Islet hypertrophy following pancreatic disruption of Smad4 signaling

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Simeone, Diane M., Lizhi Zhang, Mary K. Treutelaar, Lanjing Zhang, Kathleen Graziano, Craig D. Logsdon, and Charles F. Burant. Islet hypertrophy following pancreatic disruption of Smad4 signaling. Am J Physiol Endocrinol Metab 291: E1305–E1316, 2006.—To investigate the role of transforming growth factor (TGF)-β family signaling in the adult pancreas, a transgenic mouse (E-dnSmad4) was created that expresses a dominant-negative Smad4 protein driven by a fragment of the elastase promoter. Although E-dnSmad4 mice have normal growth, pancreas weight, and pancreatic exocrine and ductal histology, beginning at 4–6 wk of age, E-dnSmad4 mice show an age-dependent increase in the size of islets. In parallel, an expanded population of replicating cells expressing the E-dnSmad4 transgene is found in the stroma between the enlarged islets and pancreatic ducts. Despite the marked enlargement, E-dnSmad4 islets contain normal ratios and spatial organization of endocrine cell subtypes and have normal glucose homeostasis. Replication of cells derived from primary duct cultures of wild-type mice, but not E-dnSmad4 mice, was inhibited by the addition of TGF-β family proteins, demonstrating a cell-autonomous effect of the transgene. These data show that, in the adult pancreas, TGF-β family signaling plays a role in islet size by regulating the growth of a pluripotent progenitor cell residing in the periductal stroma of the pancreas.

the mechanism by which this occurs is not known. In addition, the identity and location of putative islet progenitor cells is not known nor is it known whether the putative adult islet progenitor cell differentiates by recapitulation of the pathway used during embryogenesis (19).

Transforming growth factor (TGF)-β family signaling is important in regulating the development, growth, and repair of a number of tissues and specific TGF-β family members, such as GDF8 in skeletal muscle (31), and GDF11 in neurons in the olfactory epithelium (50) appear to modulate tissue size by regulating precursor cell proliferation and/or differentiation. The TGF-β family of proteins are an important class of morphogens in early pancreatic development (8, 30, 41, 51) and during islet formation (12, 43). TGF-β and all related members of the superfamily signal through specific serine/threonine kinase receptors via phosphorylation of receptor-associated Smad protein isoforms (34). Phosphorylated Smad proteins form obligate heterodimers with the common mediator Smad4, resulting in nuclear translocation and transcriptional activation. Disruption of Smad4 function either by mutation (10) or expression of a dominant-negative protein (22) inhibits signaling by TGF-β family members.

To examine the role of TGF-β in the growth of the pancreas, we constructed a transgenic mouse expressing a dominant-negative Smad4 (dnSmad4) protein driven by the elastase promoter to disrupt pancreatic TGF-β signaling. Expression the dnSmad4 protein had no effect on exocrine or ductal portions of the pancreas; however, enlargement of structurally and functionally normal islets was observed, beginning at 4–6 wk of age. In parallel, replicating clusters of cells expressing the dnSmad4 transgene were observed located between ducts and the enlarged islets. Identical cells were also identified in in vitro duct-derived cultures from wild-type animals. These data suggest that, in the adult animal, signaling by the TGF-β family regulates the growth of the adult islet, potentially by regulation of a population of these progenitor cells in the adult mouse. These data suggest that TGF-β family signaling plays a regulatory role in determining adult islet size.

MATERIALS AND METHODS

Consortion and Breeding of Transgenic Mice

E-dnSmad4 mice. The Flag-dnSmad4 transgene was constructed by placing a 510-bp EcoRI-HindIII segment of the promoter/enhancer region of the rat elastase I gene (Dr. Raymond J. MacDonald, University of Texas Health Science Center) upstream of a 1.6-kb human Flag-dnSmad4-(1–514) cDNA in pcMV5 (Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center). dnSmad4 was gen-

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regulated by truncating 39 amino acids at the COOH terminal of wild-type Smad4, disrupting DNA-binding activity (22, 53). At the 3′-end of the transgene, a 2.1-kb BamHI-SpeI fragment containing human growth hormone (hGH) sequences lacking exon 5 in pck-hGH (Dr. Raul Urrutia, Mayo Clinic, Rochester, MN) was added to provide a polyadenylation signal. The fidelity of the sequences was confirmed by sequencing. The resulting 4.2-kb transgene was isolated, purified, and microinjected into the pronuclei of (C57Bl/6×SJL) F1 mouse ova in the Transgenic Mouse Core at the University of Michigan to generate founder transgenic mice. Transgenic mice were identified by generating a 590-bp PCR product from tail-derived DNA using a forward primer specific to the sequence encoding the Flag epitope 5′-ATG-GACTAACAAGAGCAAGATGACAAAGGC3′ and a reverse primer 5′-GCACGTAATTGTTGGATGCTGAGTCTGTGTT-3′. From 11 dams, 8 of 98 pups were transgenic. The studies were performed on two founder lines, 27 and 51, which had four and six copies of the transgene, respectively, and demonstrated the highest expression levels of Flag-dnSmad4 by Western blotting of whole pancreas. The mice were backcrossed on a C57Bl/6G background and have been maintained on a C57Bl/6 background. Experiments described in this report are from two founder transgenic mice. Transgenic mice were identified by generation 2 and provided with food and water ad libitum. The University of Michigan under a controlled 12:12-h light-dark cycle.

To test the ability of mice to undergo normal rates of cell proliferation as controlled by the TGF-β superfamily, groups of animals were weighed intermittently, and random blood glucoses were determined. Pancreatic weight of 10- to 12- to 23- to 25-wk-old animals were determined at necropsy. Elastase-Cre×Rosa26 mice. C57Bl/6J-Gt(ROSA26) Cre (Jackson Laboratories) were mated with Elastase-Cre mice (a gift of Eric Sandgren, University of Wisconsin) that carry a 200-bp elastase promoter/enhancer fragment that drives the expression of Cre recombinase (18). Pancreata of the β-gal+/Cre mice were evaluated at 6–8 wk of age.

All mice were maintained in a specific pathogen-free facility at the University of Michigan under a controlled 12:12-h light-dark cycle and provided with food and water ad libitum. The University of Michigan Institutional Animal Care and Use Committee approved all experiments. RT-PCR Detection of mRNA Expression

Total RNA of pancreas, liver, colon, lung, and spleen from wild-type and transgenic animals was isolated by a modified acid guanidinium-thiocyanate-phenol-chloroform extraction as previously described (52). RT-PCR to detect the specific expression of Flag-dnSmad4 was performed by using the primers described above and the Access RT-PCR System (Promega).

Western Blotting

Pancreata were lysed by sonication for 10 s in 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitors. The lysate was centrifuged at 14,000 g for 15 min at 4°C. Supernate protein (100 μg) was resolved on 10% SDS-PAGE, transferred to nitrocellulose membranes, probed with goat polyclonal anti-Flag at 1:1,000 dilution (Santa Cruz, Santa Cruz, CA), and visualized using the enhanced chemiluminescence Detection System (Amersham, Arlington Heights, IL). Preparation of Pancreatic Acini and [3H]Thymidine Incorporation

The preparation of pancreatic acini was performed as previously described (52). The dispersed acini were separated into aliquots on 24-well plates. Cells were maintained in a humidified atmosphere of 5% CO2 in air at 37°C during incubation times. The rate of DNA synthesis in cultured pancreatic acini was measured using a [3H]thymidine incorporation assay, as previously described (52). Acini were exposed to 50 nM fibroblast growth factor (FGF)-2 with or without 100 nM TGF-β1 (R&D Systems, Minneapolis, MN) for indicated times after which 0.1 μCi/ml [3H]thymidine was added for an additional 24 h. [3H]thymidine incorporation was expressed as a percentage of total counts per minute in control cells.

Immunostaining

Sections of pancreata were probed for the presence of various proteins using the following individual antisera (dilution): guinea pig anti-insulin (1:1,000), rabbit anti-glucagon (1:1,000) and rabbit anti-pancreatic polypeptide (1:100; Linco Research, St. Charles, MO), rabbit anti-somatostatin (1:500; Dako, Carpenteria, CA), pancreatic polypeptide (1:500; Sigma, St. Louis, MO), rabbit anti-Pdx-1 (1:3,000; Christopher Wright, Vanderbilt University), mouse anti-flag (1:50; Sigma), rabbit and mouse anti-proliferating cell nuclear antigen (PCNA, 1:50; Santa Cruz), mouse anti-nestin (1:100; Pharmingen, San Diego, CA), and mouse anti-Isl-1 (1:200). To identify pancreatic ducts, a FITC-labeled Dolicibio biflorus agglutinin (DBA) lectin was used at 2 μg/ml. Biotinylated goat anti-rabbit IgG, goat anti-guinea pig IgG, goat anti-mouse IgG, and avidin-labeled peroxidase were obtained from Vector Laboratories (Burlingame, CA). Texas red-conjugated donkey anti-mouse IgG and fluorescein-conjugated donkey anti-rabbit were purchased from Santa Cruz.

For immunohistochemical staining of insulin, glucagon, and somatostatin, the Vectastain ABC-Peroxidase Kit (Vector Laboratories) was used according to the manufacturer’s instructions. Peroxidase activity was visualized by applying diaminobenzidine solution containing 0.05% hydrogen peroxide for 1–3 min at room temperature. Sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted. When monoclonal antisera were used, a Mouse on Mouse Immunodetection Kit (Vector Laboratories) was employed.

For double immunofluorescent staining, primary antibodies from rabbit and mouse were used. Sections were treated with Vector Antigen Unmasking Solution for 15 min in a microwave oven. After being blocked with 5% donkey serum in PBS with 0.1% Triton X-100, the sections were incubated with the dilutions of the antibodies described above overnight at 4°C. After being rinsed, fluorescein-conjugated donkey anti-rabbit secondary antibodies (1:200) were applied for 90 min at room temperature. After being washed with PBS, the second-step immunofluorescent staining with mouse primary antibodies was performed using the Mouse on Mouse Immunodetection Fluorescein Kit described above. After being stained, the sections were mounted with VECTASHIELD Mounting Medium. Images were obtained using a Carl Zeiss fluorescence microscope (Carl Zeiss, New York, NY).

Quantitative Islet Histomorphometry

Pancreata were fixed in 10% buffered formalin overnight and embedded in paraffin. Serial sections (150 μm) from wild-type and E-dnSmad4 mice were stained with insulin as described above. Quantitative islet histomorphometry was performed by outlining digitalized images of whole pancreas and islets, and the relative areas were quantified using National Institutes of Health Image1.62 software. The relative islet area was the ratio of the area occupied by the islet/whole pancreatic area. At least 10 random sections from each of four to five animals at each age from wild-type and E-dnSmad4 mice were quantified. Cell density was determined by measuring the area of the islet and counting the number of nuclei within the proscribed area as previously described (47). The number of nuclei was quantified in four to five random sections from five different mice at the indicated ages.

Bromodeoxyuridine Labeling

In initial experiments, groups of three wild-type and E-dnSmad4 mice at 20 wk of age were injected with 50 mg/kg bromodeoxyuridine (BrDU; Sigma) in sterile saline. After 6 h, pancreata were harvested, and four random sections were stained with BrdU antisera. The number of BrdU+ nuclei associated with a morphologically determined islet was quantified. At least 1,200 nuclei were counted in each mouse.
Glucose Tolerance Tests and Plasma Insulin Levels

Glucose tolerance was performed on animals fasted overnight. Oral glucose tolerance was determined by administering 2 g/kg dextrose in water to 24-wk-old wild-type and E-dnSmad4 mice. Tail vein glucose was determined at 0, 15, 30, 60, and 120 min after administration of glucose with a Freestyle glucometer (Therasense, Alameda, CA). An intraperitoneal tolerance test was performed on 12- to 16-wk-old wild-type and E-dnSmad4 mice by injection of 1 g/kg dextrose in water. At 0, 30, and 60 min after the injection, blood was obtained by retroorbital vein sampling. Whole blood glucose levels were determined by the glucose oxidase method with a glucometer (Lifescan). Plasma insulin concentrations were assayed using the rat insulin RIA Kit from Linco Research.

Localization of β-Galactosidase Activity in Elastase-Cre×Rosa26 Mice

Pancreata from 6- to 8-wk-old F1 mice (double-transgenic or Rosa26 mice) were fixed, and β-galactosidase activity was localized in whole mount sections as described previously (46). After development, the sections were mounted in paraffin and immunostained for insulin or glucagon as described above. Alternatively, Ficol-purified islets (45) obtained from 10-wk-old Rosa26 or Rosa26×Elastase-Cre mice were developed with X-gal (46).

Primary Duct Cultures

Ducts were isolated by collagenase digestion (P2; Boergering-Mannheim) from 16- to 20-wk-old wild-type or E-dnSmad4 mice as previously described (46). The ducts were minced, plated on tissue culture dishes (Primaria; Falcon), and cultured in DMEM-F-12 medium (1:1) with 10% FBS and antibiotics at 37°C. After 5–8 days of growth, the cultures were overlaid with 10% matrigel, which was removed after 24 h, and the cultures were then grown for an additional 14–21 days in the same medium.

Growth inhibition by TGF-β family members was determined by establishing explant duct cultures for 5 days and then adding TGF-β1, activin A, bone morphogenic protein-2 (BMP2), or BMP4 (Cell Signaling, Beverly, MA) at the indicated concentrations in DMEM-F-12 media with 1% FCS for 48 h. In some cultures, BrdU (0.5 mM) was added for the last 6 h of incubation.

For immunocytochemistry, primary duct cultures were fixed in 4% paraformaldehyde and blocked for 30 min in 10% of the appropriate normal serum supplemented with 0.3% Triton X-100. Primary antisera nestin (1:100), insulin (1:2,000), and Flag (1:100) were applied for 2 h at room temperature, washed, and incubated for 45 min with fluorescein-conjugated second antibodies (1:400 dilution; Molecular Probes, Eugene, OR). Nuclei were counterstained with DAPI in VectaShield (Vector Laboratories).

Statistical Analysis

The unpaired Student’s t-test or ANOVA was used to compare differences between the groups where appropriate. P values of <0.05 were considered significant.

RESULTS

Development of Mice Carrying a dnSmad4 Protein

We created transgenic mice that express a flag-tagged, dominant-negative Smad4 protein under control of the elastase promoter and the “A” element of the elastase enhancer region (20). A 1.6-kb Smad4 fragment containing an NH2-terminal Flag tag sequence and a COOH-terminal truncation of 39 amino acids, which creates a dominant-negative protein (52), was fused to a 0.5-kb fragment containing the pancreas-specific elastase promoter/enhancer (Fig. 1A). The linearized construct was microinjected in fertilized mouse eggs, and animals positive for the transgene were bred to C57BL6/J females. Eight founder lines expressing the transgene were created. Detailed analysis was performed on the F1 generation of two independent lines (designated E-dnSmad4). The two lines contained four and six copies of the transgene as determined by real-time quantitative PCR (data not shown) and showed identical phenotypes; thus, the data are presented as a composite of both lines. RT-PCR (Fig. 1B) and Western blotting (Fig. 1C) confirmed pancreas-specific expression of Flag-tagged dnSmad4. Pancreata of E-dnSmad4 mice appeared grossly normal. Immunohistochemical staining of the pancreata from 4-wk-old mice using an anti-Flag antibody demonstrated a mosaic transgene protein expression in acinar cells (Fig. 1D) with ~30% of the pancreas expressing the transgene, as has been described previously with this promoter (18). Importantly, there was no evidence of transgene expression in the islets of any animal tested.

Functional expression of the transgene in acini was determined by measuring TGF-β-mediated growth inhibition of isolated acinar cells. Primary cultures from wild-type and E-dnSmad4 mice treated with 1 nM FGF-2 showed a threefold increase in [3H]thymidine incorporation into DNA (Fig. 1E). Addition of TGF-β inhibited [3H]thymidine incorporation by 70% in acini isolated from wild-type mice; however, TGF-β treatment was ineffective in inhibiting DNA synthesis in acini from E-dnSmad4 mice (Fig. 1E).

No apparent abnormalities in the growth or development were observed in the E-dnSmad4 mice of either sex up to 12 mo of age (Fig. 1F). Additionally, there were no differences in the weight of major organs, including the pancreas (Fig. 1G and data not shown).

Islet Hyperplasia in E-dnSmad4 Mice

Histological examination of pancreata of 2-wk-old E-dnSmad4 showed no abnormalities. However, as the animals aged, there was a progressive increase in islet size when compared with wild-type littermates. Statistically significant changes in relative islet area were identified by 6 wk of age, and by 18 wk of age there was nearly a fivefold increase in relative islet area compared with nontransgenic littermates (Fig. 2, A and B). Islet enlargement was apparent in both male and female mice (data not shown). E-dnSmad4 mice at 4 wk of age had a similar number of cells in each islet, but at this age there were a few islets that were already enlarging (Fig. 2C). A significant increase in the number of cells per islet was apparent in the islets of E-dnSmad4 mice at 24 wk of age (Fig. 2C). There was no change in islet number (data not shown) or the number of nuclei/islet area in the E-dnSmad4 mice (119 ± 22 and 106 ± 35 nuclei/µM2 in the wild type and E-dnSmad4, respectively, n = 12 islets in each group), indicating that the increase in islet size was because of hyperplasia of islet cells and little or no contribution by cellular hypertrophy. There was no apparent change in the exocrine or duct morphology in any E-dnSmad4 animal in any of the mice at any age.

Although many rodent models of islet hyperplasia result in disordered islet architecture and concomitant alterations in glucose metabolism (2, 15, 47), this was not observed in the E-dnSmad4 mice. The overall architecture of the islets in E-dnSmad4 mice was normal, with insulin staining in the islet core and glucagon, somatostatin, and pancreatic polypeptide
staining at the periphery of the islet, identical to that found in wild-type islets, with no apparent changes in the proportions of the different endocrine cells within the islet (Fig. 3A).

The significant increase in islet mass in the older E-dnSmad4 did not lead to changes in glucose tolerance. Glucose excursion following oral glucose challenge was similar in both male and female wild-type and E-dnSmad4 mice compared with wild-type littermates (Fig. 3B), and the rise in glucose and insulin after intraperitoneal glucose injection in male E-dnSmad4 was identical to that in wild-type littermates (Fig. 3C). Thus the increased islet size in the E-dnSmad4 mouse does not appear to be a response to, nor does it lead to, alterations in glucose homeostasis.

Hyperplasia of Potential Islet Precursor Cells

In examining histological sections of older E-dnSmad4 pancreata, we noted distinct areas of cellular hyperplasia that consisted of masses of round cells located between the enlarged islets and nearby ducts (Fig. 4A). These hyperplastic cell masses were in the stroma and did not appear to distort the architecture of the ducts. Similar cell masses were not observed in littermate wild-type and E-dnSmad4 mice; n = 5–8 animals at each time point. G: pancreas weight of wild-type and E-dnSmad4 mice at the indicated ages.
and 59 islets, respectively, \( P < 0.05 \); Fig. 4E). In E-dnSmad4 pancreata, BrdU staining occurred both in the islet proper, in the periphery of the islet, and within the periductal clusters (Fig. 4F). This resulted in a significant increase in the labeling index when these cells were included in the analysis (0.15 ± 0.09 in wild type vs. 1.21 ± 0.13% in E-dnSmad4, \( P < 0.01 \)). Longer labeling periods increased the apparent number of BrdU\(^+\) cells in the islet (data not shown), but it is difficult to ascertain if this is because of replication of preexisting islet cells or migration of labeled cells from the periphery. Staining for activated caspase 3 in 24-wk-old mice did not reveal staining in islets of wild-type or E-dnSmad4 mice (data not shown), suggesting that there is minimal apoptotic activity in either group of animals.

The proliferating cells carry the dnSmad4 transgene as determined by the costaining of these for the Flag epitope on the transgene and PCNA (Fig. 4, G and H). These results suggest that proliferation of the periductal cells could provide a mechanism for the increased size of the islet. Recently, we (46) and others (14, 27) have described the presence of cells in the periductal stroma that express the intermediate filament protein nestin. In E-dnSmad4 mice, a subset of the periductal cells stained for the intermediate filament protein nestin (Fig. 4I), and nestin was coexpressed within a subset of the dnSmad4\(^+\) population (Fig. 4, J–L). Nestin was also found in linear cells within the islet, which we and others have previously determined to represent endothelial cells (27, 46).

When stained with antisera for Pdx-1, a subset of periductal cells was positive but demonstrated primarily cytoplasmic localization (Fig. 5A) while some cells showed both cytoplasmic and nuclear staining (Fig. 5A). The cells within the islet proper showed exclusively nuclear staining for Pdx-1 (Fig. 5). Costaining for nestin and Pdx-1 showed that these proteins are expressed in a distinct subset of cells (Fig. 5A). The periductal cells were devoid of staining for insulin (Fig. 5C) as well as glucagon and amylase (data not shown) and the ductal marker DBA (Fig. 5D). Thus this replicating periductal population that expressed the dnSmad4 transgene has properties of a progenitor population devoid of expression of hormones, ductal or exocrine markers.

**Lineage Tracing of Transgenic Elastase Promoter Cells**

The finding of an accumulation of replicating periductal cells expressing transcription factors associated with islet development.
in proximity to enlarging islets suggests that the periductal cells are islet precursors. The expression of the dnSmad4 protein in these cells suggests that protein is acting in a cell autonomous manner to increase the growth and differentiation of these cells. In adult animals, the elastase gene is normally expressed only in the acinar cells of the exocrine pancreas, raising the possibility that the progressive enlargement of islets in the E-dnSmad4 mice is because of a paracrine effect from acinar cells rather than a cell autonomous effect of the dnSmad4 protein in the putative islet precursor population. In our transgenic mouse, dnSmad4 protein expression is driven by a portion of the 5′/H11032 region of the elastase gene that contains multiple regulatory elements, including a domain that can direct β-cell expression of reporter proteins, but this element is normally silenced in pancreatic endocrine cells by adjacent, negative regulatory elements (36). Some transgenic mice that use these elastase elements to drive transgenes demonstrate both islet and nonislet alterations (1, 32, 37), which suggests that this element may be active in islet lineage cells. To identify transgenic elastase element-lineage cells, we performed direct tracing experiments using a mouse expressing cre-recombinase driven by a 5′-elastase promoter/enhancer element, which contains the same activator and repressor elements contained in the E-dnSmad4 transgene (18). This Elastase-cre mouse was crossed with C57BL/6J-Gtrosa26tm1Sor (Rosa26) mice carrying a genomic "floxed" β-galactosidase gene, allowing genomic recombination to eliminate an upstream stop codon in the β-galactosidase mRNA and allow production of an active β-galactosidase protein (Fig. 6A). Thus the parent cell and all daughter cells of a recombination event can be identified by assessing β-galactosidase activity using the appropriate substrate.

We assayed for the presence of β-galactosidase in endocrine pancreas by three methods. First, frozen sections from Elastase-Cre×Rosa26 mice were incubated with X-gal, which resulted in small precipitates forming in endocrine and exocrine cells (Fig. 6, B–D). High-power images show clearly that blue X-gal precipitates are localized in insulin-positive cells (Fig. 6, B–D, insets). No precipitates were seen in Rosa26 mice, which were not crossed with Elastase-cre (data not shown). We also incubated whole pancreata from Rosa26 and Elastase-Cre×Rosa26 mice in an X-gal-containing solution and then costained with glucagon. When examined histochemically, we found mottled blue staining in acinar cells and within endocrine cells of the islets in Rosa26/Elastase-Cre mice (Fig. 6, E–G), but not in Rosa26 mice (Fig. 6, H–J). The endocrine cell expression was confirmed by immunostaining the X-gal-developed pancreas for glucagon (Fig. 6, E–G). We also incubated isolated islets from the Rosa26×Elastase-Cre and Rosa 26 mice in X-gal solution. A subset of the Rosa26×Elastase-Cre islets stained blue (11/84, 13% from 2 mice; Fig. 6, K and L). As with the whole tissue, islets from the Rosa26 mice remained unstained after incubation in X-gal solution (Fig. 6M). Thus islets can arise from cells that at one point had an active elastase promoter fragment as defined by the activation of the β-galactosidase gene.

**Enhanced In Vitro Growth and Differentiation of Duct-Derived Cultures from E-dnSmad4 Mice**

To further characterize periductal cells in E-dnSmad4 mice and to determine if similar cells are present in wild-type mice...
(but whose expansion is inhibited in vivo), we exploited a culture system utilizing primary explants of pancreatic ducts that produces cultures displaying stereotypic growth and which can be induced to differentiate to produce mature endocrine cells (6, 46). Growth of primary duct cultures was uniformly heavier from E-dnSmad4 ducts, and E-dnSmad4 cultures had a slightly higher replication rate, as determined by BrdU incorporation, compared with wild-type cultures (Fig. 7A). Immunohistochemical staining with anti-Flag antisera showed that the Flag-tagged dnSmad4 protein was expressed in the majority of cells growing from E-dnSmad4 duct cultures (Fig. 7B), demonstrating that they represent the same cells present in the Flag-positive periductal cell masses seen in pancreatic sections from the E-dnSmad4 mice. As we have observed previously...
subset of the cultured cells were nestin+ (Fig. 7B), with the proportion of nestin+ cells greater in the E-dnSmad4 cultures, indicated by a doubling of nestin mRNA levels in the E-dnSmad4-derived cultures compared with wild-type cultures as determined by quantitative RT-PCR (data not shown). The periductal cultures did not stain with DBA or with antiserum to amylase (data not shown).

The similar characteristics of primary duct-derived cultures from E-dnSmad4 and wild-type mice suggest that the latter has a similar potential islet progenitor population, but the expansion of this population in vivo is suppressed by TGF-β family signaling. To determine whether there is differential sensitivity of wild-type and E-dnSmad4 mice to growth inhibition by TGF-β and related proteins, we assessed growth of primary duct explants from wild-type and E-dnSmad4 mice in the presence of TGF-β1, activin A, BMP2, and BMP4. By scoring the number of PCNA+ cells after 48 h, we found that PCNA expression was strongly inhibited by activin A and by ~50% with 2 ng/ml of TGF-β1 and 10 ng/ml of BMP4, whereas BMP2 had no effect on the PCNA expression in wild-type cells (Fig. 7C). In contrast, a small but not significant decrease in PCNA expression was found in cultures from E-dnSmad4 pancreata following treatment with the TGF-β family members. Similar results were obtained when we examined the incorporation of BrdU in the duct-derived cultures, with activin A...
and TGF-β1 causing a 50% decrease in BrdU labeling after 6 h of treatment (Fig. 7D). These results show that the dnSmad4 protein actively inhibits the signaling of TGF-β family members in this cell population.

DISCUSSION

In this report, we show that disruption of TGF-β family signaling by the expression of a dominant-negative Smad4 protein in the pancreas results in age-dependent islet hypertrophy. In parallel, we find an expansion of a population of cells in the periductal area that increases in size in parallel to the islet. In addition, the periductal population expresses the dnSmad4 transgene, is replicating, and a subset of the cells express Pdx-1, albeit in the cytoplasm. However, the dnSmad4 protein is not expressed in the mature cells of the islet but is expressed in a subset of acinar cells, which raises the possibility that paracrine signals from these periductal cells or exocrine tissue that also express the transgene are responsible for the progressive increase in islet size.

Signaling by the TGF-β family of proteins modulates the growth and development of a number of tissues, including skin [TGF-β (16)] skeletal muscle [myostatin (31)], and neurons [GDF11 (50)] by regulation of precursor cell growth and differentiation. The findings here are consistent with the hypothesis that TGF-β family signaling regulates the flux of islet precursor cells into the differentiation pathway. Previous studies have suggested a role for TGF-β signaling in the regulation of islet size. Overexpression of TGF-β1 in islets using the insulin promoter results in alterations ranging from disruption of the islet architecture with minimal changes in relative β-cell mass to significant islet atrophy (17, 30, 41). However, in these studies, there is associated pancreatic inflammation that complicates the interpretation of the studies. Homologous disruption of TGF-β1 results in enlarged islets relative to pancreatic mass; however, in these studies, there is a significant decrease in exocrine pancreas size (12), making absolute determination of TGF-β signaling difficult.

A recent study (21) in embryonic mice with targeted mutations suggest that GDF11, signaling via Smad2, negatively regulates the formation of NGN3+ cells in the developing pancreas. In these mice, which do not survive to adulthood, there is also an arrest in β-cell development, indicating that TGF-β may also be required for β-cell maturation. This is consistent with findings in an earlier study (54) using a model of islet regeneration in adult mice that demonstrated that inhibition of activin signaling resulted in expansion of pancreatic epithelial cells but decreased the numbers of differentiated β-cells.

We present evidence from lineage tracing that at least a subset of endocrine cells in the islet arise from cells that at one time expressed genes from a truncated elastase promoter that drives cre-recombinase. Previous studies suggest that both endocrine and exocrine cells of the pancreas arise from a common precursor cell population that expresses the transcription factor ptf1a (26), a protein first identified as part of a complex activating elastase gene expression (39). The ptf1a appears to interact with the A element of the elastase promoter, contained in both the E-dnSmad4 construct and the Elastase-Cre construct used in these studies, which could provide a mechanism for activation of the transgene in the developing animal (11). Although distinct enhancer elements in the elastase gene direct both β-cell and acinar cell expression, an upstream element contained in the transgenic constructs suppresses endocrine pancreas expression of the elastase (20),
which would shut off the transgene expression in the mature islet. These findings provide a potential mechanism for E-dnSmad4 transgene expression in the periductal progenitor cells and absence of expression in the mature islet.

Our lineage tracing experiments indicate that the elastase promoter/enhancer is at least transiently expressed in an endocrine progenitor. Published studies have hinted that transgenic elastase promoter/enhancer elements used in these studies are expressed in more than just mature exocrine cells. Cell lines derived from mice expressing the SV-40 T-antigen driven by the elastase promoter express markers of both endocrine and exocrine lineages (37). Bell and colleagues (1) noted hyperplasia and tumors of the islets of Langerhans, in addition to acinar cell carcinomas, in transgenic mice bearing a elastase I-SV-40 T-antigen fusion gene despite the absence of transgene expression in the islets. In a recent report, Lewis and colleagues (32) expressed TVA, the receptor for avian leukosis sarcoma virus subgroup A, under the control of the elastase promoter in transgenic mice. When the TVA virus expressing the polyoma virus middle T antigen was introduced in mice, tumors developed with both acinar and endocrine gene markers. Importantly, when the virus carried c-Myc, only endocrine tumors were formed with normal exocrine architecture. These reports and the studies reported here suggest that the proximal elastase promoter fragment used in the transgenic mice is expressed in an early pancreatic progenitor cell population for endocrine cells.

TGF-β and activin A were unable to inhibit growth of acinar cells and E-dnSmad4 duct-derived cultures, demonstrating intact function of the dnSmad4 transgenic protein, suggesting a cell autonomous effect of the transgene. The expression of the dnSmad4 protein in duct-derived cultures from the E-dnSmad4 mice and the identification of the transgene in the periductal region of the pancreas in tissue sections support the idea that these are the same cells. This is further supported by the finding of the intermediate filament protein nestin in these cell populations both in vivo and in vitro. Previously, we (46) and others (24) have determined that primary cultures grown from wild-type ducts can differentiate into hormone-expressing cells. Here we show that both wild-type and E-dnSmad4 duct cultures express transcription factors associated with islet development, albeit at low levels. This suggests that in vivo, wild-type animals harbor a small number of islet precursor cells in the periductal area, but their growth is tonically inhibited. When explanted in vitro, this inhibition is released, allowing expansion of the population.

Recent studies (13) have suggested that postnatal increase in islet size is because of replication of preexisting β-cells and not because of neogenesis, in contrast to our findings that suggest the presence of an islet progenitor cell in the mature pancreas. One potential interpretation of these conflicting results may be that these precursor cells are available for generation of new endocrine cells under different physiological conditions than tested by Dor et al. (13).

In summary, disruption of the TGF-β family signaling in a pluripotent pancreatic cell type by expression of a dominant-negative Smad4 protein results in the expansion of a normally quiescent islet precursor cell population. We suggest that the number of cells in the mature islet is controlled at the level of this fairly immature cell type through TGF-β family signaling. The identification and isolation of this multipotent cell may provide a source of islets for transplantation in humans. The regulation of these cells may also provide insight into the mechanisms regulating physiological expansion of β-cells in conditions of insulin resistance and the failure of the β-cell mass that occurs during the development of type 2 diabetes.

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