Tshz1 Regulates Pancreatic β-Cell Maturation

The homeodomain transcription factor Pdx1 controls pancreas organogenesis, specification of endocrine pancreas progenitors, and the postnatal growth and function of pancreatic β-cells. Pdx1 expression in human-derived stem cells is used as a marker for induced pancreatic precursor cells. Unfortunately, the differentiation efficiency of human pancreatic progenitors into functional β-cells is poor. In order to gain insight into the genes that Pdx1 regulates during differentiation, we performed Pdx1 chromatin immunoprecipitation followed by high-throughput sequencing of embryonic day (e) 13.5 and 15.5 mouse pancreata. From this, we identified the transcription factor Teashirt zinc finger 1 (Tshz1) as a direct Pdx1 target. Tshz1 is expressed in developing and adult insulin- and glucagon-positive cells. Endocrine cells are properly specified in Tshz1-null embryos, but critical regulators of β-cell (Pdx1 and Nkx6.1) and α-cell (MafB and Arx) formation and function are downregulated. Adult Tshz1+/− mice display glucose intolerance due to defects in glucose-stimulated insulin secretion associated with reduced Pdx1 and Clec16a expression in Tshz1+/− islets. Lastly, we demonstrate that TSHZ1 levels are reduced in human islets of donors with type 2 diabetes. Thus, we position Tshz1 in the transcriptional network of maturing β-cells and suggest that its dysregulation could contribute to the islet phenotype of human type 2 diabetes.

A reduction in functional β-cell mass underlies the progression of all forms of diabetes (1–3). The inability of exogenously administered therapeutics to replicate the finely tuned regulation of insulin secretion by pancreatic β-cells has raised the development of cell replacement strategies to a high priority. To this end, the directed differentiation of embryonic stem cells to insulin-producing β-cells and the reprogramming of differentiated non-β-cells are being aggressively pursued. The efficiency of generating mature functioning β-cells from human stem cell–derived pancreatic progenitors is low, however, paralleling a relative paucity of knowledge about factors regulating the maturation of hormone-producing cells in the pancreas (4).

Pancratic and duodenal homeobox 1 (Pdx1) is a critical regulator of pancreas formation and adult β-cell function (5–7). Pdx1 is first expressed in the mouse at embryonic day (e) 8.5 in the prepancreatic endoderm, is maintained in multipotent progenitor cells, and becomes restricted to the β- and δ-cells in the adult islet, with low levels detected in acinar tissue. Developmentally, Pdx1 is critical for maintaining pancreatic progenitors, promotion of endocrine cell specification, and β-cell proliferation (7–10). In the mature β-cell, Pdx1 is required for maintenance of the β-cell phenotype, glucose-stimulated insulin secretion, and cell survival (6,11,12). Human mutations of PDX1 cause pancreatic agenesis and monogenic forms of early- and late-onset diabetes, including neonatal diabetes, maturity-onset diabetes of the young, and late-onset type 2 diabetes (13–17). As it is such a critical regulator of pancreatic development and adult function, protocols aimed...
at differentiating embryonic stem cells to insulin-positive β-cells have relied on Pdx1 as a marker of proper cell differentiation.

We hypothesized that identification of Pdx1 transcriptional targets around the time of the secondary transition, when the principle wave of insulin+ cells is formed during embryogenesis, would lead to the discovery of novel maturation factors. To that end, we performed Pdx1 chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq), which led to the identification of the transcriptional regulator Tshz1, a member of the Teashirt zinc finger family of transcription factors that regulate cellular proliferation and differentiation and stem cell maintenance during embryonic development in Drosophila (18–21). In mammals, disruption of Tshz1 results in defects in axial skeletal, ear, and palate formation (22) as well as neuronal development and function of the olfactory bulb (23). Here, we demonstrate that Tshz1 is a direct target of Pdx1 in the endocrine pancreas, and we define the role of Tshz1 in β-cell differentiation and adult function utilizing Tshz1 null embryos and Tshz1+/− adult animals. Lastly, we identified Tshz1 as a component of the β-cell transcriptional network whose expression is altered in human islets isolated from donors with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Animals

Animals were housed at the animal care facility at the University of Pennsylvania, and all procedures were approved by the Institutional Animal Care and Use Committee. The Tshz1tm1Cor, Tshz1GFP, Pdx1loxP, and Tg(Neurog3-cre)C1Able alleles have previously been described (5,22–24). All animals were kept on a mixed CD1 background. For embryonic experiments, noon of the day of vaginal plug discovery was designated e0.5.

Animal Physiology

Glucose tolerance, insulin tolerance, and in vivo glucose-stimulated insulin secretion tests were performed on 11- to 14-week-old female animals. For glucose tolerance tests, mice were fasted for 16 h before injection of a 2 g/kg body wt 20% d-glucose i.p. Blood glucose was measured by handheld glucometer (FreeStyle/OneTouch) at 0, 15, 30, 60, and 120 min after injection. Serum was collected and circulating insulin was assayed by ELISA (Chemicon). For insulin tolerance tests, mice were fasted for 6 h prior to an injection of 1.5 units/kg insulin i.p. Radioimmunoassay was used to assess total pancreatic insulin and glucagon content from acid-ethanol extracted embryonic and adult pancreata. Samples were quantified by the Radioimmunoassay and Biomarkers Core of the University of Pennsylvania Diabetes Research Center.

Islet Isolation and Proinsulin:Insulin Measurement

Islets were isolated from 11- to 14-week-old female mice by collagenase digestion followed by three rounds of handpicking. Proinsulin and insulin content from isolated adult islets were assessed by ELISA (Chemicon). ChIP Assays and Library Preparation

Embryonic pancreata from e13.5 (n = 90) and e15.5 (n = 15) CD1 mice were isolated fixed in 1% formaldehyde and quenched in 150 mmol/L glycine. Pancreata were washed with PBS and lysed in 100 μL cold lysis buffer (10 mmol/L Tris-HCl at pH 8.0, 10 mmol/L NaCl, 3 mmol/L MgCl2, 1% NP-40, 0.1% SDS, and 0.5% deoxycholic acid) with protease and phosphatase inhibitors (Calbiochem) for 10 min. Lysates were then sonicated with a Diagenode Bioruptor (30-s on/off pulses for a total of 15 min). Immunoprecipitations were performed as previously described (25) using a goat anti-Pdx1 antiserum (kindly provided by Chris Wright, Vanderbilt University). ChIP PCR was performed with the following primers: Tshz1 intron F, 5′-AGCTCCACACTACGGGCAA-3′; R, 5′-CAGGGCTTCAATGGATTGGT-3′; Alb F, 5′-TGGGAAACCATCCTATCAGA-3′; and Alb R, 5′-CACCTTTGTGTGTGTTCCCTTTG-3′. ChIP-Seq libraries were prepared as previously described (26). Sequence alignment and statistical analysis were performed by the Functional Genomics Core at the University of Pennsylvania (http://fgc.genomics.upenn.edu/).

RNA Isolation and Quantitative RT-PCR Analysis

Embryonic pancreata from e15.5, e16.5, and e18.5 animals were isolated, stored in RNeAlater (Ambion), and homogenized in TRIzol (Invitrogen) using a TissueLyser (Qiagen). Islets were cultured overnight in islet medium (27) and homogenized in TRIzol (Invitrogen). Embryonic and islet RNA was isolated using the RNeasy Mini Kit (Qiagen). Mouse islet and embryonic RNA was reverse transcribed using SuperScript III (Invitrogen). Transcript measurement was measured using SYBR Green–based quantitative PCR (Bio-Rad), and values were normalized to HPRT transcript as an internal control. Human islet RNA was prepared and quantified as previously described using TaqMan primers specific for TSHZ1 (Applied Biosystems) (28,29). Values are expressed as fold change compared with wild-type littermate tissues. Primer sequences are included in Supplementary Table 1.

Histology

Embryonic and adult pancreata were isolated, fixed in 4% paraformaldehyde for 2 h at 4°C, and cryoembedded in OCT (Tissue-Tek). Ten-micron sections were cut, blocked in 5% donkey serum in PBS with 1% BSA, and incubated with the following primary antibodies in PBS with 1% BSA overnight at 4°C: guinea pig anti-insulin (1:500; Abcam), rabbit anti-glucagon (1:1,000; Sigma-Aldrich), goat anti-GFP (1:100; Abcam), and mouse Ki67 (1:500; BD Biosciences). Cy2, Cy3, and Cy5 secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a 1:500 dilution. DAPI was used as a nuclearcounterstain (Thermo Fisher). Images were captured on an Eclipse E600 epifluorescence microscope (Nikon) with a QIClick digital camera (QImaging). Insulin and glucagon area measurements were calculated using MetaMorph software. Area was quantified from e18.5 embryos every
100 μm and adult pancreata every 250 μm from a minimum of four animals per genotype. β-Cell proliferation was quantified on sections from e18.5 embryos every 100 μm with at least 2,000 total β-cells counted per mouse.

Statistics
Data are presented as means ± SEM. Differences between groups were analyzed using a two-tailed Student t test. Values were considered significant when the P values were less than 0.05. Two-way ANOVA was performed on glucose and insulin tolerance test data.

RESULTS
Pdx1 Regulates Tshz1 During Mouse Development
In order to identify new regulators of pancreatic endocrine maturation, we performed Pdx1 ChIP-Seq on whole pancreata at e13.5 and e15.5. These time points were chosen as Pdx1 switches from regulating genes involved in multipotency to that of the β-cell program during this time (8,30). We focused on Pdx1 targets that were occupied at both time points and are transcription factors not previously associated with endocrine cell function. From this analysis, we identified Tshz1. Pdx1 occupies Tshz1 at the proximal promoter and two intronic sites 3′ of the first exon (Fig. 1A). In order to validate the ChIP-Seq results, we performed Pdx1 ChIP on e15.5 pancreata and performed quantitative PCR using primers spanning the first intronic site, which is highly evolutionarily conserved and occupied by multiple islet factors including PDX1 in adult human β-cells (31). From this, we confirmed that Pdx1 does occupy this site at e15.5 (Fig. 1B). Tshz1 transcript is detected in neurogenin (Ngn)3-expressing pancreatic endocrine progenitors (32). To determine whether Pdx1 directly regulates Tshz1 in committed endocrine cells, we performed a loss-of-function experiment by deleting Pdx1 in endocrine precursors using the Tg(Neurog3-cre)C1Able line. Pdx1 was efficiently deleted from total pancreas RNA at e16.5 (Fig. 1C). Although Ngn3 expression was not altered, suggesting that the endocrine progenitors are still present, Tshz1 was markedly downregulated (Fig. 1C). These results demonstrate that Pdx1 regulates Tshz1 specifically in the endocrine compartment of the pancreas.

Tshz1 Is Expressed in Committed Endocrine Cells of the Developing Pancreas
We next sought to determine the expression pattern of Tshz1 beginning at the time of the secondary transition and through adulthood. To detect Tshz1, we utilized a GFP knock-in allele, which has been shown to faithfully recapitulate Tshz1 expression during olfactory bulb neuronal development (23). As Tshz1 mRNA was identified previously in Ngn3+ cells (32), we first determined whether Tshz1 is expressed in these cells. Interestingly, Tshz1 was not coexpressed with Ngn3. Rather, Tshz1 expression was tightly associated with that of the general

Figure 1—Pdx1 regulates Tshz1 during mouse pancreatic development. A: Pdx1 ChIP-Seq of e13.5 and e15.5 whole pancreas. For each time point, the input sample is on top, while the bars underneath each plot represent statistically significant peaks as assessed by HOMER (Hypergeometric Optimization of Motif EnRichment) (53). The red square demarcates the Pdx1 peak conserved between our results and those of Pasquali et al. (31). B: Pdx1 ChIP of e15.5 pancreata. Quantitative PCR was performed from three independent ChIPs. **P < 0.05, Student t test. C: Pdx1, Ngn3, and Tshz1 transcript level in the e16.5 pancreas of Pdx1loxP/loxP.Tg(Neurog3-cre)C1Able compared with control animals (Pdx1loxP/loxP). n = 4 pancreata/genotype. *P < 0.05, **P < 0.01, Student t test.
endocrine cell marker chromogranin A (Fig. 2A). To determine whether Tshz1 remains expressed in all endocrine cell types or becomes restricted to specific endocrine lineages, we assessed GFP colocalization with the endocrine hormones at both e15.5 and e18.5. At e15.5, we detected GFP in both insulin- and glucagon-positive cells; however, expression was higher in insulin-expressing cells than in glucagon-expressing cells (Fig. 2A). This pattern was maintained at e18.5 and in adult islets (Fig. 2B and C). We also noted a low level of GFP expression in somatostatin and pancreatic polypeptide–positive cells at e18.5 (Fig. 2B and C). Thus, Tshz1 expression is initiated after Ngn3 expression is extinguished and becomes relatively enriched in β-cells, with low levels of expression in α-cells, δ-cells, and pancreatic polypeptide cells.

**Tshz1 Is Not Required for Endocrine Cell Specification**

Utilizing homozygous Tshz1\(^{GFP/ GFP}\) mice, we examined the cell fate of GFP-expressing cells in the absence of Tshz1 function. We found insulin\(^+\)/GFP\(^+\) and glucagon\(^+\)/GFP\(^+\) cells in the pancreas of Tshz1\(^{GFP/ GFP}\) mice (Fig. 3A and data not shown), indicating that Tshz1 is not required for endocrine cell specification. To determine whether Tshz1 is required for the expansion of α- or β-cell mass, we assessed relative area occupied by glucagon and insulin staining in Tshz1\(^{tm1Corè/tm1Corè}\) mice. No differences were detected, and there was not any effect on β-cell replication (Fig. 3B and Supplementary Fig. 1). These results indicate that in the absence of Tshz1, endocrine cells are formed and do not undergo increased cell proliferation as has been described in olfactory bulbs lacking Tshz1 (23). The absence of an effect on β-cell proliferation in the context of normal β-cell mass suggests that β-cell survival is also not affected by Tshz1 deficiency. Finally, we assessed expression of all hormone transcripts at e18.5; all showed normal levels in Tshz1\(^{tm1Corè/tm1Corè}\) mice, with the exception of glucagon, which was reduced by \(\sim60\%\) (Fig. 3D). Collectively, we conclude that Tshz1 is an endocrine-specific transcription factor that is not involved in endocrine cell specification or growth during development.

**Tshz1 Deficiency Disrupts the Transcriptional Network of the Developing Endocrine Pancreas**

We next examined expression of members of the transcriptional network of the developing endocrine pancreas at e15.5 and e18.5. We detected no change in Ngn3 or of regulators of α- and β-cell formation at e15.5, suggesting normal size of the endocrine progenitor pool and consistent with the absence of Tshz1 expression in Ngn3\(^+\) cells and with the normal mass of α- and β-cells observed at e18.5. (Figs. 3B and 4A). In contrast, Pdx1, Nkx6.1, and MafB, key regulators of the β-cell differentiation program, were significantly reduced at e18.5, whereas MafA was unchanged (33–35) (Fig. 4B). Expression of Arx, a transcriptional regulator of the α-cell program, was also moderately reduced (36,37). Collectively, our results position Tshz1 in the transcriptional networks involved in both α- and β-cell differentiation.

**Adult Tshz1 Heterozygous Mice Display Defects in Insulin Secretion**

To study the role of Tshz1 in postnatal β-cells, we analyzed Tshz1\(^1/vtm1Corè\) mice with heterozygous Tshz1 gene disruption (22). Tshz1-null mice could not be investigated, as they die soon after birth owing to an inability to feed secondary to defects in palate formation (22). In contrast, Tshz1\(^1/vtm1Corè\) mice are viable with normal body weight (Supplementary Fig. 2). α- and β-Cell mass were not different between Tshz1\(^1/vtm1Corè\) and littermate controls (Fig. 5A). To assess a role for Tshz1 in islet function, we examined acute glucose-stimulated insulin secretion. While basal insulin secretion was normal, Tshz1\(^1/vtm1Corè\) mice exhibited a 50% decrease in glucose-stimulated insulin secretion at 3 min (Fig. 5B). The reduction in insulin secretion was associated with reduced glucose tolerance measured by intraperitoneal glucose tolerance test (Fig. 5C). Insulin sensitivity of Tshz1\(^1/vtm1Corè\) mice as assessed by insulin tolerance test was normal, indicating that diminished peripheral insulin sensitivity does not contribute to the glucose intolerance of Tshz1\(^1/vtm1Corè\) mice (Fig. 5D). These results demonstrate a specific role for Tshz1 in maintaining normal glucose homeostasis through the regulation of β-cell function.
Reduced insulin secretion could be due to defects in insulin expression or processing. Thus, we assessed insulin content, transcript, and proinsulin:insulin ratios from adult Tshz1+/tm1Corè mice, all of which were normal (Supplementary Fig. 3 and Fig. 6A). Similar to the observations during embryonic development, the transcriptional regulators Pdx1 and MafB were both significantly downregulated in adult Tshz1+/tm1Corè islets, whereas Nkx6.1 was not changed (Fig. 6A). Pdx1 has been shown to be a critical regulator of genes involved in glucose-stimulated insulin secretion including Glut2 and Tfam (11,38). To assess whether these targets are changed, as well as Clec16a, a regulator of mitophagy (39) and direct target of Pdx1 (S.A.S., personal communication), we measured transcript from wild-type and Tshz1+/tm1Corè islets. Although we did not detect any significant changes in Tfam or Glut2, Clec16a was significantly downregulated (Fig. 6A). These data demonstrate that, similar to embryonic development, Tshz1 regulates the transcriptional network of adult islets; the defects in insulin secretion
that we observe are potentially due to reduced mitochondrial function rather than to defects in insulin production or processing.

**Type 2 Diabetic Islets Have Decreased TSHZ1**

Mutations of individual members of the transcriptional network of β-cells have been identified in monogenic forms of early- and late-onset diabetes in humans (40). This may destabilize the transcriptional network of the mature cell as well as impact specific components of the secretion pathway (31). Indeed, islets from donors with type 2 diabetes have a disruption in several key regulators of the adult β-cell (29) whose expression is perturbed in Tshz1+/− islets (Fig. 6A). Therefore, we sought to determine whether TSHZ1 expression itself is affected in human diabetic and control islets. As previously described (29), PDX1 levels are decreased by ~50% (Fig. 6B). We observed a modest but significant decrease of TSHZ1 in diabetic versus control samples, suggesting that Tshz1 is a component of the β-cell transcriptional network whose expression is altered in human type 2 diabetes.

**DISCUSSION**

Here, we show that the transcription factor Tshz1 is regulated by Pdx1 in committed pancreatic endocrine cells and that Tshz1 is a component of the transcriptional network of developing and mature hormone-producing cells. Heterozygous loss of Tshz1 in adult mice leads to decreased secretory function of β-cells and glucose intolerance. Finally, we show that Tshz1 is a member of the adult β-cell transcriptional network that is disrupted in human type 2 diabetic islets.

The impact of genetic disruption of Pdx1 and Tshz1 on the expression of Tshz1 and Pdx1, respectively, in mature β-cells suggests that a positive-feedback loop involving these two factors becomes operative during islet maturation. Our data indicate that Pdx1 directly regulates Tshz1 in the developing pancreas and that Tshz1 directly or indirectly feeds back to regulate Pdx1. The similarity between the high level of Pdx1 expression and the relative enrichment of Tshz1 in maturing β-cells further supports this concept. Positive-feedback loops have been described for several factors involved in pancreas formation and function (31,41,42). It is hypothesized that perturbations in one of these factors leads to the development of diabetes through downregulation of all members of the loop and their downstream targets (43). The human TSHZ1 promoter is occupied by NKX2.2 and FOXA2 in adult human islets (31), suggesting additional layers of cross-regulation within the transcriptional network of the mature endocrine pancreas.

We found no effect of Tshz1 gene disruption on insulin gene expression, insulin content, or insulin processing. 

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**Figure 4**—Key regulators of α- and β-cell differentiation are decreased in Tshz1-null mice. Transcript levels from wild-type (WT) or Tshz1tm1Corè/tm1Corè whole pancreata at e15.5 (A) or e18.5 (B) were assessed for changes in key islet developmental regulators. n = 7–10 per group per time point. *P < 0.05, **P < 0.01, Student t test.
Neither Glut2 nor Tfam, other transcriptional targets of Pdx1 that contribute to the Pdx1 deficiency phenotype, was dysregulated in Tshz1 heterozygous islets (11,38). The role of Tshz1 in the mature β-cell may be to regulate mitochondrial function, based on the reduction in Clec16a, which we recently demonstrated to regulate insulin secretion and mitochondrial turnover by mitophagy in pancreatic β-cells (39). It is not clear whether Tshz1 directly regulates Clec16a or does so only indirectly through its regulation of Pdx1. Future studies involving a combination of ChIP-Seq and RNA-Seq will be required to comprehensively identify the direct targets of Tshz1 that mediate its regulation of mature β-cell function.

Expression analysis of human ES cells during directed differentiation into endocrine cells describes a pattern of TSHZ1 expression similar to the pattern we demonstrate here during mouse pancreas development (44). TSHZ1 is detected just after the specification of the endocrine network, as shown by NGN3 expression. Furthermore, cells that fail to differentiate into monohormonal cells have decreased levels of TSHZ1 compared with adult human islets. Thus, increasing TSHZ1 levels might be an attractive way to promote endocrine cell maturation in current stem cell differentiation protocols in part by promoting optimal expression of key transcription factors.

Our results demonstrate a role for Tshz1 in regulation of β-cell maturation and not endocrine progenitor survival or expansion. These results are interesting, as studies in Drosophila have implicated Teashirt (Tsh) in multiple developmental functions including maintenance of progenitor cells and promotion of differentiation (19,45). These studies suggest that distinct binding partners mediate the disparate functions of Tsh. For example, during Drosophila retinal development, complexes including Tsh, Eyeless (Pax6), and Hth (Meis factors) maintain the proliferation of progenitor cells while simultaneously suppressing differentiation (19). Furthermore, conditional knockout of Tshz1 in the olfactory bulb elicits increased proliferation and a derepression of Pax6, which is not noted in our knockout or heterozygous Tshz1 animals. The list of cofactors that have been identified to interact with Tsh also includes components of the Hippo pathway, Meis and Pax factors, and members of the Wnt and Hh pathways (19,21,46). These cofactors are noteworthy in that they have been shown to play distinct roles in pancreatic development from progenitor cell maintenance to cell proliferation to endocrine cell function (47–52).

Whether Tshz1 relies on interactions with a known partner to mediate its functional role in endocrine maturation or interacts with a novel cofactor(s) in this context will be

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**Figure 5**—Tshz1+/− mice are glucose intolerant owing to decreased insulin secretion. A: Islet α- and β-cell mass from 12-week-old female wild-type (WT) and Tshz1+/+mCoré mice. n = 5–6 per group. P = NS as assessed by Student t test. B: Acute glucose-stimulated insulin secretion after a glucose bolus was measured from serum at 0 and 3 min postinjection (13-week-old female mice). n = 16–18 per group. *P < 0.05. C: Intraperitoneal glucose tolerance test performed on 11-week-old female mice. n = 10–12 per group. P < 0.05, Tshz1+/+mCoré versus Tshz1+/− mice, by ANOVA. D: Insulin tolerance of female mice (11–12 weeks old). n = 10–12 per group. P = NS, Tshz1+/+mCoré versus Tshz1+/−, by ANOVA.
of significant interest and relevance to translational efforts to promote β-cell maturation.

Type 2 diabetes is a complex disease with no single gene identified in its pathogenesis. Perturbations of multiple factors involved in adult function have been demonstrated, which could contribute to the reduced functional capacity of these cells (29). Here, we show that TSHZ1 is also downregulated in type 2 diabetic islets. The similarity of the transcriptional network of Tshz1+/− islets with that of human type 2 diabetic islets highlights the potential importance of TSHZ1 in human islet function. Collectively, these studies identify Tshz1 as a new regulator of endocrine cell maturation and function that might be exploited for therapeutic benefit for diabetes.

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