Detection of β cell death in diabetes using differentially methylated circulating DNA

Eitan M. Akirav^{a,1}, Jasmin Lebastchi^a, Eva M. Galvan^a, Octavian Henegariu^a, Michael Akirav^b, Vitaly Ablamunits^a, Paul M. Lizardi^c, and Kevan C. Herold^{a,2}

^aDepartment of Immunobiology and Internal Medicine, Yale University School of Medicine, New Haven, CT 06511; ^bFaculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel; and ^cDepartment of Pathology, Yale University School of Medicine, New Haven, CT 06511

Edited* by Donald F. Steiner, University of Chicago, Chicago, IL, and approved October 12, 2011 (received for review July 8, 2011)

In diabetes mellitus, β cell destruction is largely silent and can be detected only after significant loss of insulin secretion capacity. We have developed a method for detecting β cell death in vivo by amplifying and measuring the proportion of insulin 1 DNA from β cells in the serum. By using primers that are specific for DNA methylation patterns in β cells, we have detected circulating copies of β cell-derived demethylated DNA in serum of mice by quantitative PCR. Accordingly, we have identified a relative increase of β cell-derived DNA after induction of diabetes with streptozotocin and during development of diabetes in nonobese diabetic mice. We have extended the use of this assay to measure β cell-derived insulin DNA in human tissues and serum. We found increased levels of demethylated insulin DNA in subjects with new-onset type 1 diabetes compared with age-matched control subjects. Our method provides a noninvasive approach for detecting β cell death in vivo that may be used to track the progression of diabetes and guide its treatment.

epigenetics | autoimmunity | biomarker

The β cell loss that leads to type 1 diabetes mellitus (T1D) is silent. In T1D, killing of β cells and subsequent presentation with hyperglycemia takes weeks in the nonobese diabetic (NOD) mouse model of T1D and possibly years in humans (1). Hyperglycemia occurs when the majority of β cells have been destroyed, providing only limited options for therapy (2, 3). Early detection of ongoing β cell death would allow for earlier interventions at a time before the development of hyperglycemia, when a more significant β cell mass is present. Indeed, immunotherapy is most successful in patients with residual β cell function (3–5). Measurements of insulin, proinsulin, and C-peptide responses to a variety of tests have been used as indices for β cell mass and destruction (6–9), whereas HLA genes and autoantibodies have been used as genetic indicators of high-risk individuals (10–12). However, these measurements do not identify the ongoing β cell destruction in islets. Unfortunately, the first direct evidence of β cell destruction becomes apparent only after β cell function has been compromised and glucose levels have risen in response to provocative stimuli (13, 14). Furthermore, the location of the pancreas in the abdominal cavity and the relatively small size of the islets of Langerhans pose a significant limitation for direct islet imaging and evaluation of β cell mass (15).

Epigenetic modifications of DNA are used by various cell types to control tissue-specific gene expression. These modifications include histone acetylation/deacetylation and DNA methylation (16–18). Methylation of DNA sequences occurs in CpG sites to maintain a transcriptionally repressive chromatin configuration, whereas demethylation results in a transcriptionally permissive configuration (19). Differential methylation of oncogenes has been used to identify microsatellite instability in patients with colon cancer, and detection of differentially methylated DNA in the serum of cancer patients has been used as a biomarker for cancer diagnosis (20–22). Previous studies have relied on the detection of serum-derived tissue-specific epigenetic modifications to identify DNA released from those cells when they die.

In this paper, we report a noninvasive method for the detection of circulating β cell-derived DNA in murine models of acute and chronic β cell destruction that provides a sensitive biomarker for β cell death in prediabetic mice in vivo and in

human tissues and serum. Our method can identify β cell death before the onset of hyperglycemia and soon after the onset of T1D. This strategy may prove useful for monitoring β cell destruction in individuals at risk for the development of T1D, monitoring the progression of β cell destruction in individuals with T1D, and use as a marker to guide therapy in patients with T1D with possible ongoing β cell destruction.

Results

Methylation-Specific Primers Can Detect Differentially Methylated *Ins1* Gene DNA from β TC3 and PMJ Murine Cell Lines. To identify differentially methylated CpG dinucleotides present in the *Ins1* gene in β cells, we examined the methylation patterns of the *Ins1* gene in the glucose-responsive murine insulinoma cell line β TC3 (23). As a non- β cell control, we used the PMJ macrophage cell line. DNA from both cell types was extracted and subjected to bisulfite treatment as described in *Materials and Methods*. We identified a differentially methylated CpG dinucleotide at position NUCLEOTIDE 52339278 (<http://genome.ucsc.edu/cgi-bin/hgGateway>, Feb 2009 GRCh37/hg19) on chromosome 19, corresponding to the CpG in position +177 downstream from the *Ins1* transcription start site, which was demethylated in β TC3 cells and methylated in control PMJ cells (Fig. 1A). This CpG dinucleotide is located in the coding region of the insulin mRNA residing in the proinsulin protein and is evolutionarily conserved in mouse and human insulin genes.

To verify the tissue specificity of demethylation at this site, we determined the frequency of demethylated and methylated CpG sites in products of the methylation-insensitive PCR from bisulfite-treated DNA from sorted murine insulin-positive cells isolated from MIP-GFP mice and from liver (Fig. 1B). The majority of the sites were demethylated in DNA from β cells. The CpG site at +177 was demethylated in 13 of 15 clones isolated from β cells, but in 0 of 8 clones isolated from liver ($P < 0.001$). We found that 25% of the 105 sites, or 33% of the clones, showed methylated cytosines in at least one of the seven CpG sites analyzed. In contrast, 86% of the 56 sites analyzed from liver were methylated.

The relatively low amounts of circulating DNA in the serum posed a challenge for detecting cell-specific DNA species. Thus, we designed a nested PCR in which we first amplified insulin DNA with methylation-insensitive primers between a region spanning the CpG dinucleotide of interest, followed by a second reaction with methylation-sensitive primers capable of differentiating β cell-derived and non- β cell-derived insulin DNA (Fig. 2A and Table S1). The first PCR generated a product of 204 bp that was

Author contributions: E.M.A., J.L., O.H., and K.C.H. designed research; E.M.A., J.L., E.M.G., O.H., M.A., and V.A. performed research; E.M.A. and O.H. contributed new reagents/analytic tools; E.M.A., J.L., M.A., P.M.L., and K.C.H. analyzed data; and E.M.A., O.H., V.A., P.M.L., and K.C.H. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

¹Present address: Winthrop University Hospital, Mineola, NY 11501.

²To whom correspondence should be addressed. E-mail: kevan.herold@yale.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1111008108/-DCSupplemental.

Fig. 1. DNA from β TC3 and PMJ cell lines and β and non- β cells have a differentially methylated CpG dinucleotide in the *Ins1* gene. (A) (Upper) Unmodified DNA sequence of murine *Ins1* DNA depicting the position of the differentially methylated CpG dinucleotide (arrow). (Lower) Comparison of bisulfite treated genomic DNA from either the β TC3 or PMJ cell line, demonstrating the nucleotide modification of CpG dinucleotides owing to demethylation at position 52339278. (B) Sequence analysis of product amplified in the first-step PCR. The sequence of 15 clones from murine β cells and 8 clones from murine liver cells are shown (○ indicates demethylated cytosines; ●, methylated cytosines). The locations of the methylation sites from the transcription start site are indicated. The primers of the second-step PCR were specific for methylated/demethylated cytosine at bp +177, corresponding to nucleotide 52339278.

gel-extracted to improve real-time PCR efficiency. This first-step product was used as template in a second PCR with methylation site-specific primers. Real-time PCR analysis showed a 256-fold (eight-cycle) increase in demethylated DNA levels relative to methylated DNA levels in bisulfite-treated DNA from β TC3 cells with a single melting peak (Fig. 2B). An exact inverse ratio was observed in the non- β cell line PMJ, in which PCR product from methylation-dependent primers was observed eight cycles earlier than PCR product from methylation-independent primers. The identity of the PCR products was verified by sequencing. Taken together, these data indicate the presence of a unique differentially methylated CpG dinucleotide in the coding region of the *Ins1* gene, and demonstrate our ability to detect differentially methylated DNA from either a β cell-like or non- β cell-like origin by methylation-sensitive quantitative PCR analysis.

Demethylated *Ins1* DNA Is Enriched in Primary Murine Islets and Cell-Sorted Insulin-Positive Cells. To test our assay's ability to detect methylation-specific modification of DNA from primary murine

tissues, we collected kidney, liver, brain, and islet tissues from NOD/SCID mice, which, unlike WT NOD mice, do not develop insulinitis or β cell destruction. DNA was extracted and treated with bisulfite, followed by the nested PCR analysis described above. Methylation-specific primers demonstrated a >12-fold increase in demethylated DNA in the crude islet preparations compared with liver, kidney, and brain (Fig. 3A).

To confirm that β cells were the primary source of the demethylated insulin DNA in our nested PCR, we dissociated murine islets into single cells and stained them for insulin. Insulin-positive β cells and insulin-negative cells were sorted by FACS (Fig. 3B), and the DNA was isolated and treated as described above. There was a 45-fold increase in demethylated DNA in the insulin-positive cell fraction compared with insulin-negative cells from islets (Fig. 3C). Product sequencing revealed an identical demethylated modification in insulin-positive islet cells as in the β TC3 cell line, whereas the non- β cell fraction demonstrated a methylated CpG dinucleotide, as observed in the PMJ cell line.

We next analyzed the linearity of the ratio between the two DNA species by mixing demethylated DNA (derived from β cells) and methylated DNA (derived from non- β cells) and measuring the difference in cycle threshold (Ct) values detected (Fig. 3D). The difference in the Ct values of the methylated and demethylated products of the second-step PCR were characterized using the demethylation index as described in *Materials and Methods*, which corresponds to quantitative differences in the quantity of DNA. There was a linear relationship between the log ratio of β cell-derived and non- β cell-derived DNA and a demethylation index between 100:1 and 1:100 ($r^2 = 0.957$; $P < 0.01$), suggesting that it is possible to measure the quantitative differences in the DNA species over this wide range.

Circulating Demethylated *Ins1* DNA Is Increased in Streptozotocin-Treated BALB/c Mice. To determine whether our assay can detect β cell death in vivo, we collected serum from BALB/c mice before and after treatment with high-dose (200 mg/kg) streptozotocin (STZ), and isolated, processed, and analyzed the DNA as described above. The STZ-treated mice demonstrated increased glucose levels at 24 h after STZ injection, indicating acute injury to β cells ($P < 0.001$) (Fig. 4A). Despite a modest decline in glucose levels at 8 h after treatment ($P < 0.05$), most likely reflecting loss of β cell membrane integrity and release of insulin granules, there was a 2.6-fold increase in the demethylation index at 8 h ($P < 0.05$) and a 3.8-fold increase at 24 h ($P < 0.02$) (Fig. 3C). We studied the percentage of nucleated cells in the islets after STZ treatment and found a reduced percentage of DAPI-positive, insulin-positive cells staining in the islets at 8 h after STZ treatment (UnTx = 55.1% vs. $t_8 = 41.3\%$; $P < 0.002$) (Fig. 3D). We also found a further reduction in the percentage of DAPI-positive, insulin-positive cells at 24 h after STZ treatment (Fig. 3C), which corresponded to the peak in circulating demethylated DNA and increased baseline glucose levels (UnTx = 55.1% vs. $t_{24} = 32.8\%$; $P < 0.0001$) (Fig. 3B). Taken together, these data indicate the ability of methylation-

Fig. 2. Identification of differentially methylated DNA by real-time PCR. Bisulfite-treated purified DNA from tissues, cells, or serum origin was purified and used in the first-step, methylation-insensitive reaction. The products were gel-purified and used as a template in a second-step reaction with methylation-specific primers.

Fig. 3. Demethylated *Ins1* DNA is enriched in primary islets and FACS-sorted primary insulin-positive cells. (A) Ratio of demethylated:methylated DNA in primary murine tissues. The cycle differences were normalized to the cycle difference of kidney DNA. The data are from a single experiment representative of more than five experiments. (B) FACS plot analysis showing the presence of insulin-positive and -negative cells sorted from dispersed islets. (C) Demethylated:methylated DNA levels in the sorted cell population (shown in B). The insulin-positive cell cycle difference was normalized to the insulin-negative cell cycle difference. Data are from a single experiment representative of two experiments. (D) DNA from the first-step reactions from sorted β cells and from islet-derived non- β cells were mixed in ratios of 1:1, 1:10, and 1:100 and then added to the second-step reaction. The relationship between the ratio of DNA and the demethylation index is shown ($r^2 = 0.96$; $P = 0.0038$).

specific real-time PCR to detect demethylated DNA derived from damaged β cells in the serum of STZ-treated mice.

Circulating Demethylated *Ins1* DNA Is Elevated in Prediabetic NOD Mice. We next determined whether chronic β cell destruction

Fig. 4. Demethylated *Ins1* DNA is increased in the serum after STZ treatment of mice. (A) Blood glucose levels of untreated and STZ-injected BALB/c mice ($n = 6$ animals per group) $*P < 0.05$; $^{\dagger}P < 0.02$ vs. prediabetic mice. (B) Demethylation index of the nested PCR performed on DNA from sera of the BALB/c mice. Between 16 and 18 mice were analyzed at each time point. The sera from two mice were pooled for analysis. $*P < 0.05$. The box-and-whisker plots show the minimum and maximum values. (C) Histomorphologic analysis of DAPI-positive, insulin-positive cells in the islets of the STZ-treated mice shown in B. $*P < 0.0001$; $^{\dagger}P < 0.002$. (D) Representative islets of STZ-treated mice. DAPI is in blue; insulin, in red.

could be detected in the NOD mouse model of spontaneous diabetes, a model of chronic autoimmunity in human T1D. NOD mice were challenged with an i.p. glucose tolerance test (IPGTT) beginning at 7 wk of age, during which basal glucose levels were normal, and extending through the development of overt hyperglycemia (Fig. 5A). The IPGTTs revealed subtle changes in glucose tolerance beginning at 9 wk of age that showed a statistically significant difference from the 7-wk response only at 14 wk ($P < 0.05$) (Fig. 5B). The fasting glucose levels remained normal at all time points (Fig. 5A and B) (24). The demethylation index increased significantly before the decline in insulin levels and before the increase in fasting glucose levels ($P = 0.0002$) (Fig. 5C). At 14–15 weeks, the median demethylation index was increased by 21-fold (range, 3.2- to 211-fold; $n = 12$) compared with the average of 7-wk-old mice ($P < 0.01$) (Fig. 5C and D). Interestingly, in 16- to 24-wk-old mice with overt hyperglycemia, the index declined but was still elevated compared with that in the 7-wk-old NOD mice ($P < 0.05$).

The range of increase in demethylation indices in the prediabetic mice was broad, possibly related to individual differences. To understand the relationships between β cell mass and the demethylation index, we investigated the relationship between total pancreatic insulin content and the demethylation index in a separate experiment with prediabetic NOD mice. We found a decline in pancreatic insulin content with age that was statistically lower at 15 wk compared with 7-wk-old NOD mice ($P < 0.05$). At the same time, the demethylation index increased by 13-fold at 11 wk compared with 7 wk ($P < 0.05$), and by 14-fold at 15 wk ($P < 0.01$) (Fig. 5D). To analyze the relationship between pancreatic insulin content and the demethylation index in individual mice, we compared these two parameters and found that they were significantly negatively correlated ($r^2 = 0.28$; $P < 0.05$) (Fig. 5E). Taken together, these data show a link between an increased demethylation index and β cell loss.

Demethylated *Ins* DNA Is Increased in Human Islets and in Serum from Patients with New-Onset T1D. We used a similar strategy to analyze demethylated insulin DNA in human tissues. Primers for the first-step and nested PCR reactions were prepared from the analogous sequences in human *INS* on chromosome 11 (Fig. 6A and Table S1). Total DNA was isolated and used in the first-step PCR after bisulfite treatment.

We sequenced the products of the first-step PCR and identified two peaks in the CpG site at nucleotide 2182036 (<http://genome.ucsc.edu/cgi-bin/hgGateway>, Feb 2009 GRCh37/hg19) in position +399 downstream from the transcription start site in the DNA from human islets. This double peak corresponds to methylated and demethylated cytosines. Only a single peak, corresponding to methylated cytosine, was found in human kidney DNA (Fig. 6A and B).

We then sorted primary insulin-positive human β cells from dissociated islets by staining with the zinc selective dye FluoZin-3-AM and cloned products of the first-step reaction from these cells, and compared the sequences with kidney cells (25). All of the clones (10 of 10) from purified β cells showed demethylated DNA at bp 273 and 399 in the insulin gene, compared with 0 of 12 clones from kidney ($P < 0.001$) (Fig. 6C). Moreover, CpG sites were rarely demethylated in kidney ($<25\%$ of clones), and none of the clones from kidney exhibited demethylation at all of the CpG sites, whereas all sites but one were demethylated in all 10 clones sequenced from human β cells.

We then compared the demethylation index in DNA isolated from islets, kidney, and liver as well as in unmethylated and methylated synthetic DNA (Fig. 6D). We found a significant increase in the demethylation index in islets ($P < 0.001$) compared with liver (57-fold) and kidney (91-fold). The demethylation index with islet DNA (0.729 ± 0.05) was similar to the demethylation index with synthetic unmethylated DNA (0.70 ± 0.03). The identity of products was verified by sequencing. The average interassay coefficient of variation from three separate analyses of this tissue DNA was $21.7\% \pm 6.4\%$.

Finally, we compared the demethylation index in serum samples from patients with T1D ($n = 5$; mean age, 10.8 ± 1.02 y; range, 8–14 y) within the first year (mean duration of T1D, 7.0 ± 1.30 mo;

Fig. 5. Serum-derived demethylated *Ins1* DNA is increased in prediabetic NOD mice with impaired glucose tolerance. (A) IPGTT data for prediabetic NOD mice at various ages ($n \geq 5$ per group). Note that the fasting glucose (at $t = 0$) is similar at all time points. (B) Area under the curve of IPGTT data from A. $*P < 0.05$. (C) Demethylation index measured with DNA from the sera of prediabetic (wk 7–14) and diabetic NOD mice. $P = 0.0002$ by ANOVA; $**P < 0.01$; $*P < 0.05$; $n = 5, 5, 5, 7$, and 5 mice/group. The box-and-whisker plots show the minimum and maximum values. (D and E) In a separate experiment, pancreata and serum were harvested from mice at the indicated ages ($n = 5$ mice per time point) for

range, 4–11 mo) after diagnosis with healthy control subjects who were age-matched, because demethylation might have been affected by islet growth in children (Fig. 6E). The demethylation index was significantly higher in the patients with T1D ($P < 0.02$), and the average demethylation index in the nondiabetic subjects was similar to the index with DNA isolated from liver or kidney. We also conducted a similar analysis with second-step PCR primers that target bp +329. Analysis with this primer pair resulted in overall lower demethylation indices, but we found a similar significant increase in the demethylation index ($4.42 \times 10^{-4} \pm 2.07 \times 10^{-4}$ vs. $2.37 \times 10^{-6} \pm 1.81 \times 10^{-6}$) in this second cohort of subjects with recent-onset (i.e., first 1-1/2 y) T1D ($n = 12$) compared with healthy control subjects ($n = 11$; $P = 0.015$).

Discussion

We describe a unique method for the detection of β cell death in vivo in models of autoimmune and chemically induced diabetes in mice, in human tissues, and in serum from patients with T1D. This assay identifies methylated CpG dinucleotide in the insulin DNA that is derived exclusively from β cells. Our findings indicate that this method provides a biomarker for detecting β cell loss in prediabetic mice during progression of diabetes, and suggest it does the same in patients with new-onset T1D.

Kuroda et al. (26) previously identified demethylation of CpG sites in the insulin promoter, consistent with the notion that methylation of promoters is a mechanism for controlling tissue-specific gene expression. However, our analysis targeted differentially methylated CpG dinucleotides in the *Ins1* gene in mice and the *Ins* gene itself in humans. The conservation of demethylation of this sequence across species suggests that its methylation may play an active role in regulation of insulin gene transcription. In addition, via sequencing, we showed that CpG sites both upstream and downstream of the CpG at +177 are also equally demethylated in β cell DNA, indicating that the entire region contributes to gene regulation.

Our sequence analysis revealed that unlike human *Ins*, which was completely demethylated in primary β cells, murine *Ins1* was demethylated in 75% of the CpG sites studied from murine β cells isolated from MIP-GFP⁺ mice. Based on the available data, we are not sure whether this reflects allele-specific inactivation of insulin 1 in β cells that produce insulin 2 (and vice versa), possibly in response to metabolic stress, or whether there is some genomic imprinting and silencing of only one of the parental alleles (27). The MIP-GFP transgene may account for β cell stress, given that it has been known to result in β cell death in certain lines (28). A caveat of our approach is that the death of β cells in which insulin genes are methylated as a result of stress might go undetected.

We were able to detect acute β cell death *in vivo*, as indicated by the presence of β cell-derived demethylated DNA after STZ treatment. The fact that hyperglycemia was not observed at the 8 h time point demonstrates our method's ability to detect β cell death before frank hyperglycemia occurs. This conclusion is supported by our histomorphologic analysis of the percentage of nucleated cells in the islet, which revealed a drop in the percentage of DAPI-positive, insulin-positive cells, suggesting that DNA material can be released to the surrounding tissues and can be detected in the circulation. In the model systems that we have studied, toxin- and cytolysis-induced cell death were clearly detectable, but whether other forms of β cell death that might not result in release of nuclear contents into the circulation, such as apoptosis or autophagy, will be identified is unclear (29, 30).

In autoimmune diabetes, β cell death is considered a chronic process rather than an acute process such as occurs after STZ

measurement of insulin content and demethylation index, respectively. The insulin content and demethylation index in 11- and 15-wk-old mice were compared with 7-wk-old mice. $*P < 0.05$; $**P < 0.02$ by post hoc analysis of ANOVA. (E) Relationship between pancreatic insulin content and demethylation index in individual mice. Two measurements from each mouse are plotted ($r^2 = 0.28$; $P < 0.05$).

Fig. 6. Analysis of insulin DNA sequences in human tissues and sera. (A) Unmodified DNA sequence in human *Ins* gene showing preserved CpG pair at bp +273 and +399 identified in the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>). (B) Sequence data of the first-step PCR showing methylation DNA patterns in primary human kidney and whole islets. The arrow shows the presence of demethylated CpG found in human islets at bp +399 (at position 2182036, site of the reverse primer). Note the two peaks in human islets representing both demethylated and methylated forms from β cells and non- β cells in the islets. (C) Sequence analysis of product amplified in the first-step PCR from sorted human β cells and kidney. The sequence of 10 clones from human β cells and 12 clones from human kidney cells are shown (○ indicates demethylated cytosines; ●, methylated cytosines). The base pairs are indicated downstream from the transcription start site. The primers of the second-step PCR were specific for methylated/demethylated cytosine at bp +273 and +399. (D) DNA was isolated from human kidney, liver, and islets and analyzed by nested PCR. Synthetic DNA was also analyzed in these reactions. Each dot represents a separate isolation and analysis of tissue DNA. The demethylation index was significantly greater with DNA from islets compared with liver and kidney. *** $P < 0.001$. (E) DNA was isolated from five subjects with recent-onset T1D (●) and from six healthy control subjects (■). The demethylation index was significantly higher in patients with T1D ($P = 0.017$, Mann–Whitney U test).

administration, and changes with chronic destruction may be more difficult to detect (1, 2). Nonetheless, we were able to detect a significant increase in β cell-derived demethylated DNA in the circulation of prediabetic NOD mice even when fasting glucose levels and overall glucose tolerance were not significantly impaired compared with young NOD mice. Just before the onset of hyperglycemia, however, a dramatic increase in the level of demethylated insulin DNA occurred. Our analysis of pancreatic insulin content and β cell-derived demethylated DNA in the serum revealed a statistically significant inverse correlation. In addition to the variability that is intrinsic to these biological measurements, another possible explanation for the discrepant data are that the demethylation index may rise *before* a decline in insulin content is apparent. As shown by our analysis of prediabetic NOD mice, this was clearly the case, with the greatest increase in the demethylation index seen before hyperglycemia was identified.

We have previously shown that β cells may fail to express insulin (i.e., are degranulated) at the time of onset of diabetes in NOD mice but are still viable and may recover with immunotherapy, such as anti-CD3 mAb (24). Moreover, β cell function might be affected by ambient glucose level, certain drugs, and other factors (31). With the caveat that stressed β cells may methylate insulin DNA and die without release of demethylated insulin, we do not expect our measurements to be affected by changes in β cell function, in contrast to measurements of circulating insulin mRNA in the serum of recipients of islet allografts (24, 31, 32). Thus, our approach to analysis may be useful in distinguishing β cell death from impaired function. Further studies in other disease settings are needed, however.

Interestingly, our measure of β cell death demonstrated continued release of demethylated insulin DNA after the appearance of frank hyperglycemia, but at a reduced level compared with prediabetic (i.e., 14-wk-old) mice. The decline in β cell-derived DNA after the onset of hyperglycemia suggests that the relative abundance of demethylated insulin DNA in the circulation may be reduced because of a total loss of β cell mass. For example, a higher percentage of β cells may be destroyed after diagnosis with hyperglycemia than before diagnosis, but fewer β cells actually may be destroyed (24, 31, 32).

Our method developed in mice was also able to detect circulating β cell-derived DNA in humans. We found uniform demethylation of CpG sites within the insulin gene in human β cells and methylation in non- β cells. Our tissue analysis findings are consistent with

this finding from the sequence analysis. Importantly, the average demethylation index was significantly greater in subjects with new-onset T1D, in whom β cell death occurs, than in healthy control subjects. Not all patients with T1D had an increased demethylation index, likely reflecting the heterogeneity of the disease process in individuals. Additional studies correlating the demethylation index with β cell function as well as with samples from nondiabetic subjects at high risk for T1D will help determine whether a similar relationship occurs during the progression of human disease.

Overall, our data demonstrate the usefulness of our application of methylation-specific PCR for detecting β cell death *in vivo*. The assay may provide insight into the progression of T1D, survival of β cells after transplantation, or turnover of β cells during pregnancy or growth. Further studies are needed to evaluate and validate our method's general applicability in clinical settings such as after immune interventions or in prediabetes to identify individuals at risk for progressing to T1D. Such studies in these settings will be important in assessing the applicability of this method for human studies.

Materials and Methods

Mice. Female NOD/LtJ, MIP-GFP NOD, and BALB/c mice were obtained from The Jackson Laboratory and maintained under pathogen-free conditions. Seven-wk-old NOD mice were screened for hyperglycemia every 2 wk and were diagnosed with diabetes when two consecutive glucose levels >200 mg/dL were measured in whole blood from the tail vein using a Bayer Glucometer Elite XL. The animal care protocol was approved by Yale University's Animal Use Committee.

Human Subjects. Tissues were obtained from the pathology laboratory at Yale New Haven Hospital. Serum was collected from healthy control subjects and from individuals with recent-onset (i.e., within the first 1–12 y) T1D participating in a clinical trial (NCT 00378508). Institutional Review Board approval was obtained for the collection of tissues and sera, and informed consent was obtained from subjects for the collection of sera.

STZ Treatment. Eight-wk-old BALB/c mice received a single i.p. injection of 200 mg/kg of STZ. Blood glucose levels were measured at 8 h and 24 h after STZ treatment. At designated time points, mice were killed and serum and pancreas were collected for further analysis.

Insulin Content of Pancreas. Whole pancreas was snap-frozen in liquid nitrogen (33). Insulin was extracted with precooled (-20°C) acid-ethanol, and the insulin content was measured with a mouse insulin ELISA kit (Crystal Chem).

DNA Collection and Bisulfite Treatment. For isolation of purified β cells, islets were isolated from NOD/SCID mice, and single cell suspensions were prepared by collagenase digestion. The cells were stained intracellularly with guinea pig anti-insulin antibodies, followed by a secondary FITC-conjugated donkey anti-guinea pig antibody. The stained cells were then FACS-sorted to either insulin-positive or insulin-negative fractions. Other β cells were isolated from islets from NOD MIP-GFP mice, and insulin-positive cells were sorted on the basis of GFP fluorescence. Purified human β cells were isolated from dissociated islets that were permeabilized and stained with FluoZin-3-AM (25). The β cells were sorted by gating on the upper 16% of the stained cells.

DNA from tissue, cells, and serum samples was purified using the Qiagen QIAamp DNA Blood Kit following the manufacturer-recommended protocol. Synthetic unmethylated and methylated DNA was purchased from Millipore. Purified DNA was quantitated using a NanoDrop 2000 spectrophotometer. DNA was then subjected to bisulfite treatment and purified on a DNA binding column to remove excessive bisulfite reagent using the Zymo EZ DNA Methylation Kit.

First-Step PCR and Gel Extraction. A methylation-independent reaction was carried out to increase the DNA template for PCR analysis. The forward and reverse primers and melting temperatures for the murine and human insulin genes are listed in Tables S1 and S2. For the reaction, bisulfite-treated DNA template was added to Zymo Taq Premix. The PCR conditions for murine and human reactions are given in Tables S1 and S2. The PCR products were excised from a 3% agarose gel. Negative controls without DNA did not yield products in the first-step reaction. In certain experiments, the purified product was sequenced at Yale University's Keck Biotechnology Research Laboratory.

Cloning and Sequencing of Insulin DNA. PCR products obtained using methylation-independent primers (from sorted β cells, pancreatic islet cells, and control tissue, either kidney or liver) were purified using a Qiagen PCR Purification Kit and ligated via TOPO-TA cloning into the pCR2.1-TOPO vector (Invitrogen). For the mouse sequence, primers outside the region in the nested PCR reactions (Table S3) were used to increase the number of CG sites. Competent TOP-10 bacteria cells were transformed with the products of TOPO ligation and streaked onto agar plates (ampicillin-resistance). After overnight incubation at 37 °C, between 12 and 40 colonies from each ligation were picked with clean pipette tips and individually inoculated into 96-well plates. After culture, the

bacteria were lysed and used as template DNA for real-time PCR with SYBR Green with the methylation-independent primers. Productive ligations were identified based on Ct values and melting points. The PCR products were sequenced by the Keck Biotechnology Research Laboratory.

Nested Methylation-Dependent Real-Time PCR. Gel-purified PCR products were used as a template for a quantitative PCR with primers specific for demethylated and methylated insulin 1 DNA. The conditions for the reaction with SYBR Green (Qiagen) and primers are listed in Table S1. The reaction was performed on an iQ-5 multicolor real-time PCR system (Bio-Rad), and the Ct cycle was determined for reactions with the demethylated and methylated primer pairs (Fig. S1). The relative abundance of demethylated DNA was expressed using the following equation: demethylation index = $2^{(\text{methylated cycle number}) - (\text{demethylated cycle number})}$. In some experiments (Fig. 3 A and C), the ratio of the demethylation index between tissues is presented. The second-step reaction Ct values were between 15 and 40.

Immunofluorescence. Pancreas was resected and fixed for 24 h in 2% PFA, then placed in a sucrose gradient and snap-frozen in liquid nitrogen. Noncontiguous 14- μ m pancreatic sections were stained with antibodies to insulin (Invitrogen) and DAPI. The bound anti-insulin antibody was detected by immunofluorescent secondary antibodies (Jackson ImmunoResearch). The slides were analyzed by fluorescence microscopy using an Olympus BX-51 microscope. Image analysis and postprocessing were performed using ImageJ (<http://rsb.info.nih.gov/ij/>). Numbers of single- and dual-color-labeled cells were counted using functions in ImageJ (colocalization, watershed, and analyze particles) (34).

Statistical Analyses. Data are expressed as mean $\pm$ SEM. The differences between means and the effects of treatments were analyzed by one-way ANOVA with Tukey's post hoc test using Prism 5 (GraphPad software) to identify the significance ($P < 0.05$) for all pairs of combinations. Nonnormally distributed data were analyzed using nonparametric tests.

ACKNOWLEDGMENTS. We thank Alison Johnson for her technical assistance. This work was supported by Juvenile Diabetes Research Foundation International Grants 2008-1012, P30 DK45735, and UL1RR024139.

- Akirav E, Kushner JA, Herold KC (2008) β -cell mass and type 1 diabetes: Going, going, gone? *Diabetes* 57:2883–2888.
- Bluestone JA, Herold K, Eisenbarth G (2010) Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature* 464:1293–1300.
- Waldron-Lynch F, Herold KC (2009) Advances in type 1 diabetes therapeutics: Immunomodulation and β -cell salvage. *Endocrinol Metab Clin North Am* 38:303–317.
- Bouneres PF, et al. (1988) Factors associated with early remission of type 1 diabetes in children treated with cyclosporine. *N Engl J Med* 318:663–670.
- Keymeulen B, et al. (2005) Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 352:2598–2608.
- Ludvigsson J, Heding L (1982) Abnormal proinsulin/C-peptide ratio in juvenile diabetes. *Acta Diabetol Lat* 19:351–358.
- Snorgaard O, Lassen LH, Binder C (1992) Homogeneity in pattern of decline of β -cell function in IDDM: Prospective study of 204 consecutive cases followed for 7.4 yr. *Diabetes Care* 15:1009–1013.
- Greenbaum CJ, et al.; Type 1 Diabetes Trial Net Research Group; European C-Peptide Trial Study Group (2008) Mixed-meal tolerance test versus glucagon stimulation test for the assessment of β -cell function in therapeutic trials in type 1 diabetes. *Diabetes Care* 31:1966–1971.
- Steele C, et al. (2004) Insulin secretion in type 1 diabetes. *Diabetes* 53:426–433.
- Erlach H, et al.; Type 1 Diabetes Genetics Consortium (2008) HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: Analysis of the Type 1 Diabetes Genetics Consortium families. *Diabetes* 57:1084–1092.
- Hagopian WA, et al. (1995) Glutamate decarboxylase, insulin, and islet cell antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. *J Clin Invest* 95:1505–1511.
- Verge CF, et al. (1996) Prediction of type 1 diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 45:926–933.
- Sherr J, Sosenko J, Skyler JS, Herold KC (2008) Prevention of type 1 diabetes: The time has come. *Nat Clin Pract Endocrinol Metab* 4:334–343.
- Sosenko JM, et al.; Diabetes Prevention Trial Type 1 Study Group (2007) Increasing the accuracy of oral glucose tolerance testing and extending its application to individuals with normal glucose tolerance for the prediction of type 1 diabetes: The Diabetes Prevention Trial Type 1. *Diabetes Care* 30:38–42.
- Medarova Z, Tsai S, Evgenov N, Santamaria P, Moore A (2008) In vivo imaging of a diabetogenic CD8⁺ T cell response during type 1 diabetes progression. *Magn Reson Med* 59:712–720.
- Klose RJ, Bird AP (2006) Genomic DNA methylation: The mark and its mediators. *Trends Biochem Sci* 31:89–97.
- Bartke T, et al. (2010) Nucleosome-interacting proteins regulated by DNA and histone methylation. *Cell* 143:470–484.
- Wang GG, Allis CD, Chi P (2007) Chromatin remodeling and cancer, part II: ATP-dependent chromatin remodeling. *Trends Mol Med* 13:373–380.
- Miranda TB, Jones PA (2007) DNA methylation: The nuts and bolts of repression. *J Cell Physiol* 213:384–390.
- Grady WM, Rajput A, Lutterbaugh JD, Markowitz SD (2001) Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. *Cancer Res* 61:900–902.
- Wallner M, et al. (2006) Methylation of serum DNA is an independent prognostic marker in colorectal cancer. *Clin Cancer Res* 12:7347–7352.
- Müller HM, et al. (2003) DNA methylation in serum of breast cancer patients: An independent prognostic marker. *Cancer Res* 63:7641–7645.
- Poitout V, et al. (1995) Morphological and functional characterization of β TC-6 cells: An insulin-secreting cell line derived from transgenic mice. *Diabetes* 44:306–313.
- Sherry NA, et al. (2006) Effects of autoimmunity and immune therapy on β -cell turnover in type 1 diabetes. *Diabetes* 55:3238–3245.
- Jayaraman S (2011) Assessment of beta cell viability. *Curr Protoc Cytom* 55:6.27.1–6.27.16.
- Kuroda A, et al. (2009) Insulin gene expression is regulated by DNA methylation. *PLoS ONE* 4:e6953.
- Giddings SJ, King CD, Harman KW, Flood JF, Carnaghi LR (1994) Allele-specific inactivation of insulin 1 and 2 in the mouse yolk sac indicates imprinting. *Nat Genet* 6:310–313.
- Leiter EH, et al. (2007) Unexpected functional consequences of xenogeneic transgene expression in β -cells of NOD mice. *Diabetes Obes Metab* 9(Suppl 2):14–22.
- Trudeau JD, et al. (2000) Neonatal β -cell apoptosis: A trigger for autoimmune diabetes? *Diabetes* 49:1–7.
- Fujimoto K, et al. (2010) Loss of Nix in Pdx1-deficient mice prevents apoptotic and necrotic β cell death and diabetes. *J Clin Invest* 120:4031–4039.
- Basadonna G, Montorsi F, Kakizaki K, Merrell RC (1988) Cyclosporin A and islet function. *Am J Surg* 156:191–193.
- Berney T, et al. (2006) Detection of insulin mRNA in the peripheral blood after human islet transplantation predicts deterioration of metabolic control. *Am J Transplant* 6:1704–1711.
- Best CH, Haist RE, Ridout JH (1939) Diet and the insulin content of pancreas. *J Physiol* 97:107–119.
- Collins TJ (2007) ImageJ for microscopy. *Biotechniques* 43(Suppl 1):25–30.