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## The Human Pancreatic Islet Methylome and Its Role in Type 2 Diabetes

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## Increased DNA Methylation and Decreased Expression of *PDX-1* in Pancreatic Islets from Patients with Type 2 Diabetes

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Mutations in pancreatic duodenal homeobox 1 (*PDX-1*) can cause a monogenic form of diabetes (maturity onset diabetes of the young 4) in humans, and silencing *Pdx-1* in pancreatic  $\beta$ -cells of mice causes diabetes. However, it is not established whether epigenetic alterations of *PDX-1* influence type 2 diabetes (T2D) in humans. Here we analyzed mRNA expression and DNA methylation of *PDX-1* in human pancreatic islets from 55 nondiabetic donors and nine patients with T2D. We further studied epigenetic regulation of *PDX-1* in clonal  $\beta$ -cells. *PDX-1* expression was decreased in pancreatic islets from patients with T2D compared with nondiabetic donors ( $P = 0.0002$ ) and correlated positively with insulin expression ( $\rho = 0.59$ ,  $P = 0.000001$ ) and glucose-stimulated insulin secretion ( $\rho = 0.41$ ,  $P = 0.005$ ) in the human islets. Ten CpG sites in the distal *PDX-1* promoter and enhancer regions exhibited significantly increased DNA methylation in islets from patients with T2D compared with nondiabetic donors. DNA methylation of *PDX-1* correlated negatively with its gene expression in the human islets ( $\rho = -0.64$ ,  $P = 0.000029$ ). Moreover, methylation of the human *PDX-1* promoter and enhancer regions suppressed reporter gene expression in clonal  $\beta$ -cells ( $P = 0.04$ ). Our data further indicate that hyperglycemia decreases gene expression and increases DNA methylation of *PDX-1* because glycosylated hemoglobin (HbA1c) correlates negatively with mRNA expression ( $\rho = -0.50$ ,  $P = 0.0004$ ) and positively with DNA methylation ( $\rho = 0.54$ ,  $P = 0.00024$ ) of *PDX-1* in the human islets. Furthermore, while *Pdx-1* expression decreased, *Pdx-1* methylation and *Dnmt1* expression increased in clonal  $\beta$ -cells exposed to high glucose. Overall, epigenetic modifications of *PDX-1* may play a role in the development of T2D, given that pancreatic islets from patients with T2D and  $\beta$ -cells exposed to hyperglycemia exhibited increased DNA methylation and decreased expression of *PDX-1*. The expression levels of *PDX-1* were further associated with insulin secretion in the human islets. (***Molecular Endocrinology* 26: 1203–1212, 2012**)

**P**ancreatic duodenal homeobox 1 (*PDX-1*) is a homeodomain-containing transcription factor that plays a key role in pancreas development and function (1–3). During embryonic development, *PDX-1* is expressed in endocrine, exocrine and ductal progenitors. In the mature pancreas, the gene is mainly expressed in islet  $\beta$ -cells, in which it plays an important role in glucose-dependent

regulation of insulin gene expression. Mutations in *PDX-1* can cause a monogenic form of diabetes (maturity onset diabetes of the young 4) in humans (4). Furthermore, silencing the gene in  $\beta$ -cells of mice causes diabetes (5). A study in rodents demonstrated that intrauterine growth retardation can cause epigenetic changes of the *Pdx-1* gene, resulting in reduced pancreatic *Pdx-1* expres-

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Abbreviations: BMI, Body mass index; FACS, fluorescence-activated cell sorting; FDR, false discovery rate; HbA1c, glycosylated hemoglobin; *PDX-1*, pancreatic duodenal homeobox 1; T2D, type 2 diabetes.

sion and diabetes in postnatal life (6). These epigenetic changes include both increased DNA methylation and histone modifications. Although this study demonstrates that epigenetic alterations of the *Pdx-1* gene are associated with reduced *Pdx-1* expression,  $\beta$ -cell dysfunction and diabetes in rodents, it is not established whether epigenetic alterations of the *PDX-1* gene participate in the development of type 2 diabetes (T2D) in humans. The aim of the present study was therefore to analyze DNA methylation of the *PDX-1* gene in pancreatic islets from 55 nondiabetic donors and nine patients with T2D. DNA methylation of the *PDX-1* gene was further related to *PDX-1* gene expression and glycosylated hemoglobin (HbA1c) levels. Luciferase assays were used to examine whether DNA methylation of the human *PDX-1* promoter and enhancer regions influences its transcriptional activity. Finally, we tested whether high levels of glucose affect the degree of *Pdx-1* expression and DNA methylation as well as the expression of three DNA methyltransferases in clonal rat  $\beta$ -cells.

## Materials and Methods

### Pancreatic islets

Pancreatic islets from 55 nondiabetic and nine T2D deceased donors were obtained from the Human Tissue Laboratory at Lund University Diabetes Centre and the Nordic Network for Clinical Islet Transplantation (Table 1). Islets were prepared by collagenase digestion and density gradient purification. After isolation, islets were cultured free-floating in CMRL 1066 culture medium (ICN Biomedicals, Costa Mesa, CA) supplemented with 10 mmol/liter HEPES, 2 mmol/liter l-glutamine, 50  $\mu$ g/ml gentamicin, 0.25  $\mu$ g/ml Fungizone (GIBCO BRL, Gaithersburg, MD), 20  $\mu$ g/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/liter nicotinamide at 37 C (5% CO<sub>2</sub>) before RNA and DNA preparation. Gene expression of endocrine (somatostatin and glucagon) and exocrine (pancreatic lipase, amylase  $\alpha$ 2A, and chymotrypsin 2) markers and dithizone staining were used to determine islet purity (7). Islet purity was similar for nondiabetic and T2D donors (72 vs. 68%,  $P = 0.29$ ). Glucose-stimulated insulin secretion from the human islets was measured *in vitro* in static incubations as previously described (8). The population ancestry of the human donors is not available. The donor before death or her/his relatives upon

admission to the intensive care unit had given their consent to donate organs and the local ethics committees approved the protocols.

### Gene expression analysis

Total RNA was extracted from human islets and rat clonal  $\beta$ -cells using All Prep DNA/RNA kit and cDNA was synthesized using QuantiTect reverse transcription kit (QIAGEN, Hilden, Germany). *PDX-1* mRNA levels were analyzed using TaqMan real-time PCR with an ABI Prism 7900 HT system and gene-specific probes and primer pairs (Assays-on-Demand, Hs00426216\_A1, Applied Biosystems Inc., Foster City, CA). The *PDX-1* transcript level was normalized to the mRNA level of cyclophilin A (4326316E; Applied Biosystems) and quantified using the  $\Delta\Delta$ Ct method. Insulin mRNA levels were analyzed in the human islets as previously described (9). The mRNA expression of *Pdx-1*, *Dnmt1*, *Dnmt3a*, and *Dnmt3b* was analyzed in rat clonal  $\beta$ -cells using the following Assays-on-Demand from Applied Biosystems: *Pdx-1*, Rn00755591\_m1; *Dnmt1*, Rn00709664\_m1; *Dnmt3a*, Rn01469994\_g1; and *Dnmt3b*, Rn01536414\_g1.

We further used Normfinder (10) to test whether the expression of two housekeeping genes, cyclophilin A and *HPRT* (Assay-on-Demand; Applied Biosystems), is stable in human islets and clonal rat  $\beta$ -cells exposed to hyperglycemia.

### DNA methylation analysis

Sequenom's MassARRAY EpiTYPER protocol was applied to measure DNA methylation (Sequenom, San Diego, CA). Two EpiTYPER assays were designed (EpiDesigner; Sequenom), of which one assay covered 15 CpG sites of the human distal *PDX-1* promoter and the other assay covered 14 CpG sites of the human *PDX-1* enhancer region, respectively. These assays generated successful data for 11 and 12 CpG sites, respectively. A number of CpG sites did not generate any methylation data using the EpiTYPER, due to either low or high mass of the cleavage product. Also, one assay covering 10 CpG sites of the rat *Pdx-1* promoter was designed, and it generated successful data for six CpG sites. The primer sequence and the location of the human and the rat EpiTYPER assays are given in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>. Genomic DNA was extracted using the All Prep DNA/RNA kit (QIAGEN). Five hundred nanograms of genomic DNA were bisulfite treated with the EZ DNA methylation kit (Zymo Research, Orange, CA). PCR were performed with bisulfite-specific PCR primers containing a T7-promoter tag and a 10-mer-tag on the reverse and forward primer, respectively. *In vitro* transcription

**TABLE 1.** Characteristics of the human pancreatic donors

|                                                   | Nondiabetic donors | T2D donors      | P value |
|---------------------------------------------------|--------------------|-----------------|---------|
| n (male/female)                                   | 55 (29/26)         | 9 (5/4)         |         |
| Age (yr)                                          | 56.7 $\pm$ 9.8     | 57.0 $\pm$ 13.1 | 1       |
| BMI (kg/m <sup>2</sup> )                          | 25.9 $\pm$ 3.6     | 28.5 $\pm$ 4.7  | 0.13    |
| HbA1c                                             | 5.7 $\pm$ 0.8      | 7.3 $\pm$ 1.2   | 0.00010 |
| Basal insulin secretion (ng/islet-h)              | 0.37 $\pm$ 0.27    | 0.22 $\pm$ 0.17 | 0.22    |
| Glucose-stimulated insulin secretion (ng/islet-h) | 1.42 $\pm$ 0.95    | 1.05 $\pm$ 1.56 | 0.045   |

Data are expressed as mean  $\pm$  SD.

and ribonuclease (RNase) cleavage reaction were conducted using the MassCleave kit according to the manufacturer's recommendations. The cleavage reaction was dispensed onto a 384-element SpectroCHIP bioarray, and mass spectra were acquired using a MassARRAY mass spectrometer (Sequenome, San Diego, CA). The spectra were analyzed and the methylation ratios were obtained by the EpiTYPER software version 1.0.1 (Sequenom). Due to the cleavage pattern, the following 10 CpG sites were analyzed in five CpG units; –857, –852, –746, –741, –3504, –3502, –3420, –3416, –3408, and –3404.

Pyrosequencing (QIAGEN) was used to analyze DNA methylation of the human proximal *PDX-1* promoter because it was not possible to design an assay for this region using EpiTYPER (Sequenom). PCR and sequencing primers were designed using the PyroMark assay design software version 2.0 (QIAGEN) (Supplemental Table 1). This assay covered 12 CpG sites and successful data were generated for all CpG sites. The reverse primer was biotinylated at its 5' end. Bisulfite-converted DNA was amplified by PCR using the PyroMark PCR kit (QIAGEN). Biotinylated PCR products were immobilized onto streptavidin coated beads (GE Healthcare, Uppsala, Sweden). DNA strands were separated using denaturation buffer (QIAGEN). After washing and neutralizing using the vacuum prep station (Biotage, Uppsala, Sweden), the sequencing primer was annealed to the immobilized strand. Pyrosequencing was performed using the PSQ HS96A (Biotage) and PyroMark Gold CDT kit (QIAGEN) according to the manufacturer's instructions. Data were analyzed using the Pyro Q-CpG software program (Biotage).

The DNA methylation assays were selected and designed to cover gene regions of *PDX-1* that previously have been shown to regulate gene expression due to the binding of transcription factors (11).

### Luciferase assay

Three different DNA fragments containing 908 bp of the human *PDX-1* promoter, 606 bp of the human *PDX-1* enhancer region, or 3800 bp of a sequence containing both the *PDX-1* promoter and the enhancer regions (sequences are given in Supplemental Fig. 1) were inserted into a CpG-free firefly luciferase reporter vector (pCpGL-basic) kindly provided by Dr. Maja Klug and Dr. Michael Rehli (Department of Hematology and Oncology, University Hospital Regensburg, Regensburg, Germany) (12). Amplification of the three human *PDX-1* sequences and insertion into the pCpGL-basic vector was done by GeneScript (Piscataway, NJ). The constructs were either mock methylated or methylated using two different DNA methyltransferases: *SssI* and *HhaI* (2.5 U/ $\mu$ g DNA) (New England Biolabs, Frankfurt am Main, Germany). While *SssI* methylates all cytosine residues within the double-stranded dinucleotide recognition sequence CG, *HhaI* methylates only the internal cytosine residue in CGCG sequence.

Clonal rat insulinoma-derived INS 832/13  $\beta$ -cells (kindly provided by Professor C. Newgard, Duke University Medical Center, Durham, NC) were cultured in RPMI 1640 medium with 11.1 mM glucose, which is the standard glucose concentration for culture of these cells, supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 mM HEPES, 50  $\mu$ M  $\beta$ -mercaptoethanol, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>. INS-1 832/13  $\beta$ -cells were seeded onto a 96 well plate in

100  $\mu$ l medium ( $25 \times 10^3$  cells/well) and incubated overnight. Cells were then cotransfected with 100 ng pCpGL-vector either without (control) or with respective *PDX-1* insert (see above) together with 2 ng of pRL renilla luciferase control reporter vector (pRL-CMV vector; Promega) as a control for transfection efficiency using 0.3  $\mu$ l FuGENE HD transfection reagent (Promega) in 50  $\mu$ l serum-free medium, and the mixture was incubated for 15 min at room temperature. Each construct was transfected in five replicate wells. Then 50  $\mu$ l of medium was added to the transfection complex and cells were incubated for 48 h before performing the dual-luciferase assay (Promega). One hundred microliters of 1 $\times$  Passive Lysis Buffer was first used to lyse the cells and then the cell lysate was diluted 1:10 with 1 $\times$  PLB. One hundred microliters Luciferase Assay Reagent II reagent were added to 10  $\mu$ l of diluted cell lysate to detect the luciferase signal, and 100  $\mu$ l Stop and Glo reagent was used to detect the Renilla signal using the TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity of each construct was normalized against renilla luciferase activity, and it is shown relative to the activity of the mock-methylated construct including both the enhancer and promoter regions. The results represent the mean of four independent experiments and the values in each experiment are the mean of five replicates. Furthermore, in each experiment, cells were transfected with an empty pCpGL-vector as a background control.

### Culturing rat clonal $\beta$ -cells in normal or high glucose levels for DNA methylation analysis

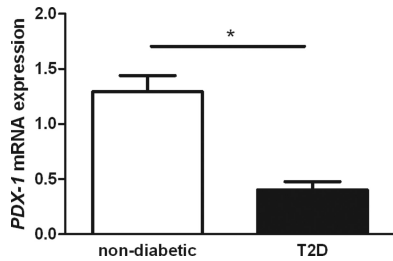
Clonal rat insulinoma-derived INS 832/13  $\beta$ -cells were cultured in RPMI 1640 medium with 11.1 mM glucose, which is the standard glucose concentration for culture of these cells, as described above. The cells were plated out in six-well plates and allowed to attach overnight. The medium was then replaced with fresh medium containing either 11.1 or 16.7 mM glucose. The cells were further cultured for 72 h, with the medium exchanged after 48 h. DNA was isolated with the All Prep kit (QIAGEN, Hilden, Germany) and kept at –20 C until analysis. DNA extracted from the cells was then used for analysis of DNA methylation.

### $\beta$ -Cell purification

$\beta$ -Cells and  $\alpha$ -cells were purified from pancreatic islets of three human donors [54, 55, and 74 yr old, with a body mass index (BMI) 21.5–23.1 kg/m<sup>2</sup>], different from the donors described in Table 1, using a method previously described (13, 14). Dissociation of islet cells was achieved by incubation with constant agitation for 3 min at 37 C in 0.05% trypsin-EDTA (Invitrogen) supplemented with 3 mg/ml deoxyribonuclease I (DNase I) (Roche, Basel, Switzerland) followed by vigorous pipetting. Labeling and fluorescence-activated cell sorting (FACS) of the  $\beta$ - and  $\alpha$ -cell fractions was performed as previously described (13, 14). Sorted  $\beta$ - and  $\alpha$ -cells were applied to microscope slides and coimmunostained for insulin and glucagon to detect the amount of  $\alpha$ -cells in the  $\beta$ -cell fraction, and vice versa. Using this method, a  $\beta$ -cell purity of  $89 \pm 9\%$  (mean  $\pm$  SD) was achieved (14).

### Statistical analysis

Differences between T2D patients and nondiabetic donors were analyzed using nonparametric Mann-Whitney *U* tests. All



**FIG. 1.** *PDX-1* mRNA expression levels in human pancreatic islets from nondiabetic donors and patients with T2D. Expression was analyzed using quantitative RT-PCR. Results are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ , nondiabetic vs. T2D islets.

compared with nondiabetic donors (T2D  $0.40 \pm 0.076$  vs. nondiabetic  $1.29 \pm 0.15$ ,  $P = 0.0002$ ; Fig. 1). Furthermore, while islet *PDX-1* mRNA expression correlated positively with insulin mRNA expression ( $\rho = 0.59$ ,  $P = 0.000001$ ) and glucose-stimulated insulin secretion ( $\rho = 0.41$ ,  $P = 0.005$ ), it correlated negatively with HbA1c levels and BMI ( $\rho = -0.50$ ,  $P = 0.0004$  and  $\rho = -0.28$ ,  $P = 0.04$ , respectively).

*P* values analyzed for the human islets were two tailed. The false discovery rate (FDR) was used to correct for multiple testing for the CpG sites analyzed using EpiTYPER in human islets, and CpG sites with a *Q* value of 0.05 or less were considered to be significantly changed. Correlations were calculated using Spearman correlation coefficient. Differences in DNA methylation between FACS sorted  $\beta$ - and  $\alpha$ -cells as well as between clonal rat  $\beta$ -cells cultured in 11.1 or 16.7 mM glucose were analyzed using paired nonparametric one-tailed tests. Differences in expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* and the luciferase data were analyzed using paired two-tailed *t* tests.  $P < 0.05$  was considered significant. Statistical calculations were performed using NCSS software (NCSS Statistical Software, Kaysville, UT) and PASW Statistics for Windows (SPSS, Chicago, IL).

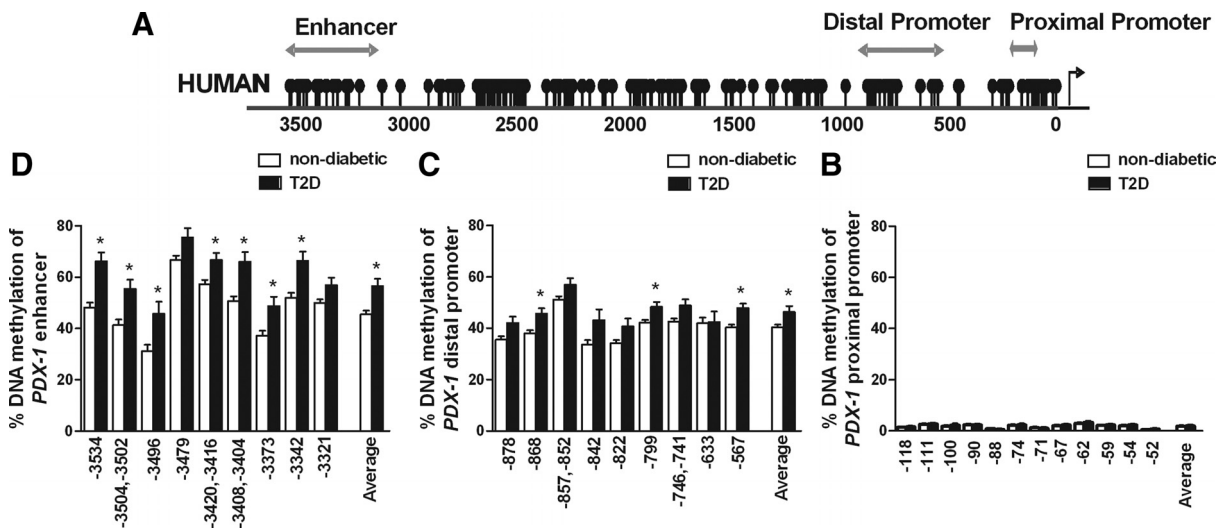
**DNA methylation of *PDX-1* in human pancreatic islets**

We next analyzed DNA methylation of *PDX-1* in pancreatic islets from patients with T2D and nondiabetic donors using three assays that cover the *PDX-1* proximal and distal promoter regions as well as the *PDX-1* enhancer region, respectively (11) (Fig. 2A). These regions of *PDX-1* were selected based on previous studies that identified regions that regulate the expression of *PDX-1* (11). All analyzed CpG sites located in the proximal promoter region close to the *PDX-1* transcription start site (Fig. 2A) showed very low levels of DNA methylation in islets from both nondiabetic and T2D donors, and there was no difference in methylation between the two groups (1.9 vs. 2.0%;  $P = 0.57$ ) (Fig. 2B). On the other hand, 10 of the analyzed CpG sites located further upstream (5') in the distal promoter region and the enhancer region of *PDX-1* showed increased DNA methylation in pancreatic islets from patients with T2D compared with nondiabetic donors (Fig. 2, C and D and Supplemental Table 2). Also, the average degree of *PDX-1* methylation of the distal promoter and enhancer regions but not the proximal pro-

**Results**

***PDX-1* expression in human pancreatic islets**

The characteristics of the donors included in this study are described in Table 1. *PDX-1* mRNA expression was decreased in pancreatic islets from patients with T2D



**FIG. 2.** Impact of T2D on DNA methylation of *PDX-1* in human pancreatic islets. A, A schematic representation of 3500 bp of the human *PDX-1* promoter and enhancer region. The three regions analyzed for DNA methylation, representing 93 bp of the proximal promoter, 436 bp of the distal promoter, and 475 bp of the enhancer region, are visualized. DNA methylation of the proximal *PDX-1* promoter (B), distal *PDX-1* promoter (C), and enhancer region (D) in human pancreatic islets of nondiabetic donors (white bars) and patients with T2D (black bars). Results are expressed as mean  $\pm$  SEM. FDR was used to correct for multiple testing with \*,  $Q < 0.05$  nondiabetic vs. T2D islets.

moter region was increased in T2D compared with non-diabetic islets (Fig. 2, B–D). The absolute increase in degree of DNA methylation in T2D islets ranged between 6.2 and 18.0% for the analyzed regions, representing fold changes between 1.15 and 1.47 (Supplemental Table 2).

### Impact of DNA methylation on gene expression of *PDX-1*

Because increased DNA methylation has been associated with transcriptional silencing, we further tested whether the degree of *PDX-1* DNA methylation correlated negatively with *PDX-1* mRNA expression in islets of all studied subjects. Indeed, for a number of analyzed CpG sites, *PDX-1* DNA methylation correlated negatively with its gene expression (Table 2). The strongest correlations were found for CpG sites located in the enhancer region, proposing a key role for this region in the regulation of *PDX-1* expression. An example of the neg-

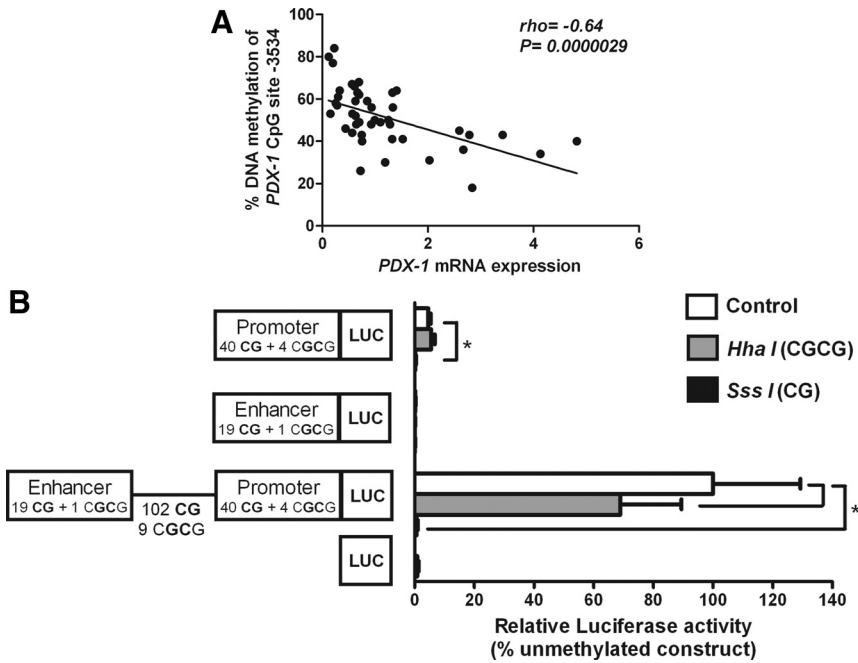
ative correlation between *PDX-1* expression and DNA methylation of one of the CpG sites is illustrated in Fig. 3A. These data suggest that increased DNA methylation may suppress *PDX-1* gene expression.

To further examine if DNA methylation of the *PDX-1* promoter is associated with reduced expression, three reporter gene constructs were made by inserting the human *PDX-1* promoter and/or enhancer sequences into a luciferase expression plasmid pCpGL that completely lacks CpG dinucleotides (12) and that hence could be used to study the effect of promoter DNA methylation in transfection assays (Fig. 3B and Supplemental Fig. 1). Each construct was then mock methylated or methylated with the methyltransferases *Hha1* and *Sss1*. While *Sss1* methylates all CpG sites, *Hha1* methylates only the internal cytosine residue in a CGCG sequence. Hence, *Sss1* results in totally methylated constructs and *Hha1* gives point meth-

**TABLE 2.** Correlations between DNA methylation of the proximal and distal promoters as well as enhancer regions of *PDX-1* and gene expression and HbA1c levels in human donors

| CpG site          | <i>PDX-1</i> gene expression |                  |                 | HbA1c levels |                |               |
|-------------------|------------------------------|------------------|-----------------|--------------|----------------|---------------|
|                   | $\rho$                       | <i>P</i> value   | Q value         | $\rho$       | <i>P</i> value | Q value       |
| Proximal promoter |                              |                  |                 |              |                |               |
| –52               | –0.16                        | 0.26             | 0.34            | 0.15         | 0.28           | 0.40          |
| –54               | –0.22                        | 0.11             | 0.18            | 0.16         | 0.27           | 0.40          |
| –59               | –0.13                        | 0.35             | 0.44            | 0.093        | 0.52           | 0.68          |
| –62               | –0.23                        | 0.10             | 0.18            | –0.064       | 0.66           | 0.79          |
| –67               | –0.074                       | 0.59             | 0.64            | 0.0049       | 0.97           | 0.99          |
| –71               | –0.10                        | 0.49             | 0.59            | 0.11         | 0.45           | 0.61          |
| –74               | –0.24                        | 0.082            | 0.15            | 0.014        | 0.92           | 0.99          |
| –88               | –0.073                       | 0.60             | 0.64            | 0.048        | 0.74           | 0.82          |
| –90               | –0.39                        | <b>0.0046</b>    | <b>0.014</b>    | 0.079        | 0.60           | 0.75          |
| –100              | –0.28                        | <b>0.042</b>     | 0.10            | 0.18         | 0.20           | 0.32          |
| –111              | –0.18                        | 0.18             | 0.25            | –0.052       | 0.72           | 0.82          |
| –118              | –0.062                       | 0.66             | 0.68            | 0.0011       | 0.99           | 0.99          |
| Distal promoter   |                              |                  |                 |              |                |               |
| –567              | –0.36                        | <b>0.010</b>     | <b>0.027</b>    | 0.45         | <b>0.0024</b>  | <b>0.028</b>  |
| –633              | 0.035                        | 0.81             | 0.81            | 0.24         | 0.11           | 0.18          |
| –746, –741        | –0.28                        | <b>0.047</b>     | 0.10            | 0.39         | <b>0.010</b>   | <b>0.038</b>  |
| –799              | –0.29                        | <b>0.043</b>     | 0.10            | 0.41         | <b>0.0057</b>  | <b>0.028</b>  |
| –822              | –0.090                       | 0.56             | 0.64            | 0.37         | <b>0.020</b>   | <b>0.046</b>  |
| –842              | –0.27                        | 0.12             | 0.19            | 0.30         | 0.088          | 0.16          |
| –857, –852        | –0.29                        | <b>0.048</b>     | 0.10            | 0.41         | <b>0.0066</b>  | <b>0.028</b>  |
| –868              | –0.21                        | 0.17             | 0.24            | 0.44         | <b>0.0029</b>  | <b>0.028</b>  |
| –878              | –0.21                        | 0.14             | 0.21            | 0.34         | <b>0.023</b>   | <b>0.049</b>  |
| Enhancer          |                              |                  |                 |              |                |               |
| –3321             | –0.43                        | <b>0.0022</b>    | <b>0.0094</b>   | 0.42         | <b>0.0038</b>  | <b>0.028</b>  |
| –3342             | –0.50                        | <b>0.00034</b>   | <b>0.0034</b>   | 0.42         | <b>0.0046</b>  | <b>0.028</b>  |
| –3373             | –0.49                        | <b>0.00047</b>   | <b>0.0035</b>   | 0.37         | <b>0.014</b>   | <b>0.041</b>  |
| –3408, –3404      | –0.50                        | <b>0.00081</b>   | <b>0.0041</b>   | 0.38         | <b>0.014</b>   | <b>0.041</b>  |
| –3420, –3416      | –0.47                        | <b>0.00065</b>   | <b>0.0039</b>   | 0.36         | <b>0.017</b>   | <b>0.042</b>  |
| –3479             | –0.45                        | <b>0.0026</b>    | <b>0.0098</b>   | 0.40         | <b>0.015</b>   | <b>0.041</b>  |
| –3496             | –0.63                        | <b>0.00022</b>   | <b>0.0033</b>   | 0.36         | 0.068          | 0.14          |
| –3504, –3502      | –0.47                        | <b>0.0047</b>    | <b>0.014</b>    | 0.28         | 0.091          | 0.16          |
| –3534             | –0.64                        | <b>0.0000029</b> | <b>0.000087</b> | 0.54         | <b>0.00024</b> | <b>0.0072</b> |

FDR was used to correct for multiple testing and to generate Q values. Bold values represent significant P and Q values after the FDR analysis.



**FIG. 3.** Impact of DNA methylation on gene expression of the human *PDX-1* gene. A, An inverse correlation between *PDX-1* mRNA expression and DNA methylation of CpG site –3534 in human pancreatic islets. B, A diagram of the three luciferase reporter plasmids used to test the effect of DNA methylation on *PDX-1* promoter activity and the empty vector is visualized. The three plasmids contain either 908 bp of the human *PDX-1* promoter, 606 bp of the human *PDX-1* enhancer region, or 3800 bp of a sequence containing both the *PDX-1* promoter and the enhancer region inserted into a pCpGL-basic vector. Methylated (gray and black bars) or mock-methylated (white bars) *PDX-1* promoter and/or the enhancer constructs were transfected into clonal rat  $\beta$ -cells for 48 h before luciferase assay. The data were normalized with cotransfected renilla luciferase control vector and are the average from four separate experiments of five replicates each. Data are presented as relative expression compared with the nonmethylated construct including both the enhancer and promoter region. Results are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ .

ylated constructs in which only a fraction of the CpG sites are methylated. The number of CpG sites that may be methylated by these enzymes in the respective construct is shown in Fig. 3B. Clonal rat  $\beta$ -cells were then transfected with the mock-methylated or methylated constructs. The highest reporter gene expression was generated by the mock-methylated construct including both the promoter and enhancer regions ( $100 \pm 29\%$ ). However, when the promoter region alone was mock methylated, the reporter gene expression was much lower ( $4.6 \pm 0.8\%$ ). The reporter gene expression level generated by the mock-methylated enhancer region alone was similar to the empty pCpGL vector, proposing that the enhancer region alone does not induce *PDX-1* expression. Furthermore, as shown in Fig. 3B, methylation of the human *PDX-1* promoter and/or enhancer region suppressed reporter gene expression. While point methylation by *Hha1* of the *PDX-1* promoter and enhancer regions suppressed reporter gene expression to  $69 \pm 20\%$ , total methylation of this construct by *Sss1* suppressed reporter gene expression to  $0.66 \pm 0.45\%$  in the clonal rat  $\beta$ -cells ( $P \leq 0.05$ ).

On the other hand, for the promoter region alone, reporter gene expression was reduced (from  $4.6 \pm 0.8$  to  $0.24 \pm 0.21\%$ ;  $P = 0.023$ ) only when the construct was totally methylated by *Sss1*, and there was no effect on gene expression by point methylation by *Hha1* ( $P = 0.26$ ).

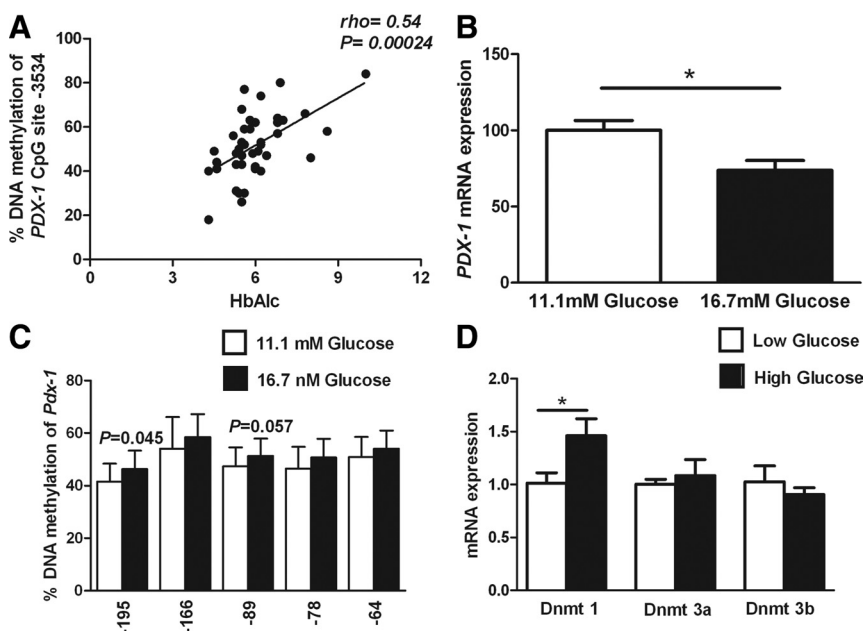
### Impact of hyperglycemia on DNA methylation of *PDX-1*

We next examined whether HbA1c levels, which are used as long-term measurements of blood glucose levels, correlate with the degree of *PDX-1* DNA methylation in the human islets. HbA1c levels correlated positively with the degree of DNA methylation for a number of analyzed CpG sites, indeed suggesting that hyperglycemia may increase DNA methylation of *PDX-1* (Table 2). A correlation between HbA1C and *PDX-1* DNA methylation is shown in Fig. 4A. To examine whether hyperglycemia has a direct impact on gene expression and DNA methylation of *Pdx-1*, we cultured clonal rat  $\beta$ -cells in normal and high levels of glucose for 72 h. While mRNA expression of *Pdx-1* decreased ( $P = 0.016$ , Fig. 4B), DNA methylation of *Pdx-1* increased nominally ( $P = 0.045$ ,

Fig. 4C) in  $\beta$ -cells exposed to high glucose. We next examined whether high levels of glucose could affect the expression of three key DNA methyltransferases compared with cells cultured in low levels of glucose. While mRNA expression of *Dnmt1* increased ( $P = 0.015$ ), mRNA expression of *Dnmt3a* and *Dnmt3b* did not change ( $P = 0.52$  and  $P = 0.27$ , respectively) in the clonal  $\beta$ -cells exposed to high compared with low levels of glucose (Fig. 4D).

### DNA methylation of *PDX-1* in $\alpha$ - and $\beta$ -cells

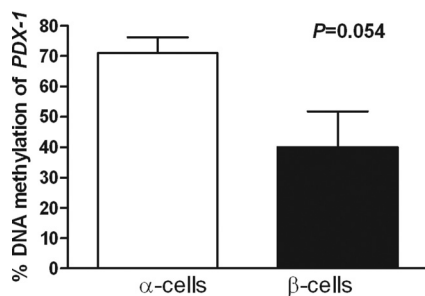
Finally, because epigenetic factors are known to regulate cell specific gene expression and because it is established that the mature pancreas mainly expresses *PDX-1* in pancreatic  $\beta$ -cells, we examined whether the degree of DNA methylation of *PDX-1* differed in FACS-sorted  $\alpha$ - and  $\beta$ -cell fractions from islets of three human donors. Although all three donors showed increased *PDX-1* DNA methylation in  $\alpha$ - compared with  $\beta$ -cells, the increase was only nominally significant (Fig. 5).



**FIG. 4.** Impact of hyperglycemia on DNA methylation of *PDX-1* and gene expression of *Pdx-1* and *Dnmt1*. A, The HbA1c level correlated positively with DNA methylation of CpG site –3534 in human pancreatic islets. Clonal rat  $\beta$ -cells cultured in 16.7 mM glucose (black bars) for 72 h exhibit (B) decreased *Pdx-1* mRNA expression and (C) nominally increased DNA methylation of two CpG sites of the *Pdx-1* promoter compared with  $\beta$ -cells cultured in 11.1 mM (white bars). Results are expressed as mean  $\pm$  SEM and represent six independent experiments. D, High (16.7 mM) compared with low (2.7 mM) glucose for 48 h increased the mRNA expression of *Dnmt1* but not *Dnmt3a* or *Dnmt3b* in clonal rat  $\beta$ -cells ( $n = 4$ ). Results are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ .

## Discussion

To our knowledge, this study presents the first analysis of DNA methylation of *PDX-1* in pancreatic islets from human donors with or without T2D. We found that CpG sites of the distal *PDX-1* promoter and its enhancer exhibited increased DNA methylation with a concomitant decrease in *PDX-1* mRNA expression in islets from patients with T2D. It is well established that a decrease in *PDX-1* expression results in impaired insulin secretion (4, 5, 15, 16). It has also been previously shown that *PDX-1* regulates insulin gene transcription through binding to the insulin promoter in  $\beta$ -cells (17–19). In agreement with these previous studies, we found that both glucose-stim-



**FIG. 5.** DNA methylation of *PDX-1* in  $\alpha$ -cells (white bars) and  $\beta$ -cells (black bars). Cells were isolated from pancreatic islets of three human donors. Results are expressed as mean  $\pm$  SEM.

ulated insulin secretion and insulin mRNA expression correlated positively with *PDX-1* expression in the human islets. It is hence possible that the low levels of *PDX-1* expression found in islets from donors with T2D contribute to the impaired insulin expression and secretion seen in these patients (9).

Park *et al.* (6) have previously shown that an adverse intrauterine environment leads to reduced pancreatic *Pdx-1* expression and hence impaired insulin secretion and diabetes in adult rats. An explanation for this phenotype is that epigenetic modifications of *Pdx-1*, including both increased DNA methylation and histone modifications, are associated with reduced gene transcription in rat islets (6). Our study proposes that epigenetic modifications, *i.e.* increased DNA methylation, of *PDX-1* may also be associated with reduced gene expression and consequently impaired insulin secretion and diabetes in humans. In contrast to the rodent studies, in which epigenetic

modifications of the proximal promoter close to the transcription start site seem critical for reducing islet *Pdx-1* expression, our data propose that increased DNA methylation of the enhancer region and a more distal part of the promoter may be critical in reducing *PDX-1* expression in human islets, *i.e.* the strongest inverse correlations between DNA methylation and gene expression, were found for CpG sites located in the enhancer region. Moreover, the CpG sites located in the *PDX-1* enhancer region showed the largest increase in DNA methylation in islets from patients with T2D compared with nondiabetic donors. In support for this theory, when we tested whether DNA methylation affects expression of the human *PDX-1* promoter using several reporter gene constructs, we found that methylation of the construct including both the enhancer and promoter regions indeed reduced the transcriptional activity of the human *PDX-1* gene the most. It is previously known that the enhancer region plays a key role for transcriptional activity of the human *PDX-1* gene (11, 20). Ma *et al.* (21) have recently shown that the reduced expression of *PDX-1* seen in gastric cancer may be due to increased DNA methylation and reduced histone acetylation of the *PDX-1* gene, further supporting the importance of epigenetic regulation of *PDX-1* in human diseases.



Hyperglycemia has previously been associated with decreased expression of *PDX-1* in  $\beta$ -cells (22). Here we identified an inverse correlation between HbA1c levels, representing the time-averaged mean levels of glycemia *in vivo*, and *PDX-1* mRNA expression, proposing a negative effect of hyperglycemia on *PDX-1* expression in human islets. We further demonstrate that high levels of glucose decrease *Pdx-1* expression in clonal rat  $\beta$ -cells. Previous studies have shown that hyperglycemia can have direct effects on the epigenetic pattern, which may result in transcriptional changes (9, 23). The positive correlations we identified between HbA1c levels and DNA methylation of *PDX-1* in the human islets indicate that this may also be the case for the *PDX-1* gene. In support of this, we found nominally increased DNA methylation of the *Pdx-1* gene in clonal rat  $\beta$ -cells exposed to high levels of glucose. Furthermore, glucose increased mRNA expression of one DNA methyltransferase, *Dnmt1*, but not *Dnmt3a* and *Dnmt3b* in the clonal  $\beta$ -cells. Using chromatin immunoprecipitation, Park *et al.* (6) have previously shown that *Dnmt1* is the primary DNA methyltransferase associated with the *Pdx-1* gene in islets from rats exposed to an adverse intrauterine environment, proposing that *Dnmt1* may affect the level of DNA methylation of *Pdx-1*. The binding of *Dnmt1* to *Pdx-1* was prevented by treatment with exendin-4, a glucagon-like peptide-1 analog used in diabetes treatment (24). This resulted in decreased DNA methylation and increased expression of *Pdx-1* (24). However, pancreatic islets from patients with T2D exhibit only a small but not significant increase in *DNMT1* expression (data not shown), and future studies are needed to test whether DNA methylation changes of *PDX-1* precede the manifestation of T2D.

In mature pancreatic islets, *PDX-1* is mainly expressed in  $\beta$ -cells. However, some *PDX-1* expression has also been found in islet  $\delta$ -cells and in some other tissues including the developing brain and in the adult hypothalamus (25–27). Epigenetic mechanisms can be used to control cell and tissue-specific gene expression. In this study we had access to FACS-sorted  $\beta$ - and  $\alpha$ -cell fractions from islets of only three human donors; nevertheless, our data suggest that DNA methylation may be involved in controlling cell-specific *PDX-1* expression in the human islets. In this context, it was recently reported that *Dnmt1*-mediated methylation of the  $\alpha$ -cell-specific transcription factor *Arx* in  $\beta$ -cells contributes to their specific phenotype (28). Here  $\beta$ -cells deficient in *Dnmt1* converted to  $\alpha$ -cells, resulting in reduced expression of  $\beta$ -cell specific genes including *Pdx-1* and insulin (28). Likewise, suppression of *Pdx-1* function in insulin-secreting cells favors an  $\alpha$ -cell-like phenotype (29). In contrast to the study by

Dhawan *et al.* (28), we found increased *Dnmt1* expression in parallel with decreased expression and increased DNA methylation of *Pdx-1* in clonal  $\beta$ -cells exposed to high levels of glucose. The discrepancy between our studies may be explained by the conversion of  $\beta$ - to  $\alpha$ -cells in the study by Dhawan *et al.* (28).

Even though we cannot fully rule out that the changes we find in *PDX-1* expression and DNA methylation are due to altered cell composition in islets from patients with T2D, our data from the clonal rat  $\beta$ -cells demonstrate that hyperglycemia specifically increases *Pdx-1* promoter DNA methylation and decreases *Pdx-1* expression in  $\beta$ -cells. Moreover, if a reduced  $\beta$ -cell mass would cause increased *PDX-1* methylation in diabetic islets due to the higher *PDX-1* methylation in  $\alpha$ - compared with  $\beta$ -cells, then all analyzed CpG sites of *PDX-1* would show increased methylation in islets from diabetic compared with nondiabetic donors. However, this is not the case. While the proximal *PDX-1* promoter shows similar very low levels of methylation in islets from both diabetic and nondiabetic donors, the distal promoter and enhancer regions of *PDX-1* show differential DNA methylation due to T2D. It is hence unlikely that the differences we see in DNA methylation are due to a reduced  $\beta$ -cell number in diabetic islets. In addition, our luciferase experiments provide functional confirmation that increased DNA methylation reduces the transcriptional activity of the *PDX-1* gene. Moreover, while some investigators have found reductions in  $\beta$ -cell number in human T2D islets (30), others have not seen any changes (31), and this is still a controversial issue. Another study recently reported differential DNA methylation in islets from patients with T2D, which was not associated with a reduced  $\beta$ -cell content (32).

Recent studies from our group and others demonstrate that epigenetic modifications influence genes with important roles in insulin secretion and action, *i.e.* we found increased DNA methylation of the insulin promoter in pancreatic islets from patients with T2D (9, 33–43). Epigenetic modifications may be passed on from one cell generation to the next (mitotic inheritance) and/or between generations of a species (meiotic inheritance) (44). In plants, epigenetic modifications are known to be inherited from one generation to the next (45). However, there is still limited information about the inheritance of epigenetic traits between generations in mammals (39, 46, 47).

The data from this study demonstrate that epigenetic modifications of *PDX-1* may reduce its expression in human diabetic islets, which may lead to impaired insulin expression and secretion. Our data further suggest that hyperglycemia may be a factor behind increased DNA methylation and decreased expression of *PDX-1*.

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