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

Dayeh, Tasnim

2016

Link to publication

Citation for published version (APA):
Dayeh, T. (2016). The Human Pancreatic Islet Methylome and Its Role in Type 2 Diabetes Epigenetics and Diabetes
Increased DNA Methylation and Decreased Expression of PDX-1 in Pancreatic Islets from Patients with Type 2 Diabetes

Beatrice T. Yang,* Tasnim A. Dayeh,* Petr A. Volkov, Clare L. Kirkpatrick, Siri Malmgren, Xingjun Jing, Erik Renström, Claes B. Wollheim, Marloes Dekker Nitert, and Charlotte Ling

Department of Clinical Sciences (B.T.Y., T.A.D., P.A.V., S.M., X.J., E.R., C.B.W., M.D.N., C.L.), Lund University Diabetes Centre, Scania University Hospital, 205 02 Malmoe, Sweden; and Department of Cell Physiology and Metabolism (C.L.K., C.B.W.), University Medical Center, 1211 Geneva 4, Switzerland

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 β-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 β-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 β-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 β-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 β-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)

Pancreatic 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 β-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 β-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-

---

* B.T.Y. and T.A.D. contributed equally to this work.
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, β-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 influence 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 methyltransfereases in clonal rat β-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 μg/ml gentamicin, 0.25 μg/ml Fungizone (GIBCO BRL, Gaithersburg, MD), 20 μg/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/liter nicotinamide at 37°C (5% CO2) before RNA and DNA preparation. Gene expression of endocrine (somatostatin and glucagon) and exocrine (pancreatic lipase, amylase α2A, 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 β-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 ΔΔCt 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 β-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 β-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-Seqenom), 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 Supplementary 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 ± 9.8 | 57.0 ± 13.1 | 1.000 |
| BMI (kg/m²) | 25.9 ± 3.6 | 28.5 ± 4.7 | 0.13 |
| HbA1c | 5.7 ± 0.8 | 7.3 ± 1.2 | 0.00010 |
| Basal insulin secretion (ng/islet·h) | 0.37 ± 0.27 | 0.22 ± 0.17 | 0.22 |
| Glucose-stimulated insulin secretion (ng/islet·h) | 1.42 ± 0.95 | 1.05 ± 1.56 | 0.045 |

Data are expressed as mean ± 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/μg 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 β-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 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 95% air and 5% CO2. INS-1 832/13 β-cells were seeded onto a 96 well plate in 100 μl medium (25 × 103 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 μl FuGENE HD transfection reagent (Promega) in 50 μl serum-free medium, and the mixture was incubated for 15 min at room temperature. Each construct was transfected in five replicate wells. Then 50 μl 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× Passive Lysis Buffer was first used to lyse the cells and then the cell lysate was diluted 1:10 with 1× PLB. One hundred microliters Luciferase Assay Reagent II reagent were added to 10 μl of diluted cell lysate to detect the luciferase signal, and 100 μl 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 β-cells in normal or high glucose levels for DNA methylation analysis**

Clonal rat insulinoma-derived INS 832/13 β-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.

**β-Cell purification**

β-Cells and α-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/m2], different from the donors 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 β- and α-cell fractions was performed as previously described (13, 14). Sorted β- and α-cells were applied to microscope slides and communostained for insulin and glucagon to detect the amount of α-cells in the β-cell fraction, and vice versa. Using this method, a β-cell purity of 89 ± 9% (mean ± SD) was achieved (14).

**Statistical analysis**

Differences between T2D patients and non-diabetic donors were analyzed using nonparametric Mann-Whitney U tests. All
**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 compared with nondiabetic donors (T2D 0.40 ± 0.076 vs. nondiabetic 1.29 ± 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).

**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-

---

**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 ± SEM. *, *P* < 0.05, nondiabetic vs. T2D islets.

**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 ± SEM. FDR was used to correct for multiple testing with *, *P* < 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 negative 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 HhaI and SssI. While SssI methylates all CpG sites, HhaI methylates only the internal cytosine residue in a CGCG sequence. Hence, SssI results in totally methylated constructs and HhaI 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 | PDX-1 gene expression | HbA1c levels |
|----------|------------------------|--------------|
|          | ρ          | P value | Q value | ρ          | P 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     | 0.0046  | 0.014   | 0.079    | 0.60    | 0.75    |
| −100     | −0.28     | 0.042   | 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     | 0.10    | 0.027   | 0.45     | 0.0024  | 0.028   |
| −633     | 0.035     | 0.81    | 0.81    | 0.24     | 0.11    | 0.18    |
| −746,−741| −0.28     | 0.047   | 0.10    | 0.39     | 0.010   | 0.038   |
| −799     | −0.29     | 0.043   | 0.10    | 0.41     | 0.0057  | 0.028   |
| −822     | −0.090    | 0.56    | 0.64    | 0.37     | 0.020   | 0.046   |
| −842     | −0.27     | 0.12    | 0.19    | 0.30     | 0.088   | 0.16    |
| −857,−852| −0.29     | 0.048   | 0.10    | 0.41     | 0.0066  | 0.028   |
| −868     | −0.21     | 0.17    | 0.24    | 0.44     | 0.0029  | 0.028   |
| −878     | −0.21     | 0.14    | 0.21    | 0.34     | 0.023   | 0.049   |
| **Enhancer** |          |         |         |          |         |         |
| −3321    | −0.43     | 0.0022  | 0.0094  | 0.42     | 0.0038  | 0.028   |
| −3342    | −0.50     | 0.00034 | 0.0034  | 0.42     | 0.0046  | 0.028   |
| −3373    | −0.49     | 0.00047 | 0.0035  | 0.37     | 0.014   | 0.041   |
| −3408,−3404| −0.50   | 0.00081 | 0.0041  | 0.38     | 0.014   | 0.041   |
| −3420,−3416| −0.47  | 0.00065 | 0.0039  | 0.36     | 0.017   | 0.042   |
| −3479    | −0.45     | 0.0026  | 0.0098  | 0.40     | 0.015   | 0.041   |
| −3496    | −0.63     | 0.00022 | 0.0033  | 0.36     | 0.068   | 0.14    |
| −3504,−3502| −0.47  | 0.00047 | 0.014   | 0.28     | 0.091   | 0.16    |
| −3534    | −0.64     | 0.0000029 | 0.000087 | 0.54     | 0.00024 | 0.0072  |

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.
On the other hand, for the promoter region alone, reporter gene expression was reduced (from 4.6 ± 0.8 to 0.24 ± 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).
the insulin promoter in regulates insulin gene transcription through binding to 5, 15, 16). It has also been previously shown that PDX-1 patients with T2D. It is well established that a decrease in exhibited increased DNA methylation with a concomitant sites of the distal promoter and its enhancer 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 β-cells (17–19). In agreement with these previous studies, we found that both glucose-stimulated 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 increased DNA methylation, i.e. 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.

**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 β-cells (17–19). In agreement with these previous studies, we found that both glucose-stimulated 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 increased DNA methylation, i.e. 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 β-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 β-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 β-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 β-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 β-cells. However, some PDX-1 expression has also been found in islet δ-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 β- and α-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 α-cell-specific transcription factor Arx in β-cells contributes to their specific phenotype (28). Here β-cells deficient in Dnmt1 converted to α-cells, resulting in reduced expression of β-cell specific genes including Pdx-1 and insulin (28). Likewise, suppression of Pdx-1 function in insulin-secreting cells favors an α-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 β-cells exposed to high levels of glucose. The discrepancy between our studies may be explained by the conversion of β- to α-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 β-cells demonstrate that hyperglycemia specifically increases Pdx-1 promoter DNA methylation and decreases Pdx-1 expression in β-cells. Moreover, if a reduced β-cell mass would cause increased PDX-1 methylation in diabetic islets due to the higher PDX-1 methylation in α-compared with β-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 β-cell number in diabetic islets. In addition, our luciferase experiments provide functional conformation that increased DNA methylation reduces the transcriptional activity of the PDX-1 gene. Moreover, while some investigators have found reductions in β-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 β-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.
Acknowledgments

Human pancreatic islets were provided by the Nordic Network for Clinical Islet Transplantation by the courtesy of Professor O. Korsgren (Uppsala University, Uppsala, Sweden). We are grateful to Professor P. Marchetti (University of Pisa, Pisa, Italy) and Dr. Bosco (Geneva University Hospital, Geneva, Switzerland) for the provision of human islets for the β- and α-cell sorting. We also want to acknowledge Mona Śvardh and Anna-Maria Ramsay for their expert technical support. The author contributions included the following: B.T.Y., T.A.D., C.L.K., S.M., X.J., E.R., C.B.W., M.D.N., and C.L. designed the research; B.T.Y., T.A.D., C.L.K., S.M., X.J., and M.D.N. performed the research; C.L.K. and C.B.W. contributed the new reagents or analytic tools; B.T.Y., T.A.D., P.V., S.M., M.D.N., and C.L. analyzed the data; T.A.D. and C.L. wrote the paper; and B.T.Y., T.A.D., and C.L. are guarantors for this study.

Address all correspondence and requests for reprints to: Charlotte Ling, Department of Clinical Sciences, Unit of Epigenetics and Diabetes, Lund University Diabetes Centre, Scania University Hospital, 205 02 Malmoe, Sweden. E-mail: charlotte.ling@med.lu.se.

This work was supported by grants from the Swedish Research Council, Region Skåne, Scania University Hospital, Knut and Alice Wallenberg Foundation, EFS-D-Lilly, Novo Nordisk Foundation, The Swedish Diabetes Foundation, Kungliga Fysiografiska Sällskapet in Lund, Pålhlsson, Söderberg, Linne Grant B31 5631/2006, Exodiab, the Swiss National Science Foundation, and EuroDia 6th Framework Program.

Disclosure Summary: The authors have nothing to declare.

References

1. Kaneto H, Miyatsuka T, Kawamori D, Yamamoto K, Kato K, Shiraia T, Takataki N, Yamazaki Y, Matsushita M, Matsuoka TA 2008 PDX-1 and MafA play a crucial role in pancreatic β-cell differentiation and maintenance of mature β-cell function. Endocr J 55:235–252
2. Dutta S, Gannon M, Peers B, Wright C, Bonner-Weir S, Montminy M 2001 PDX-PBX complexes are required for normal proliferation of pancreatic cells during development. Proc Natl Acad Sci USA 98:1065–1070
3. Noguchi H, Matsushita M, Matsumoto S, Lu YF, Matsui H, Bonner-Weir S 2005 Mechanism of PDX-1 protein transduction. Biochem Biophys Res Commun 332:68–74
4. Stoffers DA, Ferrer J, Clarke WL, Habener JF 1997 Early-onset type-II diabetes mellitus (MODY4) linked to IFP1. Nat Genet 17:138–139
5. Ahlgren U, Jonsson J, Jonsson K, Simu K, Edlund H 1998 Β-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the β-cell phenotype and maturity onset diabetes. Genes Dev 12:1763–1768
6. Park JH, Stoffers DA, Nicholls RD, Simmons RA 2002 Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. J Clin Invest 118:2316–2324
7. Olsson AH, Yang BT, Hall E, Taneera J, Salehi A, Nitter MD, Ling C 2011 Decreased expression of genes involved in oxidative phosphorylation in human pancreatic islets from patients with type 2 diabetes. Eur J Endocrinol 165:589–595
8. Rosengren AH, Jokubka R, Tojdar O, Granhall C, Hansson O, Li DQ, Nagarav V, Reinbotho TM, Tuncel J, Eliasson L, Groop L, Rorsman P, Salehi A, Lyskenso V, Luthman H, Renström E 2010 Overexpression of α2A-adrenergic receptors contributes to type 2 diabetes. Science 327:217–220
9. Yang BT, Dayeh TA, Kirkpatrick CL, Taneera J, Kumar R, Groop L, Wollheim CB, Nitert MD, Ling C 2011 Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA1c levels in human pancreatic islets. Diabetologia 54:360–367
10. Andersen CL, Jensen JL, Órntoft TF 2004 Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250
11. Melloul D, Marshak S, Cerasi E 2002 Regulation of pdx-1 gene expression. Diabetes 51(Suppl 3):S320–S325
12. Klug M, Rehli M 2006 Functional analysis of promoter CpG methylation using a CpG-free luciferase reporter vector. Epigenetics 1:127–130
13. Parraud N, Bosco D, Berney T, Pattou F, Kerr-Conde J, Donath MY, Bruun C, Mandrup-Poulsen T, Billestrup N, Halban PA 2008 Proliferation of sorted human and rat β cells. Diabetologia 51:91–100
14. Kirkpatrick CL, Marchetti P, Purrello F, Pirro S, Bugliani M, Bosco D, de Koning EJ, Engelse MA, Kerr-Conde J, Pattou F, Wollheim CB 2010 Type 2 diabetes susceptibility gene expression in normal or diabetic sorted human α and β cells: correlations with age or BMI of islet donors. PLoS ONE 5:e11053
15. Brissova M, Shiota M, Nicholson WE, Gannon M, Knobil SM, Piston DW, Wright CV, Powers AC 2002 Reduction in pancreatic transcription factor PDX-1 impairs glucose-stimulated insulin secretion. J Biol Chem 277:11225–11232
16. Hui H, Perfetti R 2002 Pancreas duodenum homeobox-1 regulates pancreas development during embryogenesis and islet cell function in adulthood. Eur J Endocrinol 146:129–141
17. Qiu Y, Guo M, Huang S, Stein R 2002 Insulin gene transcription is mediated by interactions between the p300 coactivator and PDX-1, BET2A, and E47. Mol Cell Biol 22:412–420
18. Al-Quobaii F, Montenarh M 2008 Pancreatic duodenal homeobox factor-1 and diabetes mellitus type 2 (review). Int J Mol Med 21:399–404
19. Ohneda K, Mirmira RG, Wang J, Johnson JD, German MS 2000 The homeodomain of PDX-1 mediates multiple protein-protein interactions in the formation of a transcriptional activation complex on the insulin promoter. Mol Cell Biol 20:900–911
20. Ben-Shushan E, Marshak S, Shoshkes M, Cerasi E, Melloul D 2001 A pancreatic β-cell-specific enhancer in the human PDX-1 gene is regulated by hepatocyte nuclear factor 3β (HNF-3β), HNF-1α, and SP transcription factors. J Biol Chem 276:17533–17540
21. Ma J, Wang JD, Zhang WJ, Zou B, Chen WJ, Lam CS, Chen MH, Pang R, Tan VP, Hung IF, Lan HY, Wang QY, Wong BC 2010 Promoter hypermethylation and histone hypoacetylation contribute to pancreatic-duodenal homeobox 1 silencing in gastric cancer. Carcinogenesis 31:1552–1560
22. Hribal ML, Perego L, Lovari S, Andreozzi F, Menghini R, Perego C, Finzi G, Ussellini I, Placidi C, Capella C, Guzzi V, Lauro D, Bertuzzi F, Davalli A, Pozza G, Pintori R, Fedrici M, Lauro R, Brunetti A, Folli F, Sesti G 2003 Chronic hyperglycemia impairs insulin secretion by affecting insulin receptor expression, splicing, and signaling in RIN β cell line and human islets of Langerhans. FASEB J 17:1340–1342
23. El-Osta A, Brasacchio D, Yao D, Pocai A, Jones PL, Roeder RG, Cooper ME, Brownlee M 2008 Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. J Exp Med 205:2409–2417
24. Pinney SE, Jaekle Santos LJ, Han Y, Stoffers DA, Simmons RA
2011 Exendin-4 increases histone acetylase activity and reverses epigenetic modifications that silence Pdx1 in the intrauterine growth retarded rat. Diabetologia 54:2606–2614
25. Leonard J, Peers B, Johnson T, Ferreri K, Lee S, Montminy MR 1993 Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. Mol Endocrinol (Baltimore, Md) 7:1275–1283
26. Schwartz PT, Perez-Villamil B, Rivera A, Moratalla R, Vallejo M 2000 Pancreatic homeodomain transcription factor IDX1/IPF1 expressed in developing brain regulates somatostatin gene transcription in embryonic neural cells. J Biol Chem 275:19106–19114
27. Song J, Xu Y, Hu X, Choi B, Tong Q 2010 Brain expression of Cre recombinase driven by pancreas-specific promoters. Genesis 48: 628–634
28. Dhawan S, Georgia S, Tschen SL, Fan G, Bhushan A 2011 Pancreatic β cell identity is maintained by DNA methylation-mediated repression of Arx. Dev Cell 20:419–429
29. Wang H, Maechler P, Ritz-Laser B, Hagenfeldt KA, Ishihara H, Philippe J, Wollheim CB 2001 Pdx1 level defines pancreatic gene expression pattern and cell lineage differentiation. J Biol Chem 276:25279–25286
30. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC 2003 β-Cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110
31. Clark A, Jones LC, de Koning E, Hansen BC, Matthews DR 2001 Decreased insulin secretion in type 2 diabetes: a problem of cellular mass or function? Diabetes 50(Suppl 1):S169–S171
32. Volkmar S, Dedeuwaarder S, Cunha DA, Ndlovu MN, Defrance M, Deplus R, Calonne E, Volkmar U, Igoillo-Esteve M, Naamane N, Del Guerra S, Masini M, Bugliani M, Marchetti P, Cnop M, Deplus R, Calonne E, Volkmar U, Igoillo-Esteve M, Naamane N, Del Guerra S, Masini M, Bugliani M, Marchetti P, Cnop M, Eizirik DL, Fuks F 2012 DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients. EMBO J 31:1405–1426
33. Ling C, Poulsen P, Simonsson S, Rönn T, Holmkvist J, Almgren P, Hagert P, Nilsson E, Mabey AG, Nilsson P, Vaag A, Groop L 2007 Genetic and epigenetic factors are associated with expression of respiratory chain component NDUFB6 in human skeletal muscle. J Clin Invest 117:3427–3435
34. Ling C, Del Guerra S, Lapi R, Rönn T, Granhall C, Luthman H, Masiero P, Marchetti P, Groop L, Del Prato S 2008 Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. Diabetologia 51:615–622
35. Ling C, Groop L 2009 Epigenetics: a molecular link between environmental factors and type 2 diabetes. Diabetes 58:2718–2725
36. Rönn T, Poulsen P, Hansson O, Holmkvist J, Almgren P, Nilsson P, Tuomi T, Isomaa B, Groop L, Vaag A, Ling C 2008 Age influences DNA methylation and gene expression of COX7A1 in human skeletal muscle. Diabetologia 51:1159–1168
37. Brons C, Jacobsen S, Nilsson E, Rönn T, Jensen CB, Storgaard H, Poulsen P, Groop L, Ling C, Astrup A, Vaag A 2010 Deoxyribo-nucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. J Clin Endocrinol Metab 95:3048–3056
38. Barrès R, Oster ME, Yan J, Rude A, Fritz T, Caidahl K, Krook A, Zierath JR 2009 Non-CpG methylation of the PGC-1α promoter through DNMT3B controls mitochondrial density. Cell Metab 10:189–198
39. Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA, Morris MJ 2010 Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. Nature 467:963–966
40. Gauton KJ, Nammo T, Pasquali L, Simon JM, Giresi PG, Fogarty MP, Panhuis TM, Miczekowski P, Secchi A, Bosco D, Berney T, Montanya E, Mohlke KL, Lieb JD, Ferrer J 2010 A map of open chromatin in human pancreatic islets. Nat Genet 42:255–259
41. Bhadare R, Schug J, Le Lay J, Fox A, Smirnova O, Liu C, Naji A, Kaestner KH 2010 Genome-wide analysis of histone modifications in human pancreatic islets. Genome Res 20:428–433
42. Stitziel NL, Sethupathy P, Pearson DS, Chines PS, Song L, Erdos MR, Welch R, Parker SC, Boyle AP, Scott LJ, Margulies EH, Bochinke M, Furcy TS, Crawford GE, Collins FS 2010 Global epigenomic analysis of primary human pancreatic islets provides insights into type 2 diabetes susceptibility loci. Cell Metab 12:443–455
43. Sandovici I, Smith NH, Nietert MD, Ackers-Johnson M, Urbic-Lewis S, Ito Y, Jones RH, Marquez VE, Cairns W, Tadayyon M, O’Neill LP, Murrell A, Ling C, Constância M, O’zanne SE 2011 Maternal diet and aging alter the epigenetic control of a promoter-enhancer interaction at the Hnf4α gene in rat pancreatic islets. Proc Natl Acad Sci USA 108:5449–5454
44. Danchin E, Charmantier A, Champagne FA, Mesoudi A, Pujol B, Blanchet S 2011 Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. Nat Rev Genet 12:475–486
45. Cubas P, Vincent C, Coen E 1999 An epigenetic mutation responsible for natural variation in floral symmetry. Nature 401:157–161
46. Chong S, Whitelaw E 2004 Epigenetic germline inheritance. Curr Opin Genet Dev 14:692–696
47. Anway MD, Cupp AS, Uzumcu M, Skinner MK 2005 Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466–1469