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# PANDER-induced cell-death genetic networks in islets reveal central role for caspase-3 and cyclin-dependent kinase inhibitor 1A (p21)

Brant R. Burkhardt [a,c,*], Scott R. Greene [a], Peter White [b], Ryan K. Wong [c], John E. Brestelli [b], Jichun Yang [c], Claudia E. Robert [c], Todd M. Brusko [d], Clive H. Wasserfall [d], Jianmei Wu [a], Mark A. Atkinson [d], Zhiyong Gao [a], Klaus H. Kaestner [b], Bryan A. Wolf [a,c]

[a] *Department of Pathology and Laboratory Medicine, 803D Abramson Research Center 3516 Civic Center Blvd. Children's Hospital of Philadelphia, Philadelphia, PA 19104-4318, United States*
[b] *Departments of Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, United States*
[c] *Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, United States*
[d] *Department of Pathology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL 32610, United States*

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**Abstract**

PANcreatic DERived factor is an islet-specific cytokine that promotes apoptosis in primary islets and islet cell lines. To elucidate the genetic mechanisms of PANDER-induced cell death we performed expression profiling using the mouse PancChip version 5.0 in conjunction with Ingenuity Pathway Analysis. Murine islets were treated with PANDER and differentially expressed genes were identified at 48 and 72 h post-treatment. 64 genes were differentially expressed in response to PANDER treatment. 22 genes are associated with cell death. In addition, the genes with the highest fold change were linked with cell death or apoptosis. The most significantly affected gene at 48 h was the downregulated cyclin-dependent kinase inhibitor 1A (CDKN1A or p21). Approximately half of the genes impacted at 72 h were linked to cell death. Cell death differentially expressed genes were confirmed by quantitative RT-PCR. Further analysis identified cell death genetic networks at both time points with 21 of the 22 cell death genes related in various biological pathways. Caspase-3 (CASP3) was biologically linked to CDKN1A in several genetic networks and these two genes were further examined. Elevated cleaved CASP3 levels in PANDER-treated β-TC3 insulinoma cells were found to abrogate CDKN1A expression. Levels of CDKN1A were not affected in the absence of cleaved CASP3. PANDER-induced downregulation of CDKN1A expression coupled with induced CASP3-activation may serve a central role in islet cell death and offers further insight into the mechanisms of cytokine-induced β-cell apoptosis.


*Keywords:* PANDER; Caspase-3; p2l; Islet; Apoptosis; PancChip

## 1. Introduction

PANcreatic DERived factor (PANDER, FAM3B in humans (acc. No. AF494379) and Mus musculus cytokine-like protein 2-21 in mice (acc. No. AF494380)) is a recently discovered 235-amino acid protein that is predominantly expressed and secreted by the islets of Langerhans of the pancreas (Zhu et al., 2002). PANDER was discovered using a computational algorithm known as ostensible recognition of folds (ORF) that uses predicted secondary structures to search for novel cytokines (Aurora and Rose, 1998). This approach resulted in the discovery of a novel cytokine family consisting of 4 genes,

*Abbreviations:* PANDER, Pancreatic Derived Factor; IL-1β, Interleukin-1β; TNF-α, Tumor necrosis factor-α; IFN-γ, Interferon-γ; IPA, Ingenuity pathway analysis; IPKB, Ingenuity pathways knowledge database; RT-PCR, Reverse transcription-polymerase chain reaction; CDK's, Cyclin-dependent kinases.

* Corresponding author. Department of Pathology and Laboratory Medicine, 803D Abramson Research Center 3516 Civic Center Blvd. Children's Hospital of Philadelphia, Philadelphia, PA 19104-4318, United States. Tel.: +1 215 590 7212; fax: +1 215 590 7377.
*E-mail address:* burkhardtb@email.chop.edu (B.R. Burkhardt).

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one of which (FAM3B) was later named PANDER (Cao et al., 2003).

PANDER is capable of inducing apoptosis in both rodent and human islets (Cao et al., 2003). This biological effect on islets resembles that of other cytokines such as interleukin-1β, tumor necrosis factor-α, and interferon-γ that induce β-cell death by necrosis or apoptosis (Rabinovitch and Suarez-Pinzon, 1998; Lortz et al., 2000). Apoptosis is the primary mechanism of pancreatic islet β-cell death in autoimmune diabetes (Kurrer et al., 1997; O'Brien et al., 1997; Augstein et al., 1998). These Th1 cytokines activate various transcriptional factors such as NF-κB, STAT1, and Fas within islets that appear to ultimately lead to apoptosis (Eizirik and Mandrup-Poulsen, 2001). However, PANDER does not impact the levels of these typical apoptotic signals (Cao et al., 2005). This finding suggested that PANDER acts via other uncharacterized cell death related genes and biological pathways.

To elucidate the genetic mechanisms responsible for PANDER-induced islet cell death we have taken a comprehensive microarray approach, using the mouse PancChip version 5.0. This chip is enriched for genes expressed in the pancreas, containing over 13,000 cDNAs from EST clones expressed at various stages of pancreatic development and in diabetes-related pathways. In addition, our study has evaluated the genotypic impact of PANDER on primary murine islets rather than pancreatic β-cell lines. We also employed novel computational tools to identify PANDER-induced biological networks that regulate β-cell apoptosis. Here we present the first report to evaluate the genetic mechanisms of PANDER-induced islet cell death. The microarray and genetic pathway analysis has identified several cell death networks that provide a mechanism for PANDER-induced islet apoptosis and potential elucidation of novel pathways for cytokine-induced islet cell death.

## 2. Materials and methods

### 2.1. Preparation and PANDER treatment of murine islets

Pancreatic islets from C57Bl/6 mice (Charles River Laboratories) were isolated as described previously (McDaniel et al., 1983; Gao et al., 2000). Approximately 100 murine islets were placed into each dish and cultured for 24 h at 37 °C, 5% $CO_2$ to allow for islet recovery. Each day of treatment, 4 nM of exogenous murine PANDER was added per 100 islets in each 35-mm dish to the experimental samples. Untreated islets were supplemented with media only. Islets were exposed to PANDER for 48 or 72 h and each condition was performed in quadruplicate.

### 2.2. Preparation and amplification of islet RNA

After 48 to 72 h of treatment, islets were transferred to a 1.5 ml RNAse free tube (Eppendorf) and collected by centrifugation (400 *g*). Islets were then lysed by addition of 1 ml 4 °C TRIzol® Reagent (Invitrogen) containing 20 μg of glycogen (Roche). Tubes were then inverted and incubated at room temperature for 5 min. RNA was then extracted using standard procedures as previously described. All RNA samples were analyzed by the Agilent Bioanalyzer 2100 Lab-On-A-Chip Nano 6000 chip to determine the integrity and concentration. Only samples with a ratio of the 28S to 18S RNA ≥ 2.0 were used in the next step. In order to have sufficient sample for the microarray analysis, the total RNA was amplified using the MessageAmp™ aRNA kit (Ambion). The purity of the amplified RNA (aRNA) was also evaluated using the Bioanalyzer. All aRNA demonstrated successful and robust amplification with the average peak around 1000–1500 nt. Quantitative RT-PCR was performed to assess islet purity among samples. Samples were matched according to the islet purity based on the insulin/amylase ratio as determined by RT-PCR (Lantz et al., 2004).

### 2.3. Microarray expression profiling

2 μg of amplified RNA was labeled using amino-allyl dUTP and random hexamers to prime reverse transcription. The fluorescent label was coupled to the cDNA and subsequently hybridized to the mouse PancChip version 5.0 (Scearce et al., 2002; Kaestner et al., 2003). Median intensities of each spot were measured by an Agilent scanner using the GenePix software. Data were normalized using print tip lowess normalization with the Statistical Microarray Analysis package in "R" (Dudoit and Fridlyand, 2002). Blank spots were used to determine the background intensity (90th percentile plus two standard deviations). All spots whose intensity fell below this cutoff were assigned the cutoff value of 100. Spots that had been flagged by the GenePix software as absent were also set to this threshold.

### 2.4. Gene analysis

Two methods of analysis were employed to generate ranked lists of PANDER-induced differentially regulated genes at the 48 and 72 h time points. In total 4 biological replicates were used for each condition at each time point for the analysis. Logarithm of the odds (LOD) or B-statistic in conjunction with a Student *t*-test was performed to generate a ranked list of differentially expressed genes that had a $p < 0.05$ with a fold difference of at least 1.4. Cell death genes were identified and networks were compiled through the use of Ingenuity Pathway Analysis (www.Ingenuity.com), a web-delivered application that allows the visualization and analysis of biologically relevant themes and networks.

Expression data sets containing gene identifiers (Entrez Gene ID) and their corresponding expression values as fold changes were uploaded as a tab-delimited text file. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). The genes identified as differentially expressed as described above were included in the analysis. These genes, named Focus Genes, were utilized to search for biological networks. The application program searches the IPKB for interactions between Focus Genes and all other genes contained in the IPKB, and generates a series of networks. In addition, the program calculates a statistical score for each network according to the fit of the network to the set of