. After 24 h of incubation, islets were harvested, washed with phosphate-buffered saline, and processed for RNA isolation and quantitative gene expression.

**Plasmids**—pGL3INS-0.15kb-Luc was generated by deletion of the entire region upstream of 0.15 kb of the pGL3INS2.9-Luc. Information on plasmids SBE-Luc, CAGA12-Luc, FLAG-Smad3, FLAG-Smad4, CA-Smad3, CA-Smad2, S3-MH1, S3-MH2, S3-MH1-linker, S3-MH2-linker, TβRII, TβRI, CA-ALK5/ TβRI, and Smad3(SA) is published elsewhere (15, 20–26). Anti-Smad3 antibody was from Zymed Laboratories Inc., Inc. Inhibitor of the TGF-β receptor 1 kinase (SB431542) was from Sigma.

**Transfections and Luciferase Assays**—Cells in 6-well plates were transfected with 1 μg of reporter plasmid (hINS-Luc, CAGA12-Luc, SBE2-Luc with/without Renilla luciferase) with/without 0.2 μg of TβRII or Smad expression vectors using FuGENE 6 (Roche). Two days after transfection cells were replaced with medium containing 0.2% FBS with 11 mM glucose and left untreated or stimulated with 2–5 ng/ml TGF-β1 for 24 h before harvesting. Reporter activity was measured using the enhanced luciferase assay kit (BD Biosciences) and normalized to total protein measured by the BCA kit (Pierce) or to Renilla luciferase activity. Reporter assays were repeated at least 4–5 times, with each repeat inclusive of triplicates of each transfection or condition (such as siRNA, drug, or ligand treatments). Average of triplicates along with standard deviation is provided for each representative experiment.

**Chromatin Immunoprecipitation (ChIP) Assay**—INS-1E or βHC9 cells, with or without transfection with Smad3 + Smad4 expression vectors, were used in ChIP assays that were performed using a commercial kit from Upstate Biotechnology. Briefly, proteins and chromatin were cross-linked with 1% formaldehyde for 10 min at 37 °C, and sonicated in SDS lysis buffer. Nonspecific binding was eliminated via preincubation with salmon sperm DNA and Protein A-agarose beads at 4 °C for 30 min. The supernatant was incubated with anti-Smad3, anti-Pdx1, or control antisera overnight at 4 °C. Beads were collected and histone complex was eluted and reverse cross-linked. Recovered DNA was amplified by PCR using the following primer pairs: pair 1, −316 bp (5′-GGAATGTGTTGGAAAAATGTCTAGCAGCAA-3′), and −131 bp (5′-CTTGCCTGTGCTAGTCTCGAGAAA-3′) from the transcriptional start site of rat insulin II promoter to yield a 185-bp fragment in INS-1E cells; pair 2, −327 bp (5′-GGAATGTGTTGGAAAAATGTCTAGCAGCAA-3′) and −143 bp (5′-CTTGCCTGTGCTAGTCTCGAGAAA-3′) from the transcriptional start site of mouse II insulin promoter to yield an 186-bp fragment in βHC9 cells.

**RNA Oligonucleotides and siRNA Transfection**—siRNA duplexes targeting Smad3 were obtained from Dharmacon, Inc. The sense strand sequence of the siRNA was 5′-TCCGCACTGAGCTTCTGAA-3′. To determine the effect of Smad3 siRNA on reporter constructs, cells were transfected with 25–100 nM Smad3 siRNA or 42% GC control siRNA together with 1 μg of reporter using Lipofectamine 2000 (Invitrogen) in RPMI complete medium (with 10% FBS). Cells were harvested 3 days after transfection and luciferase activity was measured.

**Insulin and C-peptide Assay**—Cells were transfected with Smad3 siRNA or 42% GC control siRNA in RPMI complete media. After 72 h of transfection cells were preincubated for 1 h in RPMI medium containing 0.2% FBS and 11 mM glucose, then
replaced with fresh medium and incubated for 2 h. Released insulin/C-peptide was measured from the collected medium by enzyme-linked immunosorbent assay (Alpco). Total insulin from cells or primary islets was extracted with acid-ethanol overnight at −20 °C and the insulin level was normalized to protein content. Pancreatic insulin was prepared by homogenizing the whole pancreas in acid-ethanol and extracted overnight at −20 °C. Primary islets were transduced with Ad-LacZ, Ad-GFP, Ad-Smad3, and Ad-DN-Smad3 in RPMI complete media with 11 mm glucose. Released or total insulin/C-peptide was measured as described above.

Recombinant Adenovirus—Smad3WT and DNSmad3 fragments were subcloned into shuttle vector Ad5CMV-K NpA (ViraQuest). Recombinant adenovirus were amplified and purified by CsCl2 ultracentrifugation. Ad-eGFP and Ad-LacZ were used to monitor the transduction efficiency and as control virus. Transduction efficiency was monitored by evaluating GFP fluorescence (supplemental Fig. S1). Cells were infected at a multiplicity of infection of 6–50 viral particles/cell. Primary islets were transduced with 75–100 viral particles/cell in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS with 11 mm glucose. Released or total insulin/C-peptide was confirmed by Western blot analyses (data not shown). Each transduction experiment was performed on multiple preparations (n = 5 each) of non-human primate or human cadaver donor islets. Each experiment included triplicates of individual adeno viral transduction (Ad-GFP, Ad-LacZ, Ad-Smad3, or Ad-DN-Smad3). Average of triplicates along with standard deviation is provided for each representative experiment.

Animals—Mouse maintenance, breeding, and genotyping were as described elsewhere (27). Unless mentioned otherwise, all animals were fed ad libitum on a standard pellet diet with free access to water and were kept in a room in which a 12-h light-dark cycle (6 a.m. to 6 p.m.) was enforced. All experiments were conducted on 2–4-month-old age and sex-matched littermates. All animal experiments were approved by the NIH animal care and use committee.

Glucose Tolerance Test—Glucose tolerance test was performed on 4-month-old male and female mice after overnight fast. Glucose (2 g/kg of body weight) was injected intraperitoneally. Tail blood samples were drawn before (time 0) and at the indicated times after injection for automated glucose measurement (AscensiaELITE, Bayer, IN).

Insulin Secretion Assay—To examine glucose-stimulated insulin secretion, 2-month-old female mice were fasted for 16 h and then injected intraperitoneally with glucose (4 g/kg of body weight). Blood was collected at the indicated time points in heparinized tubes and plasma insulin levels were measured using an enzyme-linked immunosorbent assay kit.

Histological and Immunohistochemistry Assessment—Pancreas was removed and fixed in 10% formalin. Sections were prepared at 5-μm thickness and stained with hematoxylin and eosin for histological examination. Sections were immunostained with guinea pig anti-insulin (1:1000, Dako), rabbit anti-glucagon (1:200, Sigma), and rabbit anti-Smad3 (1:200, Zymed Laboratories Inc.) antibodies. For insulin immunohistochemistry, biotin-labeled anti-guinea pig IgG was used followed by the ABC-AP (alkaline phosphatase) system from Vector Laboratories. 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium from Sigma was used as AP substrate to develop a blue-purple color. For immunofluorescence staining, Alexa Fluor secondary antibodies were purchased from Molecular Probes and Invitrogen.

Measurement of the β-Cell Mass Ratio—Quantitative evaluation of the β-cell area was performed on insulin-stained sections using image analysis software (Metamorph). The ratio of the β-cell mass was calculated by dividing the entire insulin-positive β-cell area in the whole pancreatic section by the total area of these fields. The analysis was done in three sections (200 μm apart) from one animal and four animals from each group.

Islet Perfusion—200 islets from 2-month-old male Smad3+/+ and Smad3−/− mice were handpicked under a dissecting microscope and placed in a plastic perfusion chamber. The perfusate was Krebs buffer (pH 7.4) containing 2.2 mmol/liter Ca2+, 0.25% of bovine serum albumin and then equilibrated with 95% O2 and 5% CO2. Islets were cultured for 3–5 days in 10 mm glucose and then perifused. Islets were maintained for the first 20 min at 0 mm glucose. From 20 to 60 min a physiological mixture of amino acids was introduced. At 50 min, a glucose ramp was initiated where glucose was added on top of the amino acids at 0.83 mm/min. At 80 min all stimuli were removed until 100 min where 30 mm KCl was added until 120 min. At the end of the experiment, islets were tested for maximum insulin secretion through KCl-mediated depolarization by introducing 30 mmol/liter KCl. Experiments were performed three times as described earlier with minor modifications (28) and results from representative experiments are presented as nanograms of insulin/200 islets/min.

Western Blot Analyses—Protein extracts for Western blots were prepared from freshly isolated islets in lysis buffer containing 10 mmol/liter Tris, pH 7.6, 1% Triton X-100, 0.5% Nonidet P-40, 150 mmol/liter NaCl, 10 mmol/liter sodium orthovanadate, 10 mmol/liter sodium pyrophosphate, 100 mmol/liter sodium fluoride, 1 mmol/liter EDTA, 1 mmol/liter EGTA, and a mixture of protease inhibitors (Roche) as described elsewhere (29). Twenty-five μg of protein was fractionated onto a 10% denaturing SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Blots were incubated with the primary antibody, followed by incubation with horseradish peroxidase-coupled secondary antibody before final detection by chemiluminescence and autoradiography. α-Tubulin was used as loading control. Antibodies used were: p-Akt, Akt, p-FoxO1, FoxO1, p-ERK1/2, ERK1/2, and phospho-Smad3 antibody from Cell Signaling Technology; α-tubulin was from Sigma. Total Smad3 antibody was from Zymed Laboratories Inc., Inc.

Real Time Reverse Transcription-PCR—Islets and INS-1E cells were washed once with phosphate-buffered saline and processed for RNA isolation using the RNeasy® kit (Qiagen, IN) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed to complementary cDNA using the ABI reverse transcription kit (Applied Biosystems). Quantitative gene expression was analyzed by using TaqMan Express Plates (Applied Biosystems). For experiments with islets, three inde-
Smad3 Regulates β-Cell Function

Smad proteins are the established intracellular effectors of TGF-β signaling (5). Upon TGF-β ligand binding, the activated TβRI/ALK-5 receptors recruit and phosphorylate receptor-Smads, i.e. Smad2 and Smad3. We thus examined the role of Smad2 and Smad3 in mediating repression of the insulin promoter. Interestingly, Smad3, but not Smad2, repressed the insulin promoter (Fig. 2A). Wild-type Smad3 and constitutively active Smad3 (CA-Smad3), but not a phosphorylation-defective mutant of Smad3 (SA), activated CAGA12-Luc reporter activity (Fig. 2B). Smad2 or constitutively active Smad2 (CA-Smad2) was unable to activate the CAGA12-Luc reporter (supplemental Fig. 5) that is specific to Smad3-mediated activation (20, 23). Wild-type and constitutively active Smad3 (CA-Smad3), but not constitutively activated Smad2 (CA-Smad2), repressed hINS-Luc (Fig. 2C). Dominant-negative Smad3(SA), which cannot be phosphorylated by activated TβRI, was also unable to repress hINS-Luc (Fig. 2C). The MH1 and MH2 domains of Smad3 facilitate its nuclear import and are critical for both the DNA binding activity and the ability of Smad3 to interact with transcription factors (4, 5). As expected, we observed activation of CAGA12-Luc by wild-type Smad3, but not Smad3 with deletions in the MH1, MH2, or linker domains (Fig. 2D). In contrast, hINS-Luc activity was effectively repressed by wild-type Smad3, but not by Smad3 with deletions within the MH1, MH2, and linker domains (Fig. 2E), suggesting that these

RESULTS

TGF-β/Smad3 Represses Insulin Gene Transcription—To examine the potential regulatory effects of TGF-β on insulin gene transcription, we studied a 2.9-kb human insulin promoter-driven luciferase reporter (hINS-Luc). Primary rat islets respond to TGF-β as evidenced by a robust activation of the TGF-β responsive reporters, CAGA12-Luciferase and Smad-binding element (SBE)-Luciferase (Fig. 1A). Interestingly, under identical conditions, TGF-β potently repressed hINS-Luc in primary rat islets (Fig. 1A). β-Cell lines INS-1E, βHC9, and NIT exhibit a diminished TGF-β response due to reduced TβRII expression (data not shown). Exogenous TβRII expression restored TGF-β responsiveness to INS-1E cells as demonstrated by enhanced CAGA12-Luc activity (Fig. 1B). In contrast, TβRII effectively repressed basal and TGF-β-mediated hINS-Luc reporter activity (Fig. 1B). Interestingly, TβRII transfection resulted in a dramatic inhibitory effect on hINS-Luc activity independent of ligand (Fig. 1B). Pre-treatment of INS-1E cells with small molecule inhibitors of the TβRI kinase blunted insulin promoter repression by TGFβ1/TβRII (Fig. 1C). Interestingly, the suppressive effect of TβRII in the absence of ligand (Fig. 1B) was not completely relieved by the small molecule TβRI kinase inhibitor (Fig. 1C), suggesting that TβRII may have a TGFβ1/TβRII-independent effect on insulin promoter activity. To further examine the role of TGF-β in repression of hINS-Luc, we utilized a constitutively active TβRII kinase (CA-activin-like kinase 5; CA-ALK5 or CA-TβRII) that bypasses the need for phosphorylation-dependent activation of TβRI by TβRII. As expected, CA-ALK5 expression enhanced CAGA12-Luc reporter activity and, in contrast, CA-ALK5 effectively repressed the insulin promoter (Fig. 1D). Together, these studies indicate that TGF-β signaling represses insulin promoter activity.

Smad3 Regulates β-Cell Function
**FIGURE 2.** Smad3 represses insulin promoter activity. 

**A,** repression of hINS-Luc by Smad3, but not Smad2, in INS-1E cells. 

**B** and **C,** wild-type Smad3 and constitutively active Smad3 (CA-Smad3), but not CA-Smad2 or a phosphorylation-defective mutant of Smad3 (SA), can activate the CAGA12-Luc reporter (**B**) and repress the insulin promoter (**C**) in INS-1E cells. 

**D** and **E,** activation of the CAGA12-Luc reporter (**D**) and repression of insulin promoter (**E**) in INS-1E cells by wild-type Smad3, but not Smad3 with deletions in MH1, MH2, or linker domains. Average values ± S.D. are shown for representative experiments (error bars). 

**F,** ChIP assay using non-stimulated INS-1E and HC9 cells. Smad3 or Pdx1 bound to the endogenous insulin promoter in untransfected cells (lanes 1–11) and in INS-1E cells transfected with Smad3 and Smad4 expression vectors (lanes 12–15) was immunoprecipitated using Smad3 antibodies (lanes 3, 7, and 14) or Pdx1 antibodies (lanes 10 and 13), respectively. Positive controls (input lanes 1, 2, and 12) and negative controls (immunoprecipitation without primary antibody, lanes 5 and 9, or with IgG, lanes 4, 8, 11, and 15) are shown. Expected insulin promoter amplification (arrowhead) is shown. 

**G,** schematic depicting the proposed mechanism of inhibition of insulin transcription by TGF-β/Smad3. Upon TGF-β ligand binding, TβRII gets phosphorylated and in turn phosphorylates Smad3 which, along with the common Smad4, translocates to the nucleus to bind and repress the insulin promoter. Also shown are key regulatory elements (32, 33) on the human insulin promoter proximal to the transcriptional start site (+1). Regulators of insulin gene transcription are identified on the bottom and top of the respective interacting element. Note that primers used in ChIP assay to detect Smad3 binding flank the putative Smad box elements (●). The core rightward 5’-GTCT-3’ (→) and leftward 5’-AGAC-3’ (←) motifs of the SBEs are identified.
domains are required for repression. Taken together, these results illustrate the importance of TβRI-phosphorylated Smad3 in insulin gene repression.

TGF-β signaling is propagated via recruitment of Smad transcription factors to promoters of the TGF-β target genes, often in complex with co-activators or co-repressors (4, 5, 30). Smad transcription factors bind DNA elements that comprise the core sequence 5′-GTCT-3′. In addition, to the GTCT element, many TGF-β target gene promoters contain Smad box-like elements (31). Furthermore, the core GTCT sequence is oriented either “rightward” as 5′-GTCT-3′ or “leftward” as 5′-AGAC-3′ motifs (31). Interestingly, sequence analysis of the first 300 bases of the insulin promoter, starting from the transcription start site, revealed several Smad box and Smad box-like elements, in close proximity to elements that facilitate interaction with distinct regulators of insulin transcription (32, 33). Four motifs at positions −156 to −159 bp, −217 to −220 bp, −241 to −244 bp, and −273 to −276 bp harbored the core sequence 5′-GTCT-3′ or 5′-AGAC-3′ (Fig. 2G, supplemental Fig. S3A). Deletion of the human insulin promoter up to −150 bp, which eliminates all the aforementioned putative Smad3 binding sites, resulted in a significantly reduced repression of hINS-Luc by Smad3 (supplemental Fig. S3, A and B).

As our results were indicative of an important role for Smad3 in regulation of insulin gene transcription, we next inquired whether endogenous Smad3 transcription factors are present in the protein complexes bound to the native insulin promoter in β-cells. To directly examine Smad3 promoter occupancy, we performed chromatin immunoprecipitation (ChIP) assays using anti-Smad3 antibodies and primers that amplify regions of the human insulin promoter up to −450 bp. We found endogenous Smad3 bound to the insulin promoter in non-stimulated INS-1E and βHC9 cells. In similar ChIP assays using an anti-Pdx1 antibody, we detected binding of the transcription factor Pdx1 (Fig. 2F), which is suggestive of simultaneous promoter occupancy of a putative repressor (Smad3) and a bona fide activator (Pdx1) on the insulin promoter. Co-transfection of exogenous Smad3 and Smad4, which mimics active TGF-β signaling (34–36), resulted in loss of Pdx1 occupancy, whereas Smad3 binding was retained (Fig. 2F). Taken together, these results identify insulin as a target of TGF-β/Smad3-mediated gene repression (Fig. 2G).

Inhibition of Smad3 Results in Enhanced Insulin Content and Insulin Secretion—Our results thus far suggested that abrogating Smad3-driven TGF-β signaling might enhance insulin levels. We examined this possibility by utilizing small interfering RNA against Smad3 (siSmad3). Treatment of INS-1E and β-HC9 cells with Smad3-siRNA resulted in a substantial reduction of endogenous Smad3 protein level (Fig. 3A). In
agreement, treatment of non-stimulated INS-1E cells with Smad3-siRNA reduced CAGA12-Luc activity (Fig. 3B). In contrast, Smad3-siRNA relieved repression of hINS-Luc (Fig. 3C). Importantly, siSmad3 treatment of INS-1E cells resulted in a dose-dependent increase in intracellular and secreted insulin (Fig. 3, D and E).

Considering the dissimilarities that exist between the rodent and human insulin promoters (32), we next examined whether regulation of insulin content and secretion by Smad3 is conserved in higher order mammalian species. Adenoviral (Ad) transduction of Smad3 (Ad-Smad3) into non-human primate islet preparations and into islets isolated from human cadavers, compared with islets transduced with control Ad-LacZ or Ad-GFP, lead to reduction in intracellular and secreted C-peptide levels (Fig. 3, F and G). In contrast, transduction of dominant negative Smad3 adenovirus, Ad-DN-Smad3, resulted in elevated intracellular C-peptide levels (Fig. 3F). Collectively, these data suggest that enhanced TGFβ/Smad3 signaling suppresses the insulin level and its secretion, whereas, reduced TGFβ/Smad3 signaling relieves that repression.

Smad3-deficient Mice Exhibit Enhanced Glucose Tolerance and Glucose-stimulated Insulin Secretion—Immunofluorescence staining demonstrated that Smad3 is localized either in the perinuclear region or predominantly within the nucleus of -cells of the pancreatic islets (supplemental Fig. S5). To determine whether Smad3 influences -cell function in vivo, we next characterized mice with an inactivating mutation in the Smad3 locus (Smad3−/− mice). At birth, Smad3−/− mice are indistinguishable from their wild-type littermates and survive to adulthood although they exhibit a growth retardation phenotype, forelimb malformation, and mild immune deficiency (27, 37). Pancreatic weights were proportional to the diminished body weight (data not shown). Furthermore, the pancreatic histology, islet morphology, -cell mass, and insulin immunoreactivity in Smad3−/− mice was similar to that observed in Smad3+/+ mice (Fig. 4, A and B, supplemental Fig. S5, A and B). To examine -cell functionality, we first monitored insulin and glucose levels, in fed and fasting states, in Smad3−/− mice and their control Smad3+/+ littermates. Interestingly, we observed that the Smad3−/− mice displayed moderately increased serum insulin levels (Fig. 4, C, D, G, and H) and lower glucose levels (Fig. 4, E, F, I, and J), whereas, glucose levels of Smad3+/+ animals (fasting glucose levels of 93.0 ± 7.4 mg/dl p, 0.62 versus WT) were comparable with that seen in Smad3+/+ mice. Furthermore, pancreatic insulin content was increased in Smad3−/− mice relative to littermate Smad3+/+ mice (Fig. 5A). Together, these results support a role for Smad3 regulating insulin and glucose levels in vivo.

We next assessed the potential of Smad3 in regulating whole body glucose disposal by evaluating glucose tolerance. Intraportal glucose injection evoked a rapid increase in glycaemia, and a gradual return to near normal levels over 120 min in Smad3+/+ mice (Fig. 5, B and C). Comparatively, Smad3−/− mice exhibited a lowered increase in glycaemia with glucose levels significantly lower at all time points between 15 and 90 min (Fig. 5, B and C, supplementary Table SA). Male Smad3−/− mice were more glucose tolerant than female mice as evidenced by the significantly lower (p = 0.009) area under the curve (supplementary Table SB).

Interestingly, an elevated glucose challenge of 4 g/kg body weight induced a significantly increased glucose-stimulated insulin secretion (GSIS) response in vivo in Smad3−/− mice compared with Smad3+/+ mice (Fig. 5E). A statistically significant difference (p < 0.0001) in GSIS was observed in the Smad3−/− mice within 15 min of glucose stimulation (Fig. 5F, supplementary Table SC). Furthermore, ex vivo glucose-stimulated insulin release (GSIR) was enhanced in perfused Smad3−/− islets, compared with Smad3+/+ islets, when glucose was increased progressively from 0 to 25 mM (Fig. 5G). The insulin signaling pathway is a key regulator of normal -cell function (38), and we examined the status of this pathway in Smad3−/− islets. Increased phosphorylation of insulin signaling pathway intermediates Akt, Foxo1, Erk1, and Erk2 was observed in Smad3−/− islets compared with that in Smad3+/+ islets (Fig. 5D). These results, taken together, indicate that
Smad3 deficiency improves glucose tolerance and enhances GSIS by activating the insulin signaling pathway.

**TGF-β/Smad3 Signaling Regulates Expression of Genes Involved in β-Cell Function**—To further evaluate the role of TGF-β/Smad3 signaling in β-cell function, we examined the expression level of genes involved in insulin biosynthesis, pro-insulin processing, glucose sensing, glucose metabolism, incretin signaling, insulin exocytosis, and GSIS. We first assayed the expression levels of these genes in Smad3−/− islets and compared them to those in Smad3+/+ islets. The expression levels of Insulin 1 and Insulin 2 and that of the proinsulin processing enzymes PC1/3 and PC2 were elevated in Smad3−/− islets compared with that in Smad3+/+ islets (Fig. 6A). Similarly, expressions of the glucose transporter, Glut2, and that of the key rate-limiting enzyme in glucose metabolism, Glucokinase, were elevated in Smad3−/− islets compared with that in Smad3+/+ islets (Fig. 6A). Also, we observed that genes involved in regulating glucose sensing, insulin exocytosis, and GSIS such as SUR1, FOXO1, GIPR, Kir 6.2, Rab27a, Snap25, Uncp2, and Ntx 6.1 are overexpressed in Smad3−/− islets compared with that in Smad3+/+ islets (Fig. 6A).

These results suggested that loss of TGF-β/Smad3 signaling enhances expression of genes involved in β-cell function. They further suggested that amplified TGF-β/Smad3 signals could potentially be detrimental to expression of these genes and we next examined this possibility. We established INS-1E cells that express the constitutively active TβRI (CA-TβRI) or CA-ALK5. CA-TβRI expression lead to increased phosphorylation of Smad3 independent of exogenously added TGF-β ligand (supplemental Fig. S5), which allows examination of the effects of activated TβRI/Smad3 signaling on β-cell function. We examined the effects of CA-TβRI infection on INS-1E cell growth and viability by monitoring the gene expression of control genes (β-actin and GAPDH). In addition, we monitored the expression of Cdk4 and Cyclin D2, two genes that promote β-cell growth and proliferation (39–41). As shown in supplemental Fig. S7, we observed similar expression levels of β-actin, GAPDH, Cdk4, and Cyclin D2 in CA-TβRI-infected INS-1E cells, compared with control cells, suggestive of no detrimental effects of CA-TβRI expression. In contrast, CA-TβRI robustly suppressed expression of genes involved in insulin biosynthesis, pro-insulin processing, glucose sensing, glucose metabolism, incretin signaling, insulin exocytosis, and GSIS (Fig. 6B).

To further confirm the role of TGF-β in regulating expression of genes that control β-cell function, we examined the effects of TGF-β ligand stimulation in primary islets. Normal
islets isolated from wild-type mice were stimulated with TGF-β followed by real-time reverse transcriptase-PCR analyses. TGF-β stimulation resulted in suppression of the majority of genes that regulate β-cell function such as SUR1, Anrt, Capn10, FOXO1, GLP-1R, Ins1, Ins2, Kir 6.2, Nkx 6.1, PC1, PC2, Pdx-1, Rab27a, NeuroD1, and Klf-11 (Fig. 6C). In contrast, expression levels of select β-cell-specific genes (MafA, MafB, Glucokinase, GIP1R, Glut-2, Nkx 6.7, and UCP-2) and control genes β-actin and GAPDH were not changed upon TGF-β stimulation under the conditions used in this assay. Taken together, these results indicate that TGF-β/Smad3 signaling regulates genes involved in β-cell function: whereas, reduction of Smad3 signaling increases their expression, amplified TGF-β/Smad3 signals suppress these genes.

DISCUSSION

The TGF-β superfamily, inclusive of TGF-β, activins, and BMPs, regulates the developmental programs of many, if not all, diverse cell types (5, 6, 30). Whereas it is known that TGF-β signaling plays a critical role in early pancreatic development and pancreatic diseases (7–9), the role of this pathway in adult pancreatic function has been largely obscure. Here, we illustrate that TGF-β signaling, via the transcription factor Smad3, regulates insulin gene transcription and adult β-cell function. TGF-β/Smad3 represses the insulin promoter across species and suppresses insulin level and secretion. In contrast, inhibition of Smad3-mediated signaling results in elevated insulin content and insulin secretion. Glucose-stimulated insulin release was elevated ex vivo in perifused Smad3-deficient primary islets and in vivo in Smad3−/− mice. Enhanced β-cell...
function in Smad3−/− islets was accompanied by activated insulin receptor signaling and increased gene expression of factors that promote β-cell function. In contrast, amplified TGF-β signals repressed expression of genes that promote insulin biosynthesis, pro-insulin processing, glucose sensing, glucose metabolism, incretin signaling, insulin exocytosis, and GSIS. Taken together, these data identify TGF-β/Smad3 signaling as an important regulator of insulin gene transcription and β-cell function.

Short-term regulation of insulin secretion, such as in response to food intake, occurs primarily at the level of exocytosis (42), whereas, the long term preservation of adequate intracellular insulin stores depends on the regulation of insulin biosynthesis at the transcriptional and translational levels (32, 33). In contrast to the extensive knowledge of transcriptional activation of the insulin gene, very little is known about the transcriptional repression mechanisms that regulate insulin gene expression. Although here we have exclusively utilized the 2.9-kb human insulin promoter, we observe similar TGF-β/Smad3-mediated repression of the mouse and rat insulin promoters.4 Furthermore, many of the Smad binding sites found within the first 300 bp of the human insulin promoter region, containing the core rightward 5′-GTCT-3′ or leftward 5′-AGAC-3′ sequence, are conserved in mouse and rat species. Insulin transcriptional control is conferred by cis-acting regulatory sequences believed to be located within the first 300–400 bp from the transcription start site, which bind β-cell-restricted and ubiquitous transcription factors (32, 33). The transcription factor PDX-1 is a primary regulator of insulin expression along with Mafa, E47/β2, and cAMP-associated regulation (43). C/EBPβ and PAX4 negatively regulate the insulin promoter (44, 45) and an inhibitory sequence (−279 to −258) referred to as the negative regulatory element lies within the glucose sensing Z element (−243 to −292) on the human insulin promoter (32, 33). Interestingly, the negative regulatory element acts as a potent glucose-responsive transcriptional enhancer in primary cultured islet cells and as a transcriptional repressor in immortalized β-cells, non-β-cells, and fibroblasts (32). The negative regulatory element as well as the Pax4 and C/EBPβ binding sites are in close proximity to the putative Smad3-binding sites that we identify here (Fig. 2G). Further work is needed to determine the plausible interactions between these elements in regulating insulin gene transcription. Deletion of the human insulin promoter region that harbors the putative Smad binding sites led to reduced repression by Smad3 (supplemental Fig. S3B). Although this suggests an important role for Smad3 in insulin promoter repression, it is plausible that altered binding of other repressors and activators affects the activity of the insulin promoter. However, our finding that insulin transcripts are elevated in Smad3−/− islets, together with the decreased insulin transcripts in cells with activated TβRII/Smad3 signaling (Fig. 6), supports the notion that TGF-β/Smad3 signals regulate insulin gene expression. We have not studied either the rate of insulin gene transcription or its synthesis and thus cannot comment whether TGF-β/Smad3 controls the kinetics of insulin transcription or the rate of insulin synthesis.

Interestingly, we observe that TβRII transfection has a dramatic inhibitory effect on hiNS-Luc activity independent of ligand (Fig. 1B), which is not relieved by a small molecule TβRI inhibitor (Fig. 1C). It is plausible that TGF-β secreted by β-cells or the relatively minor amounts of TGF-β (~0.01–0.02 ng/ml majority of which is in latent form) in the 0.2% fetal bovine serum may reconstitute the TGF-β signaling pathway once TβRII is exogenously expressed. Furthermore, we observe that Smad3 can repress the insulin promoter in a TGF-β/TβRII independent manner (Fig. 2A–E). Taken together, we infer that in addition to the TGF-β/Smad3-dependent mechanism other TGF-β independent but TβRII/Smad3-mediated mechanisms of insulin promoter repression could exist.

Our finding that endogenous Smad3 occupies the native insulin promoter classifies insulin as a bona fide TGF-β target gene. It is intriguing that a "repressor" Smad3 occupies the insulin promoter in unstimulated cells along with an "activator" Pdx1 (Fig. 2F). It is plausible that in unstimulated cells Smad3 binds to the insulin promoter by a TGF-β/TβRII independent mechanism. TGF-β independent activation of Smads, particularly phosphorylation of Smad3 by kinases other than TβRI has been demonstrated (46). Last, although unlikely, trace amounts of TGF-β signaling in β-cell lines may be sufficient to activate Smad3 and allow it to bind the insulin promoter. As seen in supplemental Fig. S4, in β-cells Smad3 is localized within the nucleus, along the perinuclear region and in the cytoplasm consistent with its ability to shuttle between the nuclear and cytoplasmic compartments (47). It is feasible that this mechanism allows residual nuclear localization of Smad3 in β-cells. Interestingly, co-expression of Smad3 and Smad4, which constitutes an activated TGF-β signaling complex, displaces Pdx1 from the insulin promoter, whereas Smad3 binding is retained (Fig. 2F). The precise mechanism that results in elimination of Pdx1 binding upon Smad3 + Smad4 transfection is presently unclear and further experiments are needed to address this issue. We conclude that an activator (Pdx1) and repressor (Smad3) may occupy the insulin promoter in unstimulated cells such that a repressor:activator ratio determines the extent of gene expression. In contrast, active TGF-β signaling, in the form of a Smad3 + Smad4 complex, may displace Pdx1 from the insulin promoter. Similar observations where an activator (E2F1) and repressor (E2F4) of gene transcription simultaneously occupy target gene promoters have been described (48, 49).

We find that inhibition of Smad3 signaling leads to elevated GSIS ex vivo and in vivo (Fig. 5, E–G). It is plausible that the effects of TGF-β/Smad3 on insulin gene transcription may be independent of its role in GSIS. Levels of expression of genes involved in insulin biosynthesis, pro-insulin processing, glucose sensing, glucose metabolism, incretin signaling, insulin exocytosis, and GSIS are significantly elevated in Smad3−/− islets and, conversely, significantly repressed in cells harboring a constitutively active TβRII/Smad3 pathway (Fig. 6). Interestingly, we observe an activated insulin receptor signaling pathway in Smad3−/− islets (Fig. 5D), which is consistent with the enhanced β-cell function in Smad3−/− mice. However, the precise mechanisms whereby deficient Smad3 signaling results in

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(i) enhanced phosphorylation of insulin signaling pathway proteins, and (ii) increased expression of genes that regulate β-cell function in the islet, are unclear. It is plausible that the mild hyperinsulinemia in the Smad3-/- mice may promote activation of the insulin signaling pathway. Alternatively, it is probable that TGF-β/Smad3 signaling interacts with the insulin signaling pathway in the islet. Further studies using tissue-specific Smad3 mutant mice are addressing these issues.

TGF-β appears to exert a bimodal effect on GSIS depending on the dose, time of exposure, and concentration of coexisting glucose (50, 51). Therefore, at low glucose concentration TGF-β stimulates insulin release, whereas at a high glucose concentration the same dose of TGF-β significantly inhibits GSIS (50, 51). Further studies are needed to determine the physiological basis of the potential TGF-β-mediated dual effects on GSIS and β-cell function and to establish whether this phenomenon is regulated by Smad3. Interestingly, dual and opposing roles of TGF-β in cancer are believed to suppress primary tumors and paradoxically promote cancer metastases (52). Recent reports of the role of BMP4/BMPR1A, ALK7, and Smad7 in β-cell function further underscore the important role of TGF-β superfamily signaling in this cell type (11, 53, 54). BMP4-BMPR1A signaling plays a key role in insulin secretion by positively regulating genes involved in glucose sensing, glucose-metabolism-coupled secretion, incretin signaling, proinsulin processing, and insulin exocytosis (11). Our findings of TGF-β/Smad3-mediated regulation of genes associated with β-cell function are consistent with an important role of the TGF-β superfamily signaling in pancreas development, particularly β-cell function.

The findings that amplified TGF-β/Smad3 signals repress insulin transcription and reduce insulin level and insulin secretion, whereas ablation of Smad3 leads to improved β-cell function suggest that abnormal TGF-β signaling may promote pathogenesis of dysfunctional β-cells. We propose that TGF-β/Smad3 pathways may regulate β-cell function in settings of increased insulin demand, including insulin resistance, obesity, and during β-cell injury. Furthermore, our results that reduced TGF-β/Smad3 signaling markedly enhance insulin content and insulin secretion suggest that pharmacological inactivation of TGF-β/Smad3 signaling might be useful for promoting β-cell differentiation and ameliorating β-cell failure during diabetes.

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