The diabetes gene Hhex maintains δ-cell differentiation and islet function

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The homeodomain transcription factor HHEX (hematopoietically expressed homeobox) has been repeatedly linked to type 2 diabetes mellitus (T2DM) using genome-wide association studies. We report here that within the adult endocrine pancreas, Hhex is selectively expressed in the somatostatin-secreting δ cell. Using two mouse models with Hhex deficiency in the endocrine pancreas, we show that Hhex is required for δ-cell differentiation. Decreased somatostatin levels in Hhex-deficient islets cause disrupted paracrine inhibition of insulin release from β cells. These findings identify Hhex as the first transcriptional regulator specifically required for islet δ cells and suggest compromised paracrine control as a contributor to T2DM.

Results and Discussion

As a prerequisite toward understanding the potential role of Hhex in glucose homeostasis, we characterized its expression pattern in the adult pancreas. Coimmunofluorescent labeling of Hhex and individual hormone markers in the 9- to 12-wk-old mouse pancreas revealed Hhex nuclear protein in pancreatic ducts and Sst-secreting δ cells (Fig. 1A). Contrary to expectation, we did not detect any Hhex immunoreactivity in insulin-producing β cells or other endocrine cell types (Fig. 1B–D). In fact, 89% of Hhex-expressing cells coexpressed Sst, and, conversely, 94% of Sst-positive cells were also Hhex-positive. Similar to our observation in mice, in the adult human pancreas, a strong nuclear HHEX signal was detected in Sst-expressing cells (Fig. 1E) rather than other endocrine cell types (Fig. 1F–H).

The δ cell, which comprises 6% of mouse and 11% of human islet cells (Brissova et al. 2005), contributes to glucose homeostasis in a paracrine fashion. In response to stimuli such as glucose and tolbutamide, the δ cell releases Sst, which binds to the Sstr receptor 2 (Sstr2) on adjacent α cells and Sstr5 on β cells to inhibit glucagon and insulin secretion, respectively (Moller et al. 2003). Islets from Sst-/- mice show elevated insulin and glucagon secretion in response to various secretagogues (Hauge-Evans et al. 2009). In addition, Sstr2-deficient mice, which lack paracrine inhibition specifically in α cells, are hyperglycemic due to elevated glucagon secretion (Singh et al. 2007).

In order to study the contribution of Hhex to islet function, we ablated Hhex in islet δ cells in mice. Due to the lack of a δ-cell-specific Cre line, we used Pdx1-CreER (Gu et al. 2002) mice, in which Cre recombinase is active in both β and δ cells upon induction by tamoxifen (Guz et al. 1995; Serup et al. 1995; Schafer et al. 2011). We reasoned that because Hhex is not expressed in β cells,
these mice, once crossed with $Hhex^{\text{loxP/loxP}}$ mice, would allow us to analyze the $\delta$-cell-specific functions of Hhex. As predicted, when we ablated Hhex in 9- to 12-wk-old animals using Pdx1-CreER, by 1 wk of tamoxifen treatment, insulin- and glucagon-producing cell numbers and islet architecture were normal, as evidenced by hormone immunofluorescence staining [Fig. 2A–B'] and quantification of the endocrine cell area [Fig. 2E, F]. In addition, immunoreactivity of two key $\beta$-cell signature genes, Pdx1 and Glut2, was indistinguishable between controls and mutants (Fig. 2C–D'). Consistent with this finding, we detected comparable mRNA levels of a spectrum of $\beta$-cell marker genes in control and mutant islets [Fig. 2G]. As expected from the absence of Hhex expression in $\alpha$ and $\beta$ cells, these results confirm that Hhex does not act cell-autonomously in these cells.

In contrast to the lack of effect on $\alpha$- and $\beta$-cell number, Sst immunolabeling revealed that mutant islets lost $>$75% of $\delta$-cell area compared with controls (Fig. 3A–C). This finding was confirmed on the transcript level, where Sst expression in mutant islets was only 25% of that in the controls (Fig. 3D). In addition, Hhex mutant islets secreted 80% less Sst than control islets under both low (1 mM) and high (20 mM) glucose conditions, congruent with the reduced number of $\delta$ cells (Supplemental Fig. S1). Furthermore, expression of the cholecystokinin B receptor (Cckbr), a $\delta$-cell marker (Morisset et al. 2000), was significantly down-regulated in Hhex-deficient islets (Fig. 3D), indicating $\delta$-cell dedifferentiation in the absence of Hhex. Of note, the remaining $\delta$ cells in Hhex mutants resulted from incomplete Hhex ablation, as most of them maintain Hhex expression (Fig. 3B'). Collectively, these data demonstrate that Hhex is required for the maintenance of the $\delta$-cell phenotype in the adult islet.

To determine whether Hhex also controls $\delta$-cell differentiation during fetal development, we employed Ngn3Cre [Schonhoff et al. 2004] to inactivate Hhex in endocrine progenitors. $Hhex^{\text{loxP/loxP}}$ mice displayed a complete loss of Sst$^+$ cells in islets at embryonic day 16.5 (E16.5) and 16 wk of age, indicating an essential role of Hhex in $\delta$-cell differentiation [Fig. 4]. Note that, similar to our adult gene ablation model, $\beta$-cell number was not affected by Hhex deficiency.

Next, we explored the functional consequences of Hhex deficiency in adult $\delta$ cells. To evaluate glucose responsiveness, we challenged control and Hhex mutant mouse islets with a glucose ramp (0–25 mM) in the islet perfusion assay. Compared with controls, mutant islets exhibited both a lower glucose response threshold and enhanced overall insulin secretion [Fig. 3E]. The unbridled insulin secretion in mutants stemmed from Hhex deficiency rather than ectopic expression of Cre in the $\beta$ cell from the Pdx1-CreER transgenes, whose lack of effect on insulin secretion has been validated in several reports [Lee et al. 2011; Schaffer et al. 2011; Gao et al. 2014].

To examine glucagon secretory capacity, we employed static islet cultures. At the basal condition (16.7 mM glucose), glucagon release from control and mutant mouse islets was indistinguishable [Fig. 3F]. However, when stimulated by lowering the glucose concentration from 16.7 mM to 2.8 mM and simultaneously adding an amino acid mixture as a glucagon secretagogue, mutant islets secreted twice as much glucagon as the controls [Fig. 3F]. As predicted, elevated

Figure 1. Hhex expression in the adult mouse and human pancreas. (A) Immunofluorescent labeling of an adult mouse pancreas reveals robust Hhex expression in $\delta$ cells. Hhex immunoreactivity is also detected in pancreatic ducts (arrow) and, at lower levels, in some acinar cells. [B–D] Hhex immunofluorescence is undetectable in insulin (Ins)-expressing $\beta$ cells, glucagon (Glut2)-expressing $\alpha$ cells, or PP [pancreatic polypeptide] cells. [E–H] In the adult human pancreas, HHEX [red] is also expressed in Sst$^+$-positive (green) $\delta$ cells rather than other endocrine cell types. Borders of islets are outlined in white.

Figure 2. Acute Hhex ablation does not adversely affect $\alpha$- and $\beta$-cell numbers. [A–B'] Normal $\alpha$- and $\beta$-cell number and islet architecture in the Hhex mutant ($Hhex^{\text{loxP/loxP}}$; Pdx1-CreER mice treated with tamoxifen) and control ($Hhex^{\text{loxP/loxP}}$ mice treated with tamoxifen) pancreas as revealed by immunofluorescent staining. Islet areas are outlined in $B$ and $B'$. [C–D'] Immunolabeling showed that expression of key $\beta$-cell markers Pdx1 and Glut2 is indistinguishable in control and Hhex mutants. [E, F] $\beta$-Cell and $\alpha$-cell areas in Hhex mutants are similar to those seen in controls ($n = 4$). [G] Transcript levels of $\beta$-cell signature genes Pdx1, NeuroD, Nkx6.1, Glut2, Ins1, and Ins2 are comparable between controls and Hhex mutants as evaluated by qRT–PCR ($n = 4–6$). Data are represented as mean ± SEM. (ns) Not significant.
glucagon secretion in mutants was partially reversed by treatment with 2.4 μM Sst, confirming that the secretory defect in Hhex mutant islets was due to β-cell dysfunction (Fig. 3F). In summary, loss of Hhex disrupts inhibitory paracrine regulation by the β cell, resulting in abnormally enhanced insulin and glucagon release in response to stimuli. These findings establish a role for Hhex in normal islet physiology by maintaining β-cell number and function. Although impaired β-cell function in 9- to 10-wk-old Hhex mutants did not compromise glucose tolerance [Supplemental Fig. S2A], likely due to the counteracting effect from elevated glucagon release, Hhex deficiency resulted in enhanced fasting and overall plasma insulin concentrations after glucose injection [Supplemental Fig. S2B], confirming dampened paracrine control on insulin secretion.

Next, we sought to investigate the molecular mechanisms of how Hhex controls β-cell number. We speculated that Hhex might contribute to β-cell survival. Therefore, we screened for apoptotic cells via TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end labeling) and cleaved caspase-3 immunolabeling in Hhex mutant pancreatic at multiple time points following the first tamoxifen injection [Supplemental Fig. S3; data not shown]). However, we did not find any evidence of increased apoptosis at any time point examined. We thus conclude that the absence of Hhex does not affect β-cell survival.

Alternatively, Hhex could be a direct transcriptional activator of the Sst gene. We addressed this possibility using multiple means. We began by assessing whether there is a direct dominant activity of Hhex on the Sst promoter. Overexpression of human HHEX protein in MIN6 (mouse insulinoma) β cells [Miyazaki et al. 1990; Ishihara et al. 1993] induced an ∼35-fold increase in endogenous Sst mRNA expression as demonstrated by quantitative RT–PCR [qRT–PCR] (Fig. 5A). In contrast, ectopic HHEX expression in nonendocrine HeLa cells failed to augment Sst levels [Fig. 5B], suggesting a context-dependent role of HHEX in controlling Sst transcription. Furthermore, chromatin immunoprecipitation (ChIP) assays in MIN6 cells overexpressing the HHEX protein demonstrated HHEX binding at two specific loci [100 base pairs [bp] and 380 bp upstream of the Sst transcriptional start site [TSS]] within the endogenous Sst promoter [Fig. 5C]. A 676-bp-long proximal element of the Sst promoter encompassing these Hhex-binding sites exhibited robust activity in luciferase reporter assays. Mutation of the core Hhex-binding motif ATTA to CCCC diminished the activity of the Sst proximal promoter by 80% [Fig. 5D]. These findings suggest that Hhex directly activates Sst transcription.

In summary, we report Hhex as the first transcription factor specifically required for β-cell maintenance. We further demonstrate that Hhex contributes to islet function not by controlling β-cell physiology directly but by maintaining the differentiated phenotype of the β cell and thus paracrine regulation of β-cell activity. These findings suggest that misregulated HHEX expression within the diabetic islet might contribute to disrupted paracrine control of insulin secretion in T2DM, leading to accelerated β-cell exhaustion and β-cell failure. In the future, it will be interesting to determine the fate of the Hhex-deficient, dedifferentiated β cells using genetic lineage tracing once a robust, β-cell-specific CreER transgenic line becomes available.

**Materials and methods**

**Mice**

The derivation of the HhexloxP allele has been described previously [Hunter et al. 2007]. Pdx1-CreER mice (Gu et al. 2002) were kindly provided by Dr. Guoqiang Gu and Dr. Doug Melton. Ngn3Cre mice were purchased from Jackson Laboratory. All mice were maintained on a mixed 129SvEv/C57BL/6 background. Genotyping was performed by PCR analysis using genomic DNA isolated from the tail tips of newborn mice. Adult mice [9–12 wk old] were used in all experiments. To induce gene ablation, 1.6 mg of tamoxifen (Sigma) was injected into HhexloxP/loxP (control) and HhexloxP/FK1-CreER (mutant) animals on three consecutive days. All experiments were performed 3 d after the last
the total pancreatic areas were measured by ImageJ (Schneider 2012). Immunostaining signal magnification. The areas of positive signal and spans was used for hormone immunoperoxidase labeling. This sampling method sections. Every sixth section (a total of three to four sections per pancreas) longitudinal sections were prepared, with 50-

To quantify the ratio of hormone area to pancreas area, whole pancreata were removed, laid flat, and fixed as above. A micrometer angular sections were prepared, with 50-µm intervals between sections. Every sixth section (a total of three to four sections per pancreas) was used for hormone immunoperoxidase labeling. This sampling method spans >50% of the pancreatic volume. In addition, care was taken to score equivalent regions of control and mutant specimens. After immunohistochemical staining, 100% of each hormone-labeled section was scanned equivalent regions of control and mutant specimens. After immunohistochemistry of Sst, slides were blocked with Avidin B, goat anti-glucagon [1:250; Santa Cruz Biotechnology], goat anti-ghrelin [1:250; Santa Cruz Biotechnology], guinea pig anti-insulin [1:250; Millipore], goat anti-ghrelin [1:250; Santa Cruz Biotechnology], and rabbit anti-cleaved caspase-3 [1:750; Cell Signaling] were diluted in Casblock and incubated overnight at 4°C. Secondary antibody incubation was performed with species-specific fluorophore-labeled secondary antibodies (1:500; Jackson ImmunoResearch Laboratories) for 2 h at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Confocal fluorescent imaging was visualized using a Zeiss LSM 510 NLO/META confocal microscope at 60×. TUNEL staining was performed using the TUNEL labeling kit from R&D Systems according to the manufacturer's instructions.

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Islet insulin and glucagon secretion

Islets were isolated from control and mutant mice using standard collagenase digestion followed by purification through a Ficoll gradient. (Gao et al. 2007). To measure insulin release in response to glucose, islet perfusion was performed as previously described [Gao et al. 2010] after overnight culture at 37°C. Insulin content was determined using radioimmunoassay by the Radioimmunoassay and Biomarkers Core at the University of Pennsylvania. Glucagon release was measured by static secretion assay. Islets were preincubated for 60 min in buffer containing 16.7 mM glucose in a 96-well plate (80 islets per well), after which islets were challenged by the following conditions at 37°C: 16.7 mM glucose for 1 h, 2.8 mM glucose with 7 mM amino acid mixture and 1 mM glutamine for 30 min, and 2.8 mM glucose with 7 mM amino acid mixture, 1 mM glutamine, and 2.4 μM Sst1-14 [Sigma] for 30 min. Glucagon content was assayed by commercial EIA kit according to the manufacturer's instructions (glucagon EIA kit, R&D Systems). For assessment of total islet hormone content, islets were sedimentered by centrifugation, washed with PBS, and sonicated in RIPA buffer.

RNA extraction and qRT–PCR

Total RNA from mouse islets or MIN6 cells was extracted using RNeasy minikit (Qiagen) and analyzed by qRT–PCR as described previously [Le Lay et al. 2009]. qRT–PCR primer sequences are available on request.

MIN6 cell culture and ChIP

MIN6 cells were maintained in DMEM containing 10% FBS and 25 mM glucose at 37°C in 5% CO2. Two micrograms of pMUG-Myc-HHEX plasmid or pMUG vector [Swinger et al. 2004] was introduced via nucleofection[Amazka Biosystems] (Liu et al. 2010). Cells were harvested

**Figure 4.** Hhex deficiency in endocrine progenitors leads to loss of δ cells. Hhex was ablated during fetal pancreatic development using the Ngn3Cre transgene. Representative images of Sst and insulin double immunolabeling in a control (Hhex<sup>+/+</sup>) and mutant (Hhex<sup>−/−</sup>; Ngn3Cre<sup>+/−</sup>) pancreas at E16.5 (A–B’) and 16 wk (C–D’). (B’, D’, D’) Note the absence of Sst<sup>+</sup> cells in Hhex-deficient mice.

**Figure 5.** Hhex maintains δ-cell number through transcriptional up-regulation of Sst rather than affecting apoptosis. (A) Ectopic expression of HHEX is sufficient to induce Sst expression in MIN6 cells transfected with a Myc-tagged HHEX expression plasmid (n = 3). (B) Overexpression of HHEX in HeLa cells fails to activate Sst (n = 3). (C) ChIP using an anti-Myc 9E10 antibody was performed on Myc-tagged HHEX-expressing MIN6 cells followed by qPCR evaluation of enrichment at three putative binding sites of Hhex within the Sst promoter. Data are presented as the fold enrichment of the target amplicon in ChIP DNA compared with input DNA (n = 2 or 3). (D) Dual-luciferase reporter assays were conducted in MIN6 cells transfected with reporter constructs containing the intact (pGL3-Sst WT) or mutated (pGL3-Sst MT) proximal promoter of Sst or the basic vector pGL3 (n = 3). Data are represented as mean ± SEM. [*] P < 0.05; [**] P < 0.001; [ns] not significant. See also Supplemental Figure S3.
48 h after transfection for ChIP assays or RNA extraction. ChIP assays were performed following the protocol in Le Lay et al. (2009) using MycE10 antibody [Abcam] followed by qRT-PCR validation with the following primers: Sst-2.2kb-F, GGAAATAATTTGACCTGCCTCT; Sst-2.2kb-R, TGAGGCTGTAGATGAAGTACC; Sst-380-F, TCCGCAAATAGGAGGCCA; Sst-380-R, TGAGGCCATAGGAGGCCAAC; Sst-100-F, CCTTCCCTACTCTGATC; and Sst-100-R, TCC ACACTCCCTCCCTTAAAA.

Luciferase reporter assays

A 676-bp fragment (~750 bp relative to the TSS) of the Sst promoter spanning the Hhex-binding sites was PCR-cloned in the NheI basic vector to build pGL3-Sst wt-luc vector construct. A mutated version of this element was generated by Eurofins MWG Operon and subsequently inserted into pGL3-Sst basic vector at the Nhel and Xhol sites, resulting in mutated pGL3-Sst. One microgram of pGL3 basic vector, pGL3-Sst wt-type, or pGL3-Sst mutant mixed with 10 ng of phRL (Invitrogen) was introduced into ~1 × 10^6 cells via Lipofectamine (Invitrogen). A dual luciferase reporter assay was performed 48 h after transfection according to the manufacturer’s instruction [Promega]. Luciferase activity was normalized for transfection efficiency by corresponding Renilla luciferase activity. Experiments were performed on three biological triplicates.

Statistical methods

Statistical analysis between two groups was performed using a two-tailed Student’s t-test unless noted otherwise. Values were considered significant when P < 0.05. Variation measurements are given as standard error of the mean.

Acknowledgments

We thank Dr. Guoqiang Gu and Dr. Douglas Lab for sharing Pdx1-CreER mice, Dr. Padma-Sheela Jayaraman for providing the pMUG-Myc-HHEX plasmid, and Dr. Joshua Friedman for valuable comments on the manuscript. We are grateful to Karrie Brondell and Tia Bernard for maintenance of the mouse colonies, and Itai Doron and Xiao Ji for technical assistance. We thank the Morfology Core of the Penn Center for Molecular Studies in Liver and Digestive Disease [P30-DK050306] for reagents and technical assistance. We appreciate the help of Dr. Franz M. Matschinsky and Wei Qin at the Islet Cell Biology Core of the University of Pennsylvania Diabetes Research Center [P30-DK19525] for islet perfusion assays. This work was funded by National Institutes of Health grant R01-DK055342 and American Diabetes Association grant number 7-12-MN-37 to K.H.K.

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