Pancrotic β-Cell Death due to Pdx-1 Deficiency Requires Multi-BH Domain Protein Bax but Not Bak*

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Diabetes develops in Pdx1-haploinsufficient mice due to an increase in β-cell death leading to reduced β-cell mass and decreased insulin secretion. Knockdown of Pdx1 gene expression in mouse MIN6 insulinoma cells induced apoptotic cell death with an increase in Bax activation and knockdown of Bax reduced apoptotic β-cell death. In Pdx1 haploinsufficient mice, Bax ablation in β-cells increased β-cell mass, decreased the number of TUNEL positive cells and improved glucose tolerance after glucose challenge. These changes were not observed with Bak ablation in Pdx1-haploinsufficient mice. These results suggest that Bax mediates β-cell apoptosis in Pdx1-deficient diabetes.

Experimental Procedures

MIN6 Cell Culture, Quantification of mRNA Levels, and Lentivirus-mediated shRNA Expression—MIN6 cell culture, RNA isolation, and first-strand cDNA synthesis, and preparation of pLKO.1-Pdx1 shRNA lentivirus were performed as previously described (6). TaqMan assay numbers were: Hmbs, Mm00466026; Pdx1, Mm00435565; Bax, Mm00432051; and Bak, Mm00432045. The pLKO-Bax shRNA (RMM4533), Bak shRNA (RMM4534) were purchased from Thermo Scientific.

Immunofluorescence Analysis of Cytochrome c—

Quantitation of Cell Death—Cell death was quantified by propidium iodide (PI) staining (7) followed by flow cytometric analyses using a FACSCaliber (BD Bioscience) and FlowJo software.

Flow Cytometric Analysis of Mitochondrial Membrane Potential—Mitochondrial membrane potential was assessed by TMRE (tetramethylrhodamine, ethyl ester) staining followed by flow cytometric analysis (8). TMRE enters cells and reversibly accumulates in the highly negatively charged mitochondrial matrix according to the Nernst equation, allowing the potential to be measured.

Immunofluorescence Analysis of Cytochrome c—After 4 days of treatment with lentiviral control or Pdx1 shRNA, MIN6 cells were fixed for 15 min in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100/PBS for 5 min and then incubated for 1 h in a 5% BSA/PBS blocking solution. Then cells were incubated overnight at 4 °C with a mouse monoclonal anti-cytochrome c IgG (Pharmlingen) followed by exposure to a goat anti-mouse Alexa488-conjugated secondary antibody (Invitrogen). Images

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3 The abbreviations used are: Pdx1, pancreas and duodenal homeobox-1; TMRE, tetramethylrhodamine, ethyl ester; DKO, double knockdown; KD, knockdown; PI, propidium iodide.
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FIGURE 1. Pdx1 knockdown induces Bax activation in MIN6 cells and islets. A, Bax and Bak mRNA levels in control and Pdx1 KD cells. 3 days after Pdx1 KD in MIN6 cells, Bax and Bak mRNA levels were not different between control and Pdx1 KD cells (n = 3). B, Western blot of Pdx1 KD cells. 3 days after Pdx1 KD in MIN6 cells, immunoblot analysis was performed to determine Pdx1, Bak, and Pdx1 in Pdx1 KD MIN6 cells. C, Bax nuclear/cytosolic translocation in Pdx1 KD MIN6 cells. 3 days after Pdx1 shRNA lentivirus infection, cytosolic and nuclear proteins were analyzed by Western blot. D, Immunoprecipitate of Bax. 3 days after Pdx1 KD in MIN6 cells, cells were lysed in 1% CHAPS and then immunoprecipitated with the 6A7 anti-BAX antibody. Immunoprecipitates were analyzed by anti-BAX (N20) immunoblots. E, effects of Pdx1 on ΔΨm. MIN6 cells were treated with Pdx1 shRNA lentivirus for 0, 4 days. Cells were stained with TMRE dye to measure ΔΨm. *** p < 0.001. F, Bax and Bak mRNA levels in islets. Bax and Bak mRNA levels were measured by real time reverse transcription-PCR in islets from 5–6-week-old male Pdx1+/− mice on normal chow (n = 3–6). *, p < 0.05 compared with wild type (WT) mice.

were obtained on an Evos microscope (Advanced Microscopy Group).

Tamoxifen Administration—In this study, over a 5-day period, 4-week-old male mice were injected intraperitoneally with 3 doses of 2.5 mg of tamoxifen (Sigma, T5648) freshly dissolved in corn oil at 10 mg/ml (9).

In vivo Characterization of Mice—The Pdx1+/− mice have been previously described (4). Bax+/−/Bak−/− mice were provided by Dr. Emily Cheng (Memorial Sloan-Kettering Cancer Center) and MIP-Cre/ERT mice (9) by Louis Philipson (University of Chicago). Male mice were fed a high-fat diet containing 42% fat (Harlan Laboratories Inc.) from 5 weeks of age and provided with water ad libitum as previously described (11). The relative β-cell area was measured from anti-insulin-stained pancreas sections counterstained with hematoxylin using ImageJ software. TUNEL and Ki-67 staining were performed as previously described (11). More than 20000 β-cells and 300 islets were counted after TUNEL and Ki-67 staining and at least three mice were counted per group. All animal experiments in this study were performed under protocols approved by the University of Chicago Animal Studies Committee.

Imaging Studies of Pancreatic Islets—Formalin-fixed pancreas sections underwent antigen retrieval in boiling citrate buffer (pH 6.0) for 10 min before labeling with antibodies against insulin (A0564; DAKO), glucagon (G2654; Sigma-Aldrich), and DAPI (P-36931; Invitrogen).

Statistical Analysis—The 2-tailed unpaired Student’s t test was used to assess the statistical significance of differences between 2 sets of data. Differences were considered significant when p < 0.05. In all experiments, the number of asterisks is used to designate the following levels of statistical significance: ***, p < 0.001; **, p < 0.01; *, p < 0.05 compared with control group or wild type (WT) group. ####, p < 0.001; ###, p < 0.01; ##, p < 0.05 compared with Pdx1 KD or Pdx1+/− group. Results are presented as mean ± S.E.

Results

Pdx1 Suppression Activates Bax in MIN6 Cells—Pdx1 KD MIN6 cells did not demonstrate a significant increase in Bax and Bak mRNA (Fig. 1A) or protein levels when compared with control cells (Fig. 1B). Since Bax is located in the cytosol until activated by a diversity of stress stimuli to induce cell death through translocation to mitochondria, cellular cytosol, and mitochondrial fractions were extracted to determine if Bax subcellular fractions were altered by Pdx1 suppression. Pdx1 KD induced an accumulation of Bax protein in the mitochondrial fraction (Fig. 1C) but no change in Bak protein levels. Protein levels of cytochrome c oxidase subunit IV (COX IV), a mitochondrial marker, were similar in Pdx1 KD and control cells (Fig. 1C).

Bax conformation was also examined using the monoclonal antibody 6A7, which only recognizes the N-terminal epitope of Bax (12). The results showed that the amount of Bax precipitated by 6A7 anti-Bax antibody was increased in Pdx1 KD cells (Fig. 1D). To determine the effect of Bax mitochondrial translocation, mitochondrial membrane potential (ΔΨm) was measured by quantifying the average mitochondrial fluorescence
intensity of TMRE. TMRE uptake into mitochondria was decreased from 92.4 ± 4.5% in control cells to 60.7 ± 1.0% in Pdx1 KD cells (p < 0.001) indicating Pdx1 KD significantly decreased mitochondrial membrane potential (Fig. 1E). Bax and Bak mRNA levels were also examined in pancreatic islets isolated from 5–6 weeks old Pdx1+/− mice. mRNA levels of Bax were increased in islets from Pdx1+/− mice (p < 0.05) (Fig. 1F). Bax mRNA levels did not increase.

**Bax Suppression Reduced β-Cell Apoptosis Induced by Pdx1 KD in MIN6 Cells**—To define the functional effects of changes in Bax expression on pancreatic β-cell death after Pdx1 suppression, shRNA was used to knock down Bax in MIN6 cells. Lentiviral Bax shRNA suppressed Bax expression by more than 60% and did not affect Bak expression (Fig. 2A).

In Pdx1 KD MIN6 cells, Bax suppression inhibited the increase in cytochrome c release from mitochondria, the key event in activating apoptosis (Fig. 2B). Western blot also showed that Pdx1 induced an increase of cytochrome c release from mitochondria into the cytosol (Fig. 2C). Bax KD inhibited the cytochrome c release induced by Pdx1 KD (Fig. 2C). Pdx1 KD increased cytochrome c by 93% compared with control group (p < 0.01) (Fig. 2C). However, Bax KD significantly inhibited cytochrome c release by 42% in Pdx1 KD cells (Fig. 2C). Pdx1 KD increased cleaved caspase 3 protein by 150% compared with control cells (p < 0.001, Fig. 2D). In Bax/Pdx1 double knockdown (DKD) cells the cleaved caspase 3 protein levels were significantly lower than in Pdx1 KD cells (80% versus 250%, p < 0.001) (Fig. 2D). Furthermore, following Pdx1 KD, 50.2 ± 2.7% of the MIN6 cells took up the PI stain. In the Pdx1/Bax DKD group, only 22.7 ± 1.1% (p < 0.001 compared with Pdx1 alone) took up the PI stain indicative of a 44% increase in cell viability (Fig. 2E). Bax knockdown had no effect on β-cell death induced by Pdx1 suppression (Fig. 2E).

**Effect of Bax Ablation in Adult Pdx1+/− Mice**—To determine the effects of Bax deficiency on β-cell death in vivo, we used mice in which Bax is conditionally deleted in islets using MIP-
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**FIGURE 3.** Bax ablation protects β-cells in adult Pdx1<sup>+/−</sup> Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. A and B, Western blot of Bax in islets from 4-month old mice with normal chow (A) and high fat diet (B). C, islet morphology in adult mouse after 12 weeks on a high fat diet. Anti-insulin and anti-glucagon antibodies were used to stain β-cells (red) and α cells (green) respectively. Scale bar, 20 μm. D, histological analysis of pancreatic islets and quantitation of group data for β-cell mass (n = 3–5 per group). **p < 0.01 compared with the Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. #, p < 0.05 compared with Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. E, TUNEL labeling of adult pancreatic β-cells. Quantitative TUNEL data are shown. *p < 0.01 compared with the Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. #, p < 0.05 compared with Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. F, Ki-67 staining of β-cells. ***, p < 0.001 compared with the Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. ###, p < 0.001 compared with Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. All group data are mean ± S.E. of n = 3.

Cre/ERT (here refers to Cre) on a Bak<sup>−/−</sup> background. The expression of Bax protein in islets from mice fed either a normal chow or a high fat diet was decreased to the extent that it became almost undetectable after tamoxifen treatment (Fig. 3, A and B). Bax protein levels in islets from Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice after 4 months on a high fat diet were 5% of the levels in islets from Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice. β-cell mass was reduced by 65% (p < 0.01) in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice and the islets contained reduced numbers of β-cells (Fig. 3C). These islets also demonstrated abnormal architecture in that α cells were distributed throughout the islets compared with islets from Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice that had a central core of β-cells ringed by a mantle of α cells (Fig. 3C). The Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice showed an increase in β-cell mass compared with Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice by 73% (p < 0.01) (Fig. 3D). The proportion of β-cells that demonstrated TUNEL labeling decreased significantly from 0.08 ± 0.01% in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice to 0.035 ± 0.004% in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice (p < 0.05) (Fig. 3E). To determine whether there was also an effect of Bax ablation on β-cell proliferation, the islets were stained for the proliferative marker Ki-67. Proliferation of β-cells was decreased in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> islets and was significantly increased following Bax ablation in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> islets (p < 0.001) (Fig. 3F).

**Pdx1<sup>+/−</sup> Mice with Bax Gene Ablation in Islets Have Improved Glucose Tolerance**—Breeding Pdx1<sup>+/−</sup> background did not result in an improvement in glucose tolerance (Fig. 4A). Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice fed high-fat diet developed increased fasting blood glucose and impaired glucose tolerance (Fig. 4B). However, Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice exhibited significantly lower fasting blood glucose and improved glucose tolerance compared with Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice (Fig. 4B). Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice showed significantly improved but not normal glucose tolerance compared with Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice (Fig. 4C). The area under the blood glucose curve (AUC) decreased 27% in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice compared with Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice (p < 0.001). The AUC in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice is higher compared with that in Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice (p < 0.05) (Fig. 4C). The reduction in blood glucose after insulin administration was similar in the four groups of mice (Fig. 4D) indicating that there were no differences in insulin sensitivity. Insulin levels were decreased in the Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice under basal conditions and following glucose challenge compared with Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice (Fig. 4E). In comparison, insulin concentrations were increased in Pdx1<sup>+/−</sup>Bax<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>−</sup> mice (Fig. 4E).
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Discussion

We have previously demonstrated that reduced β-cell mass is an essential component of the diabetic phenotype in the Pdx1-deficient mouse (2), and Bim and Puma mediate β-cell death induced by Pdx1-deficiency (6). In the intrinsic apoptosis pathway, both Bim and Puma can induce Bax or Bak activation and cause cell apoptosis (12). In the present studies, we demonstrated that Pdx1 KD induces N-terminal conformational change in Bax and translocation of Bax to the mitochondria leading to its activation, alteration in mitochondrial membrane potential and cytochrome c release. Knockdown of Bax significantly reduced β-cell apoptosis and increased β-cell survival in Pdx1 deficient cells. In contrast, deficiency of Bak had no impact on these processes. Results obtained in the Pdx1+/− mouse were consistent with the in vitro results. Bak−/− alone had no effect on glucose tolerance in the Pdx1+/− mouse. The reduction in the expression of Bax in islets preserved β-cell mass as a result of a reduction in β-cells apoptosis and an increase in proliferation of β-cells in Pdx1+/−/Bax+/−Bak−/−Cre+ mice fed a high-fat diet compared with Pdx1+/−/Bax+/−Bak−/−Cre− mice. These data suggest that Bax rather than Bak is the molecule downstream of Bim and Puma that plays a critical role in mediating β-cell apoptosis induced by Pdx1 deficiency. These results are consistent with other studies. One study showed that Bim, Puma, and Bak are required for β-cell apoptosis triggered by high glucose. Loss of the BH3-only proteins Bim or Puma, or loss of Bax markedly protected islets from glucose toxicity (13). Furthermore, in human type 2 diabetic subjects, expression levels of Bim, Puma, and Bak are increased when compared with non-diabetic donors (14). These results indicate that Bcl-2 family members involved in regulating the apoptotic pathway are implicated in β-cell death induced by Pdx1 deficiency, and also suggest possible targets to reduce β-cell apoptosis in diabetic syndromes associated with reduced Pdx1 such as MODY4.

Our approach is based on the following line of reasoning. Bak deficiency in the context of Cre expression in Bax−/−Bak+/−Cre+ mice should have no impact on these beta cell parameters. We base this conclusion on the observation that the response of Pdx1+/−/Bak−/− mice to glucose challenge is the same as Pdx1+/− alone mice, suggesting that Bak deficiency does not have a significant impact in Pdx1-induced beta cell death. Additionally, our experiments show that there are no statistical differences in beta cell mass, TUNEL+ number and Ki67+ number between Bax+/−Bak−/−Cre− and Bax−/−Bak−/−Cre+ mice (Fig. 3, D−F), thus indicating that even Bak deficiency in beta cells has no impact in these parameters when the mice do not have beta cell death induced by Pdx1 deficiency. Thus we would anticipate that beta cell mass, death, and proliferation would be no different between Bax+/−Bak−/−Cre+ and Bax−/−Bak−/−Cre+ mice.

In our experiments, to rule out the compensation of Bak after Bax deletion in the β-cells, Bax+/−Bak−/−Cre− mice were chosen as control mice because Bax and Bak can compensate for...
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each other in cells. For example, Bax and Bak can compensate for each other in MEFs, as MEFs that express either Bax or Bak are sensitive to apoptosis induced by expression of BH3-only proteins (15–16). However, Bax Bak double knock-out MEFs are highly resistant to apoptotic cell death stimuli (17). Bak can also compensate for Bax in p53-null cells to release cytochrome c for the initiation of apoptosis (18). But our data indicate that Bak and Bax do not compensate for each other in beta cells.

Interestingly, Bax deficiency in Pdx1<sup>−/−</sup>Bak<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>+</sup> mice leads to significantly improved but not normal glucose tolerance and increased β-cell mass compared with Bak<sup>F/F</sup>Bak<sup>−/−</sup>Cre<sup>+</sup> mice. One explanation is that although the expression of Bax in islets was reduced by 94%, the residual levels of Bax protein in islets might be enough to induce β-cell apoptosis. Another explanation is that Pdx1 deficiency could induce other forms of β-cell death other than apoptosis such as necrosis and autophagy that are not mediated by Bax expression (6, 11, 19).

In conclusion, we have shown that Bax plays a role in mediating β-cell apoptosis caused by Pdx1 deficiency. Genetic ablation of Bax rather than Bak protects β cells from apoptosis and preserves insulin secretion and β-cell mass in Pdx1<sup>−/−</sup> mice.

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