Loss of Nix in Pdx1-deficient mice prevents apoptotic and necrotic β cell death and diabetes

Kei Fujimoto,1 Eric L. Ford,1 Hung Tran,1 Burton M. Wice,1 Seth D. Crosby,2 Gerald W. Dorn II,3 and Kenneth S. Polonsky1

1Division of Endocrinology, Metabolism and Lipid Research, Department of Medicine, 2Genome Sequencing Center, Department of Genetics, and 3Center for Pharmacogenomics, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, USA.

Mutations in pancreatic duodenal homeobox (PDX1) are linked to human type 2 diabetes and maturity-onset diabetes of the young type 4. Consistent with this, Pdx1-haploinsufficient mice develop diabetes. Both apoptosis and necrosis of β cells are mechanistically implicated in diabetes in these mice, but a molecular link between Pdx1 and these 2 forms of cell death has not been defined. In this study, we introduced an shRNA into mouse insulinoma MIN6 cells to deplete Pdx1 and found that expression of proapoptotic genes, including NIP3-like protein X (Nix), was increased. Forced Nix expression in MIN6 and pancreatic islet β cells induced programmed cell death by simultaneously activating apoptotic and mitochondrial permeability transition–dependent necrotic pathways. Preventing Nix upregulation during Pdx1 suppression abrogated apoptotic and necrotic β cell death in vitro. In Pdx1-haploinsufficient mice, Nix ablation normalized pancreatic islet architecture, β cell mass, and insulin secretion and eliminated reactive hyperglycemia after glucose challenge. These results establish Nix as a critical mediator of β cell apoptosis and programmed necrosis in Pdx1-deficient diabetes.

Introduction

Embryonic and postnatal development of the pancreas is orchestrated by the transcription factor pancreatic duodenal homeobox (PDX1; also known as insulin promoter factor 1, IPF1). Homozygosity of a frameshift PDX1 mutation that ablates the PDX1 DNA binding domain causes the rare clinical syndrome pancreatic agenesis (1), whereas heterozygous loss-of-function PDX1 mutations are linked to common human type 2 diabetes and cause inheritable maturity-onset diabetes of the young type 4 (2–4). These human diabetic syndromes are characterized by absolute or relative insulin insufficienty without pancreatic exocrine or other endocrine insufficiency, suggestive of a unique regulatory function of PDX1 in insulin-secreting pancreatic islet β cells. Because they faithfully recapitulate the human clinical phenotypes, Pdx1-deficient mice have been widely used to characterize and define cellular mechanisms for β cell loss in diabetes. Homozygous null Pdx1 mutations cause murine pancreatic agenesis, whereas Pdx1-haploinsufficient mice exhibit characteristically small pancreatic islets with greatly reduced numbers of β cells, decreased insulin secretion, and glucose intolerance that results in diabetes (5–7).

Because Pdx1 directs pancreatic growth during development, the cellular basis for diabetes caused by Pdx1 deficiency was initially thought to be decreased pancreatic β cell genesis (6, 8–10). Recent studies have refuted this notion, however, implicating programmed β cell death rather than decreased proliferation as the causative event in diabetes caused by Pdx1 insufficiency. This issue has been made more complex as 3 mechanistically distinct pathways to programmed β cell death have been observed in murine Pdx1 haploinsufficiency, apoptosis (11–14), autophagy (15), and programmed necrosis (16). Parallel pathways to β cell death offer an explanation for intermediate phenotypes obtained when individual death pathways have been targeted in Pdx1-haploinsufficient mice (15, 16).

The molecular basis by which relative insufficiency of Pdx1, a transcription factor that directs embryonic pancreatic growth and differentiation, induces multiple pathways of β cell death is not known. Because pancreatic islet β cells are dynamically regulated throughout life in response to metabolic stress (17), we considered that in addition to its developmental function, Pdx1 may play a critical homeostatic role in the adult pancreas, balancing β cell proliferation and programmed elimination in accordance with metabolic need. According to this scenario, the β cell growth factor Pdx1 should be regulated in opposition to as-yet-unidentified β cell death factors. Here, we performed unbiased transcriptional profiling of cultured murine MIN6 β cells after Pdx1 suppression by shRNA in search of reciprocal upregulation of programmed death genes and identified NIP3-like protein X (Nix; also known as BNip3L). Preventing Nix upregulation abrogated apoptotic and programmed necrotic β cell death induced by Pdx1 deficiency in vitro, and ablation of the Nix gene was sufficient to fully prevent both the diabetic phenotype and the morphological abnormalities in Pdx1-haploinsufficient mouse islets.

Results

Transcriptional profiling of Pdx1-deficient cultured insulinoma cells. To identify gene dysregulation induced by Pdx1 insufficiency, we used microarrays to compare the transcriptional signatures of cultured MIN6 mouse insulinoma β cells after Pdx1 suppression with our previously validated lentivirus-expressed Pdx1 shRNA (15, 16). Nonbiased hierarchical clustering of the mRNA expression profile in MIN6 cells after Pdx1 suppression (Figure 1A) identified 56 upregulated and 14 downregulated mRNAs (P < 0.01; FDR, 0.05). Grouping of differentially expressed transcripts by gene ontology category revealed that apoptosis/programmed cell death mRNAs were disproportionately represented among upregulated mRNAs (Figure 1B and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI44011). Among
these upregulated transcripts were the proapoptotic Bcl2 family members Nix, Bim, Puma, and Bcl2l14 (Supplemental Table 1). Confirmatory quantitative real-time PCR demonstrated robust upregulation of Nix, Bim, Puma, and Bcl2l14 mRNAs (Figure 1C and Supplemental Figure 1). Immunoblot analysis showed a corresponding increase in Nix protein induced by Pdx1 suppression (Figure 1D). Nix upregulation preceded cell death (see below).

Nix induces programmed cell death in cultured insulinoma cells. Recent studies have implicated Nix as a transcriptionally regulated apical regulator of apoptosis, autophagy, and/or necrosis in erythropoietic and cardiac cells (18–24). To our knowledge, its presence in pancreatic β cells has not previously been described. For this reason, we determined the consequences of increased Nix expression on this cell type: Nix was expressed in MIN6 cells in which Pdx1 was not suppressed (Figure 1D). Using an adenoviral (Ad) vector (22) at MOI of 10, we detected Flag-tagged Nix immunoreactivity in 95% ± 5% of MIN6 cells (Figure 2A). Consistent with its reported ability to stimulate the mitochondrial apoptosis pathway in a Bax/Bak-dependent manner (23), Nix localized largely to a mitochondrial-enriched subcellular fraction labeled by cytochrome C oxidase IV antibody (COX IV) and with mitochondria, stimulated the release of cytochrome c into the cytoplasm, and induced caspase-3 activation without induction of autophagy, measured as accumulation of LC3-II (Figure 2, B–E).

Although apoptosis is the mechanism of programmed β cell death most often reported with Pdx1 deficiency (11–14), we recently discovered that programmed necrosis mediated by opening of the mitochondrial permeability transition pore (MPTP) also plays a role in the diabetes of murine Pdx1 haploinsufficiency (16). Therefore, we determined if Nix induced the mitochondrial permeability transition in cultured murine β cells. MIN6 cells were loaded with rhodamine 123, a mitochondrial dye whose fluorescence depends upon mitochondrial membrane potential (Δψm). MPTP opening dissipates Δψm, and loss of rhodamine 123 fluorescence can therefore indicate MPTP opening (16). Figure 2D illustrates loss of rhodamine 123 fluorescence in Nix-overexpressing MIN6 cells. Quantitative analysis by fluorescence cell sorting revealed an approximately 50% loss of rhodamine 123 fluorescence in 3 independent studies (Figure 2F). Collectively, the above studies show that Nix activates apoptosis and necrosis pathways in cultured β cells.

We interrogated the roles of apoptosis and necrosis in Nix-mediated β cell death using propidium iodide (PI) staining as a marker for both apoptotic and necrotic cell death, and comparing the effects of pharmacological inhibitors of apoptosis (the caspase-3 inhibitor DEVD-CHO) and MPTP-dependent necrosis (the MPTP blocker cyclosporin A). Ad-Nix increased PI staining, and this was almost completely prevented by both DEVD-CHO and cyclosporin A (Figure 2G).
Figure 2

Nix overexpression induces programmed MIN6 cell death. (A) MIN6 cells with overexpression (OE) of β-gal or Flag-tagged Nix were stained with anti-Flag antibody (green) and DAPI (blue). Original magnification, ×400. (B) MIN6 cells were fractionated into 10,000 g pellet (10p) or 10,000 g supernatant (10s) and immunoblotted with antibody against Nix or COX IV, a marker of the mitochondrial fraction. (C) MIN6 cells with overexpression of β-gal or Nix were fractionated as in B, followed by immunoblotting with anti-cytochrome c (Cyt c) or COX IV antibodies. (D) Confocal imaging of β-gal– or Nix-overexpressing MIN6 cells. First row, Flag-tagged Nix (green) and mitochondrial (red) colocalization; second row, cytochrome c (green) and mitochondrial (red) staining; third row, caspase-3 activity (red); fourth row, rhodamine 123 (Rh123) staining (red). DAPI staining (blue) is also shown. Original magnification, ×1,000. (E) Immunoblot of Flag-tagged Nix, LC-3, and cleaved caspase-3 in β-gal– and Nix-overexpressing MIN6 cells. (F) Fluorescence cell sorting analysis of rhodamine 123 in β-gal– and Nix-overexpressing MIN6 cells. Mean fluorescence of data from 3 independent experiments is shown. (G) PI (red) and DAPI (blue) staining in β-gal– and Nix-overexpressing MIN6 cells as a function of 10 μM DEVD-CHO and/or 1 nM cyclosporin A (CsA) treatment. Quantitation of 3 independent experiments for PI staining data is shown at right. Original magnification, ×600. Data represent mean ± SEM.
Nix upregulation is necessary for β cell death induced by Pdx1 suppression. The above results describe an association between Pdx1 downregulation and Nix upregulation and demonstrate that Nix was sufficient to cause apoptotic and necrotic death of cultured β cells. To determine whether Nix upregulation after Pdx1 suppression is the essential event inducing MIN6 cell death after Pdx1 knockdown, we used shRNA to suppress Nix in cells infected with the Pdx1 shRNA lentivirus. Nix suppression in the Pdx1-insufficient state prevented caspase-3 activation, TUNEL positivity, loss of Δψₘ, and cell death as assessed by PI staining (Figure 3, A–E). Collectively, these and the above-described findings showed that Nix is both necessary and sufficient for programmed MIN6 apoptotic and necrotic death induced by acute Pdx1 suppression.

Nix is upregulated in Pdx1-haploinsufficient pancreatic islets and induces programmed islet cell death. To determine whether Nix induction plays a role in β cell loss seen with murine Pdx1 haploinsufficiency, we assayed Nix mRNA levels in pancreatic islets isolated from Pdx1⁻/⁻ mice fed a high-fat diet. Despite having proportionately fewer β cells than normal islets (see below), Nix mRNA levels indexed to the housekeeping gene Hmbs increased more than 2-fold in Pdx1⁺/⁻ compared with control islets (Figure 4A). This recapitulates the observation from MIN6 cells that Pdx1 and Nix are antithetically regulated and shows that this regulatory pairing is maintained in the context of chronic germline Pdx1 insufficiency. Consistent with a role for Nix upregulation in β cell death, apoptotic and necrotic death were induced in normal (Pdx1⁺/+) isolated pancreatic islets infected with Ad-Nix (MOI of 10; 85% ± 4% expression; Figure 4B), as assessed by caspase-3 activation and dissipation of mitochondrial Δψₘ (Figure 4, C and D).

Nix ablation preserves β cell mass and islet architecture in Pdx1-haploinsufficient mice. Because Nix was upregulated by Pdx1 deficiency in isolated pancreatic islets and cultured MIN6 cells, and increased
Nix expression caused programmed β cell death in these systems, we determined whether preventing β cell Nix upregulation in Pdx1−/− mice also prevents β cell loss in the in vivo condition. To accomplish this, we crossed Pdx1−/− mice onto the Nix-null (Nix−/−) background (21) and performed comparative histological studies of Pdx1+/− and Pdx1−/−Nix−/− mouse pancreata. The histopathological hallmarks of diabetes in the Pdx1−/− mouse were small pancreatic islets containing reduced numbers of insulin-containing β cells and abnormal islet architecture (Figure 5 and refs. 10, 15, 16). Whereas WT and Nix−/− pancreatic islets had a central core of β cells ringed by a mantle of α cells, the small Pdx1−/− islets had α cells distributed throughout the core, resulting in a characteristic decrease in absolute β cell area and in the β cell/α cell ratio (Figure 5A) with no change in islet number per pancreas (data not shown). Pdx1+/−Nix−/− mice had normal β cell area and β cell/α cell ratio, with normal islet architecture of a central core of β cells surrounded by a mantle of α cells (Figure 5A). The area of β cells per pancreas area on normal chow and high-fat diets was not different in Nix−/− mice compared with WT mice (Supplemental Figure 2A). Programmed β cell death, as assessed by the number of TUNEL-positive β cells, was characteristically increased in islets from Pdx1−/− mice and strikingly reduced in those of Pdx1+/−Nix−/− mice (Figure 5B). Nix−/− islets showed TUNEL positivity comparable to that of WT mice (Figure 5B), which supports our conclusion that prevention of β cell death in Pdx1−/−Nix−/− mice is caused by Nix deficiency and not epiphenomena. These findings were consistent in adult mice fed a high-fat diet as well as in neonatal mice 1 day after birth (Figure 5, C and D), which indicates that increased Nix expression adversely influences islets during Pdx1−/− mouse embryonic development, and not just during reactive islet growth in the adult.

Nix gene ablation prevents diabetes in Pdx1-haploinsufficient mice. To determine whether β cell salvage by Nix ablation prevents the diabetic phenotype of Pdx1−/− mice, studies of glucose and insulin tolerance were performed on Pdx1+/− and Pdx1−/−Nix−/− mice maintained on a high-fat diet to provoke glucose intolerance (15, 16). Pdx1−/− mice exhibited characteristic glucose intolerance and decreased basal and reactive insulin levels (Figure 6, A–C). In comparison, Pdx1+/−Nix−/− mice displayed normal serum glucose profile and insulin response. On the other hand, Nix−/− mice showed comparable insulin secretion and glucose tolerance to that of WT mice fed normal chow and high-fat diets (Supplemental Figure 2, B and C). Since the Nix−/− mouse is a germline knockout (21), it is possible that increased insulin sensitivity contributed to normalization of the response to glucose challenge. However, insulin tolerance tests were similar in both groups (Figure 6D). Thus, we conclude that Nix ablation prevents the diabetic phenotype in Pdx1-haploinsufficient mice by augmenting insulin secretion.

Discussion

These studies define a single molecular mechanism for increased pancreatic β cell apoptosis and programmed necrosis induced by Pdx1 insufficiency. Using the unbiased approach of transcriptional profiling in Pdx1-suppressed mouse islet cells, we identified a molecular program for β cell suicide that included Nix. We show that Nix activated β cell signaling pathways, leading to caspase-dependent apoptosis and MPTP-dependent necrosis, and that preventing Nix upregulation induced by Pdx1 deficiency was sufficient to prevent apoptotic and necrotic programmed β cell death in vitro and in vivo. In the Pdx1-haploinsufficient mouse model of diabetes, Nix gene ablation corrected the characteristic small size and loss of β cells from pancreatic islets, consequently preserving basal and reactive insulin secretion and normalizing glucose tolerance.

The current findings indicate that Nix and Pdx1 are opposing forces within a homeostatic mechanism that maintains β cell mass through reciprocal modulation of proliferation and programmed elimination. Pdx1 stimulates pancreatic β cell proliferation. Indeed, Pdx1 expression is essential for embryonic development of the pancreas, and homozygous loss-of-function mutations result in pancreatic agenesis in humans and mice (1, 5). In our prior studies of adult murine pancreatic islets, heterozygosity of Pdx1 dramatically depresses the rate of normal β cell proliferation, measured as Ki67 labeling (15, 16). The second hit that limits β cell mass in Pdx1 deficiency is a striking increase in programmed β cell death, commonly designated as apoptosis because of caspase activation and increased TUNEL labeling (15, 16). However, caspase activity and TUNEL positivity simply reflect cytochrome c release from mitochondria, which indicates that increased Nix expression adversely influences islets during Pdx1−/− mouse embryonic development, and not just during reactive islet growth in the adult.

Nix overexpression induces programmed islet cell death. (A) Real-time PCR of mRNA for Pdx1 and Nix using islets from WT and Pdx1−/+ mice after 1 week on a high-fat diet (n = 5–6 each). (B) Normal mouse islets expressing β-gal or Flag-tagged Nix were stained with anti-Flag antibody (green) and DAPI (blue). Original magnification, ×400. (C) Immunoblot of Flag-tagged Nix and cleaved caspase-3 in mouse islets overexpressing β-gal or Nix. (D) Cell sorting analysis to detect rhodamine 123 fluorescence in β-gal− or Nix-overexpressing islet cells. Mean fluorescence of 3 independent experiments is shown at right. Data represent mean ± SEM.
Figure 5
Preservation of β cell mass and islet architecture in Pdx1+/-Nix+/- mice. (A) Islet morphology in adult WT, Pdx1+/-, Pdx1+/-Nix+/-, and Nix+/- mice (9–11 weeks of age) after 6–8 weeks on a high-fat diet; α cells are stained red (anti-glucagon) and β cells are stained green (anti-insulin). Quantitation of group data for β cell area per pancreas area and β cell/α cell ratio are shown (n = 4–6 per group). (B) TUNEL labeling of adult pancreatic β cells; quantitative TUNEL data are shown at right (n = 5, each group). (C) Pancreatic islets from 1-day-old mice comparing α cells (red; anti-glucagon) and β cells (green; anti-insulin); quantitative group data are shown at right (n = 5 per group). (D) TUNEL labeling of neonatal β cells; quantitative data are shown at right (n = 5 per group). Original magnification, ×600 (including insets). Data represent mean ± SEM.
mitochondria, which can result from outer membrane permeabilization (apoptosis) or from mitochondrial disruption as a consequence of swelling after MPTP opening (necrosis). Thus, TUNEL positivity should not be equated with apoptotic cell death. Indeed, we recently demonstrated that MPTP-dependent programmed β cell necrosis is an important contributory mechanism in the diabetes of murine Pdx1 haploinsufficiency (16). Here, we identified Nix as a critical death-promoting counterregulatory factor that opposes β cell proliferation induced by Pdx1. Indeed, suppression or genetic ablation of Nix was sufficient to prevent β cell apoptosis and programmed necrosis induced by Pdx1 deficiency.

Nix is 1 of only 4 members of the BH3-only–like subfamily of Bcl2-like mitochondrial death proteins. The functional characteristic that distinguishes pseudo–BH3-only factors (Nix, BNip1, BNip2, and BNip3) from the larger group of true BH3-only factors (Bim, Puma, Bcl2I14, and others) is induction of a unique form of cell death with features of both apoptosis and necrosis (22–25). The mechanism for dual pathway activation by Nix has recently been elucidated in detail (23). Nix localizes to mitochondrial outer membranes stimulates Bax/Bak-dependent outer membrane permeabilization, releasing cytochrome c into the cytosol, and initiating the intrinsic caspase cascade that leads to apoptosis (19, 21–25). Nix can also localize to ER, stimulating the Bax/Bak-independent activation of mitochondrial permeability pores via ER-mitochondrial calcium transfer. Opening of MPTP and consequent loss of ΔΨm reverses oxidative phosphorylation, which depletes ATP, results in generation of ROS, and ultimately produces cellular necrosis from metabolic shutdown (22–25). The rare ability of Nix to activate independent apoptotic and necrotic pathways provides a mechanistic explanation for the complete prevention of the Pdx1−/− diabetic phenotype through Nix gene ablation, whereas inhibition of programmed necrosis alone (via genetic ablation of the essential MPTP protein cyclophilin D) provided only partial prevention (16).

A third mechanism of programmed β cell death, autophagy, has recently been implicated in Pdx1 deficiency (15). Autophagy induced by intense metabolic need (i.e., starvation) is a compensatory process enabling processing of cellular proteins for acute energy production (15, 25). An autophagic marker, subcellular aggregation of LC3 protein (a constituent of autophagosomal membranes), is increased in Pdx1-deficient β cells in vitro and in vivo, and inhibition of autophagy by Beclin 1 suppression or hemizygous gene ablation enhances Pdx1-deficient β cell viability (15). However, the preponderance of data suggests that autophagy is a contributory factor, and not causative, in β cell death caused by Pdx1 insufficiency. Inhibition of autophagy delays, but does not prevent, β cell death (15), and the benefits of Nix ablation on survival of Pdx1-deficient β cells were not associated with any decrease in autophagic markers in vitro or in vivo (Figure 2E and Supplemental Figure 3).

The current studies demonstrated counterregulation of β cell mass by Pdx1 and Nix in adult pancreata after the metabolic stress of a high-fat diet as well as in neonatal pancreata not been subjected to any such metabolic stress. Thus, it is likely that Pdx1 and Nix modulate reactive islet β cell hyperplasia as well as the latter stages of embryonic pancreas development (26). Further support for an important developmental role for Nix is normalization of islet cellular architecture. Normal pancreatic islets consist of a central core of insulin-containing β cells surrounded by a peripheral mantle of glucagon-containing α cells as well as other non–insulin-secreting cells. This highly ordered islet architecture is characterized disturbed in Pdx1-deficient mice, in which loss of β cells is associated with more random distribution of α cells throughout the islet core (ref. 16 and the present study). Moderation of β cell death and increased islet size is not necessarily linked with preservation of normal peripheral α cell positioning, as recently shown in Pdx1−/− Ppif−/− mice (16). However, in the current study, Nix ablation normalized pancreatic islet architecture as well as size and β cell content. Thus, regulated expression of Nix is integral to islet development and maintenance.

The regulatory role we identified herein for Pdx1/Nix pairing in pancreatic islet β cell homeostasis is similar to that previously described for erythropoietin and Nix in erythrocyte (red blood cell) formation from erythroid precursor cells (21, 27). The process of erythroblast proliferation and progression down the differentiation pathway to erythroblasts is stimulated by erythropoietin in response to metabolic need (hypoxia or decreased red blood cell mass). Nix and antiapoptotic Bcl-xL are transcriptionally upregulated in response to erythropoietin during erythroid differentiation from

Figure 6
Genetic ablation of Nix prevents diabetes in Pdx1+/− mice. (A and B) Glucose levels (A) and respective areas under the glucose curves (B) following intraperitoneal injection of 1 g/kg dextrose. n = 7 (Pdx1+/−); 10 (Pdx1−/−); 4 (WT). *P < 0.01, **P < 0.001, Pdx1+/− versus Pdx1−/−Nix−/−. (C) Insulin levels measured fasting and 30 minutes after intraperitoneal dextrose. n = 7 (Pdx1−/−); 10 (Pdx1−/−Nix−/−); 4 (WT). (D) Glucose levels following administration of 0.75 U/kg insulin. n = 4 per group. All group data are mean ± SEM of 9–11 weeks of age.
erythroblasts. The balance between Bcl-xL and Nix expression regulates erythroblast survival, and therefore the quantity of cells that progress to fully developed erythrocytes released into the peripheral circulation. In this scenario, erythropoietin is acting on the erythroid lineage in the same manner as Pdx1 acts for β cells, as a molecule that transmits a specific form of physiological stress into a message to induce the proper target cell proliferation. Nix plays the same functional role in both pathways: balancing target cell proliferation with programmed elimination. Maintaining homeostasis in cells that are required to undergo context-specific proliferation is a general paradigm that can explain the very presence of programmed death genes in these tissues. Accordingly, the approach of interrupting programmed cell death offers therapeutic potential in any condition where normal homeostasis is unbalanced.

The mechanism by which Pdx1 deficiency regulates Nix expression likely involves 1 of the 2 pathways previously described for Nix transcriptional regulation, PKC and HIF1 (28, 29). Pdx1 targets PKCα and PKCε (28), and PKCα was previously shown to direct Nix expression in the heart (30). Pdx1 also regulates HIF1 (29), and HIF1 increases Nix expression in several tissues (31–33). We believe that Pdx1 regulation of Nix is complex and involves multiple factors. These issues are currently being addressed in additional studies.

In conclusion, we used unbiased discovery of Pdx1-regulated transcripts, gene manipulation in cultured insulinoma cells and mouse islets, and genetic complementation studies in intact mice to identify Nix as a critical molecular effector of diabetes in Pdx1 insufficiency. Nix induces 2 mitochondrial cell death pathways, apoptosis and programmed necrosis, in Pdx1-deficient β cells. Not only do these results explain diverse mechanisms of β cell loss as a result of Pdx1 deficiency, but they suggest a single molecular target for preventing diabetes caused by abnormal β cell death.

**Methods**

Microarrays. mRNA from 3 independent wells of MIN6 cells infected with Pdx1 shRNA was compared with control infected cells. MIN6 cell cDNA was prepared followed by hybridization to microarrays (Illumina MouseRef6 BeadChips, which cover approximately 48,000 transcripts derived from the MEEBO set, and RIKEN FANTOM 2).

Hierarchical clustering. Normalized expression data for mRNAs were subjected to unsupervised hierarchical clustering to provide a visual assessment of the mRNA profiles. Agglomerative hierarchical clustering was performed using Partek software (Partek Inc.). Hierarchical clustering was used to group similar objects into clusters; the algorithm represents the similarity between clusters. The methodology for this technique was described previously (34). In our experiments, Pearson distance was used to measure dissimilarity (the distance between 2 rows or columns), and average linkage (the average distance between all pairs of objects in 2 different clusters) was used as the measure of distance between 2 clusters.

Gene ontology. The official AmiGO tool (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) was used to search the gene ontology database (http://www.geneontology.org/), which consists of a controlled vocabulary of terms covering biological concepts and a large number of genes.

**PL/DAPI cell death assay.** For the last hour of incubation, 10 μg/ml PI and 20 μg/ml DAPI were added directly to the media. After this incubation, the MIN6 cells were washed 3 times with PBS and fixed with 3.7% formaldehyde for 15 minutes at 4°C. Each condition reported represents greater than 600 cells counted by randomized field selection. The percentage of cell death was calculated as the number of PI-stained nuclei per the total number of nuclei stained by DAPI.

**MIN6 cell and islet culture and Pdx1 suppression.** Methods for culture of MIN6 cells (35) and islets and the recombinant lentivirus system used to deliver shRNAs to MIN6 cells were previously reported (15, 16).

**Immunoblot analysis.** Blots were probed with antibodies against Pdx1 (catalog no. sc-14664; Santa Cruz Biotechnology Inc.), cleaved caspase-3 (catalog no. 9661; Cell Signaling Technologies), actin (catalog no. A-2066; Sigma-Aldrich), Nix (catalog no. ab8399; Abcam Inc.), LC3 (catalog no. NB100-2331; Novus Biologicals Inc.), Flag (catalog no. F-3165; Sigma-Aldrich), cytochrome c (catalog no. AP1029; Calbiochem), and COX IV (catalog no. ab14744; Abcam Inc.).

**Mitochondrial isolation.** MIN6 cells were homogenized and subjected to centrifugation at 3,000 g for 10 minutes to remove nuclei and myofibrils. The supernatant was then subjected to centrifugation at 10,000 g for 10 minutes. The resultant pellet and supernatant were labeled as 10p (mitochondrial enriched) fraction and 10s (cytosol) fractions.

**Quantification of mRNA level.** RNA isolation, first strand cDNA synthesis, and TaqMan gene expression assays were performed as previously described (15, 16). Applied Biosystems TaqMan assay numbers were as follows: Hmbs, Mm00600620_g1; Pdx1, Mm00435565_m1; Nix, Mm00786306_s1.

**Measurement of ΔΨm.** Rhodamine 123 was used as an indicator of ΔΨm. This fluorescent lipophilic cationic dye is in the mitochondrial membrane because of its charge and solubility in both the inner mitochondrial membrane and matrix space (36). In the present study, MIN6 cells or dispersed islets were loaded by incubation with 10 μg/ml rhodamine 123 for 20 minutes at 37°C. Rhodamine 123 fluorescence was excited at 490 nm and was measured at 530 nm. Mean fluorescence and cell counts were assessed by FACS analysis.

**Lentivirus-mediated shRNA expression and Ad infection studies.** shRNA targeting mouse Nix was obtained from Sigma-Aldrich. Recombinant lentiviral particles were prepared as previously described (15, 16). Recombinant Ads were created by cloning Flag-tagged Nix into pAdEasy-1 vector (Stratagene) using pShuttleCMV and by recombination in BJ5183-AD-1 cells as previously described (22). MIN6 cells and isolated mouse islets were infected with Ads at a titer of 10 PFU/cell. Efficiency of Ad infection to the islets was determined on isolated islets from WT mice that were incubated with Ad-containing media for 2 days. Efficiency of Ad infection was examined on cryosections (10 μm) of fixed islets with an anti-Flag antibody (catalog no. F-3165; Sigma-Aldrich).

**In vivo characterization of mice.** The Pdx1−/− mice have been previously described (5) and were provided by H. Edlund (University of Umea, Umea, Sweden). Nix−/− mice have been described previously (21). Male mice were fed a high-fat diet containing 42% fat (Harlan Laboratories Inc.) from 3 weeks of age and provided with water ad libitum. Intraportal glucose tolerance tests were performed after a 4-hour fast (1 g/kg dextrose). Insulin levels were measured at fasting and 30 minutes after glucose challenge. Insulin tolerance tests were performed after a 4-hour fast by administering human recombinant insulin (0.75 U/kg). We quantified β cell area from anti-insulin–stained pancreas sections counterstained with hematoxylin using the intensity thresholding function of the integrated morphometry package in Meta Morph. TUNEL labeling used the DeadEnd Fluorometric TUNEL System (Promega Corp.). All experiments in this study using animal protocols were approved by the Washington University Animal Studies Committee.

**Confocal imaging studies of pancreatic islets.** Formalin-fixed pancreas sections underwent antigen retrieval in boiling citrate buffer (pH 6.0) for 10 minutes before labeling with antibodies against insulin (catalog no. 4010-01L; Millipore) and glucagon (catalog no. G2654; Sigma-Aldrich). Images were obtained on a Nikon Personal Confocal microscope.

**Statistics.** Partek software was used to compute significance of mRNA expression changes in microarray using 1-way ANOVA at P < 0.01. Multiple experimental groups were compared using 1-way ANOVA and Tukey’s post hoc test. The 2-tailed unpaired Student’s t test was used to assess the statistical significance of differences in mean outcomes.
significance of differences between 2 sets of data. Differences were considered significant when \( P < 0.05 \). Results are presented as mean ± SEM.

**Acknowledgments**

This work was supported by NIH grants R01 HL059888 (to G.W. Dorn) and R01 DK031842 (to K.S. Polonsky), the Radioimmunossay and Morphology Cores of the Diabetes Research and Training Center of Washington University (P60 DK-20579), the Washington University Digestive Diseases Research Center Core (P30 DK052574), the Clinical and Translational Science Award to Washington University (UL1 RR024992), and the Blum Kower Foundation.

Received for publication June 14, 2010, and accepted in revised form September 1, 2010.

Address correspondence to: Kenneth S. Polonsky, Department of Medicine, Washington University School of Medicine, Campus Box 8066, 660 S. Euclid Avenue, St. Louis, Missouri 63110, USA. Phone: 314.362.8061; Fax: 314.362.8015; E-mail: Polonsky@dom.wustl.edu. Or to: Gerald W. Dorn II, Washington University Center for Pharmacogenomics, Campus Box 8220, 660 S. Euclid Ave., St. Louis, Missouri 63110, USA. Phone: 314.362.4892; Fax: 314.362.8844; E-mail: gddorn@dom.wustl.edu.