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Elevations in Circulating Methylated and Unmethylated Preproinsulin DNA in New-Onset Type 1 Diabetes

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Elevated ratios of circulating unmethylated to methylated preproinsulin (*INS*) DNA have been suggested to reflect β-cell death in type 1 diabetes (T1D). We tested the hypothesis that absolute levels (rather than ratios) of unmethylated and methylated *INS* DNA differ between subjects with new-onset T1D and control subjects and assessed longitudinal changes in these parameters. We used droplet digital PCR to measure levels of unmethylated and methylated *INS* DNA in serum from subjects at T1D onset and at 8 weeks and 1 year post-onset. Compared with control subjects, levels of both unmethylated and methylated *INS* DNA were elevated at T1D onset. At 8 weeks post-onset, methylated *INS* DNA remained elevated, but unmethylated *INS* DNA fell. At 1 year post-onset, both unmethylated and methylated *INS* DNA returned to control levels. Subjects with obesity, type 2 diabetes, and autoimmune hepatitis exhibited lower levels of unmethylated and methylated *INS* compared with subjects with T1D at onset and no differences compared with control subjects. Our study shows that elevations in both unmethylated and methylated *INS* DNA occurs in new-onset T1D and that levels of these DNA species change during T1D evolution. Our work emphasizes the need to consider absolute levels of differentially methylated DNA species as potential biomarkers of disease.

The diagnosis of type 1 diabetes (T1D) is made at a time when individuals have lost substantial β-cell mass and function (1,2). Interventions instituted at T1D diagnosis have failed to result in recovery of β-cell function, raising the possibility that earlier detection of β-cell death might provide an opportunity for preventative interventions prior to T1D onset (3). Recently, several groups have proposed the measurement of circulating unmethylated DNA encoding preproinsulin (*INS*) as a biomarker of β-cell death (4–10), since β-cells have a much higher frequency of unmethylated CpG sites compared with other cell types (6,11,12) and might release this DNA species into the circulation upon death. In these studies, unmethylated *INS* DNA was expressed as a ratio relative to methylated *INS* DNA for normalization purposes. However, because β-cells and many other cell types in the islet contain some fraction of both unmethylated and methylated *INS* (6,12,13), it remains unclear to what extent each species of *INS* might be independently informative of the underlying disease process in T1D.

Droplet digital PCR (ddPCR) uses the analysis of discrete individual PCR reactions (~20,000/sample) to identify the presence of target DNA and uses Poisson statistics to extrapolate the copy number of target DNA per sample (14). This technology enables direct quantitation of differentially methylated DNA species in serum without the need for normalization. We used ddPCR to analyze serum from individuals with new-onset T1D to test the hypothesis that absolute levels (rather than ratios) of unmethylated and methylated *INS* differ between subjects with new-onset T1D and control subjects. We also assessed longitudinal

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changes in *INS* during T1D evolution and evaluated levels of these DNA species in cohorts with established type 2 diabetes (T2D) and autoimmune hepatitis to control for hyperglycemia and autoimmunity, respectively.

RESEARCH DESIGN AND METHODS

Human Subjects and Islets

Serum samples were obtained from pediatric subjects with T1D at disease onset (within 2 days of diagnosis) and then at 8 weeks and 1 year after onset at Riley Hospital for Children. Subjects were 5–15 years of age and did not present in a state of diabetic ketoacidosis. Banked serum from healthy pediatric subjects, lean and obese adults without diabetes, adults with T2D (duration of disease 7.1 ± 1.1 years), and adults with autoimmune hepatitis was obtained for comparisons. Protocols were approved by the Indiana University Institutional Review Board. Parents of subjects provided written informed consent, and children older than 7 years provided assent for their participation. Human islets were obtained from the Integrated Islet Distribution Program.

Animals

CD1, NOD, and NOD-SCID mice were maintained under protocols approved by the Institutional Animal Care and Use Committee. Mice were fed regular chow and water ad libitum. Some mice underwent transplant of 200 human islets under the renal capsule as previously described (15). Blood was collected via tail vein for PCR assays.

DNA Extraction and Bisulfite Treatment

DNA was extracted from human islets using the genomic DNA extraction kit (Sigma-Aldrich). DNA was extracted from ~20 μL mouse serum and 30–50 μL human serum using the ZR serum DNA kit (Zymo Research) or the QIAamp DNA blood mini kit (Qiagen) with 5 μg poly-A DNA as carrier. DNA recovery was ~85%, with <10–15% variance between samples. All samples then underwent bisulfite conversion using the EZ DNA Methylation kit or the EZ DNA Methylation-Lightning kit (Zymo Research), and conversion was verified using a pre- and postconversion sample in the ddPCR.

PCR Analysis

Samples were analyzed by ddPCR using a dual fluorescent probe-based multiplex assay. For human *INS* promoter amplification, the following primers were used: 5′-GGAA ATTGTAGTTTTAGTTTTTAGTTATTTGT-3′ (forward) and 5′-AAAACCCATCTCCCCTACCTATCA-3′ (reverse) in combination with probes that detected methylation or unmethylation at bp −69: 5′-ACCCCTACCGCCTAAC-3′ (VIC) and 5′-ACCCCTACCACCTAAC-3′ (FAM). We chose this site based on prior studies, which showed that position −69 in *INS* retained its unmethylated state in β-cells even under inflammatory stress of T2D (12). Primers and probes for mouse *Ins2* DNA were described previously (4). Amplified human *INS* PCR products were sequenced to confirm the PCR product identities. ddPCR was performed using ddPCR Supermix for Probes (Bio-Rad) with the following cycling conditions: 95°C for 10 min, 94°C for 30 s, and 57.5°C for 60 s for 40 cycles. Droplets were analyzed by a QX200 Droplet Reader and QuantaSoft Software (Bio-Rad), from which a concentration (copies/μL) of methylated and unmethylated *INS* DNA was obtained. This final concentration was extrapolated to copy per microliter of serum and then log-transformed for parametric statistical analysis.

Statistics

For direct comparisons of methylated and unmethylated *INS* DNA levels, two-tailed unpaired Student *t* tests were used. For comparisons of longitudinally collected samples, two-tailed paired Student *t* tests were used. *P* values <0.05 were considered significant. Statistical calculations were performed using Prism 5.0 Software (GraphPad).

RESULTS

We developed a methylation-specific PCR (MSP) assay to simultaneously quantitate methylation or unmethylation at the CpG at *INS* position −69 bp, shown in prior studies to be preferentially unmethylated in β-cells (6,11). Control plasmids containing bisulfite-converted methylated or unmethylated *INS* DNA were used to standardize the MSP assay in ddPCR. Figure 1*A* shows the gating strategy (in two-dimensional ddPCR plots) to distinguish methylated, unmethylated, and double-positive *INS*-containing droplets. For verification of linearity and ability to distinguish simultaneous mixtures of the DNA species, mixtures of plasmids at varying ratios were subjected to ddPCR as shown in the one-dimensional plots in Fig. 1*B* and *C*. These methylation-specific plasmids were used to construct linearity curves over the range of DNA copy numbers observed in serum (Fig. 1*D*).

To test whether our MSP assay detects dying human β-cells in vivo, we transplanted human islets into healthy immunocompetent CD1 mice and allowed the islets to undergo xenorejection. Unmethylated human *INS* peaked in the serum at 6 h posttransplantation, falling to undetectable levels by 48 h (Fig. 2*A*). By contrast, only a slight (insignificant) increase in methylated *INS* was detectable at 6 h posttransplantation (Fig. 2*B*). Neither unmethylated nor methylated human *INS* was measurable in nontransplanted mice, and neither unmethylated nor methylated mouse *Ins2* was altered in transplanted mice (Fig. 2*B*).

We tested our MSP assay in a mouse model of autoimmune β-cell destruction (NOD mice). Compared with NOD-SCID and CD1 controls, NOD mice exhibit elevated levels of both unmethylated and methylated mouse *Ins2* in the prediabetes phase, with levels falling at the time of diabetes (Fig. 2*C* and *D*). To correlate these findings with humans, we next assessed subjects with new-onset T1D. Serum was obtained from 32 pediatric subjects within 48 h of T1D diagnosis. Additionally, 24 of these subjects had serum collected 8 weeks post-onset

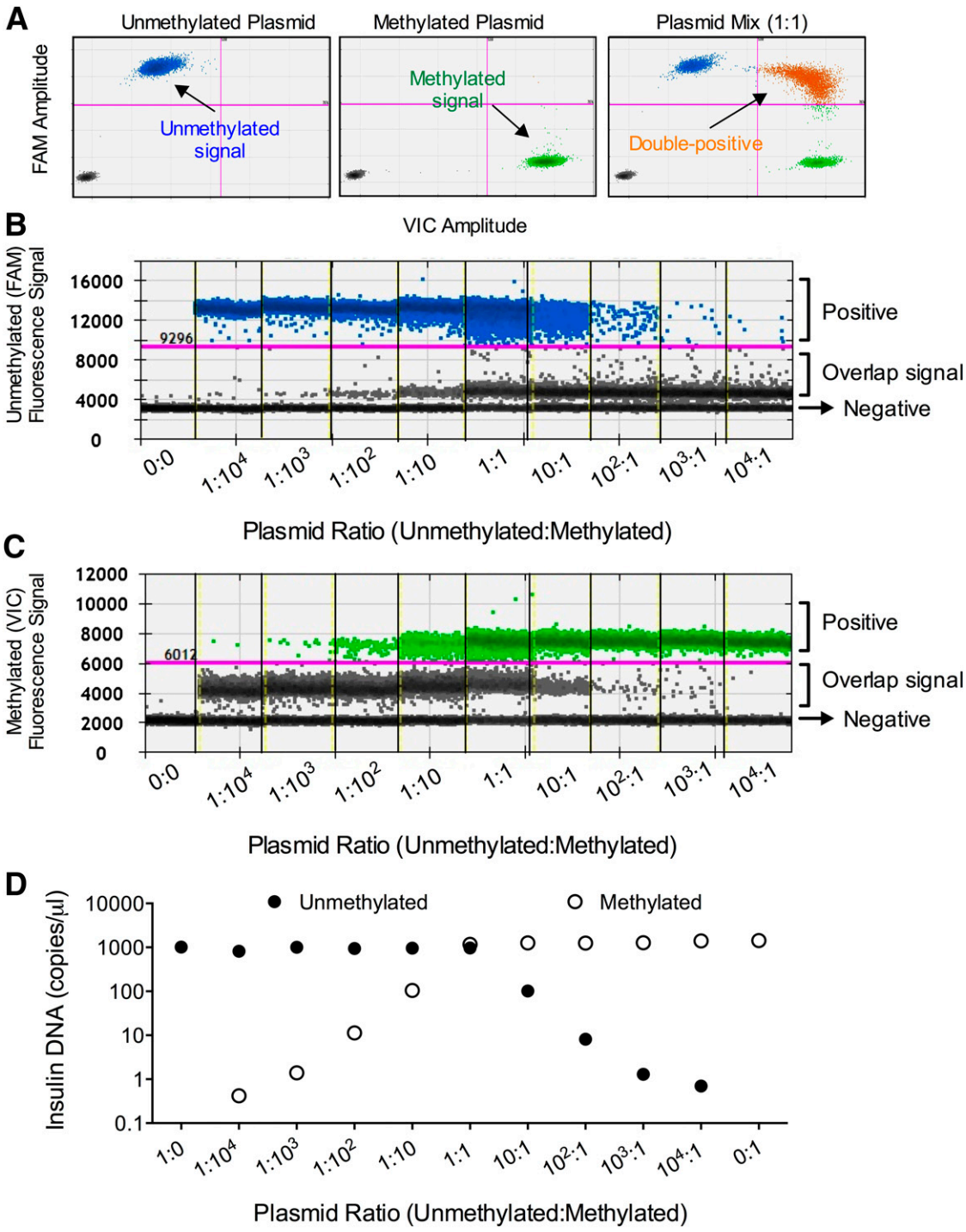

Figure 1—MSP assay specificity and validation. *A*: Two-dimensional plots using plasmid standards for unmethylated and methylated *INS* DNA and for a 1:1 mixture of the two plasmids. Arrows identify the unmethylated, methylated, and unmethylated + methylated (double-positive) *INS* DNA-containing droplets. *B* and *C*: Dilutions of plasmids containing bisulfite-converted unmethylated and methylated *INS* DNA were subjected to ddPCR; one-dimensional plots from ddPCR are shown for fluorescent probes specific for unmethylated *INS* DNA (*B*) and methylated *INS* DNA (*C*). In panels *B* and *C*, the positive, negative, and overlap (FAM probe overlapping into the VIC channel and vice versa) signals are identified. *D*: Quantitation of plasmid dilution curves, presented as copies/μL; $r^2 = 0.9818$ for unmethylated *INS* DNA and $r^2 = 0.9685$ for methylated *INS* DNA.

and 8 had serum collected 1 year post-onset. Relevant demographic and laboratory data of these subjects and control groups are presented in Table 1, and representative two-dimensional and one-dimensional plots are shown in Supplementary Fig. 1. As shown in Fig. 3*A* and *C*, levels of both unmethylated and methylated *INS* DNA were significantly higher in subjects at T1D onset compared with healthy control subjects ($P < 0.0001$), similar to NOD

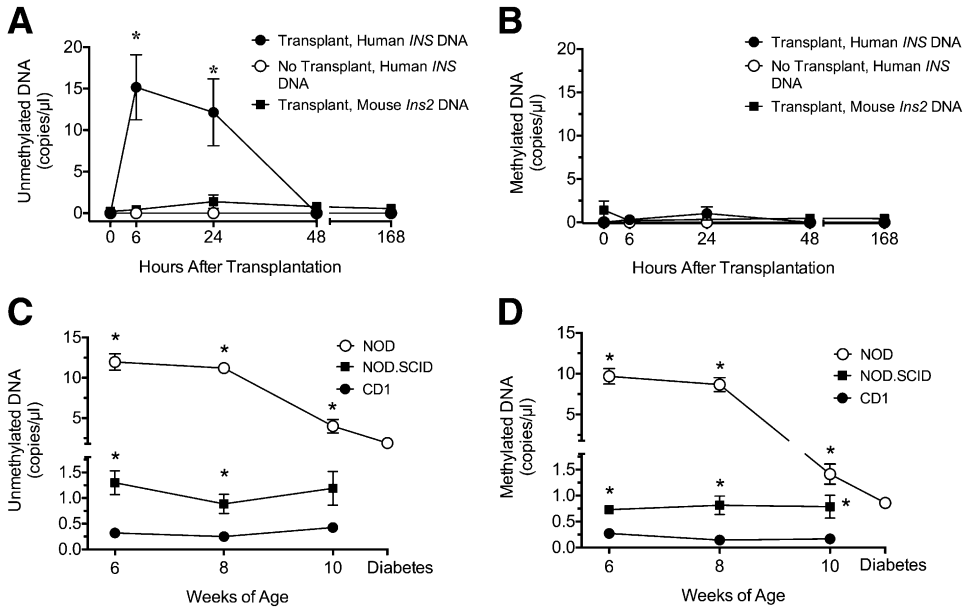

Figure 2—Circulating unmethylated and methylated human *INS* and mouse *Ins2* DNA levels in transplanted immunocompetent mice and NOD mice. *A* and *B*: CD1 mice ($n = 4$) were either transplanted (Transplant) or not (No Transplant) with 200 human islets beneath the kidney capsule. Serum was collected at the time points indicated and processed for MSP assay. Circulating unmethylated (*A*) and methylated (*B*) *INS* and *Ins2* DNA levels were measured by ddPCR. *C* and *D*: Serum from NOD, NOD-SCID, and CD1 mice ($n = 3$ per group) was collected at the ages indicated and at the age that NOD mice developed diabetes (12–14 weeks) (Diabetes) and processed for MSP assay. Circulating unmethylated (*C*) and methylated (*D*) *Ins2* DNA levels were measured by ddPCR. *$P < 0.05$ compared with time 0 in *A* and *B*, and *$P < 0.05$ compared with CD1 mice at the corresponding age in *C* and *D*.

mice. At 8 weeks after T1D onset, levels of unmethylated *INS* decreased significantly ($P < 0.0001$) and were no different than in control subjects (Fig. 3*A* and *B*). By contrast, levels of methylated *INS* DNA remained elevated 8 weeks after T1D onset compared with control subjects ($P < 0.0001$), falling below control levels 1 year post-onset ($P = 0.02$) (Fig. 3*C* and *D*). Unmethylated *INS* remained at the same levels 1 year post–T1D onset as at 8 weeks post-onset but was higher than in control subjects ($P < 0.0001$) (Fig. 3*A* and *B*).

We asked whether elevated levels of unmethylated and methylated *INS* observed in NOD mice and subjects with T1D reflects a generalized response to either autoimmunity or prevailing hyperglycemia. We performed MSP assays using serum from adults with active autoimmune hepatitis, T2D, and lean and obese healthy control subjects (Table 1). Unmethylated *INS* levels were not different between lean and obese adult subjects, and methylated *INS* DNA levels were slightly lower, but with significant overlap, in the obese control subjects ($P = 0.04$) (Fig. 3*A* and *C*). Methylated and unmethylated *INS* in both of these adult control groups was higher than in pediatric control subjects, suggesting that these circulating DNA species may exhibit age-related differences (Fig. 3*A* and *C*). Compared with both healthy adult control groups, unmethylated and methylated circulating *INS* levels were lower or no different in subjects with autoimmune hepatitis and T2D (Fig. 3*A* and *C*). Additionally, circulating unmethylated and methylated *INS* DNA were lower in subjects with autoimmune hepatitis and T2D than in subjects with T1D at onset (Fig. 3*A* and *C*). Collectively, these data suggest that elevations in unmethylated and

Table 1—Demographic and laboratory evaluation of subject cohorts

	Pediatric control	T1D at onset	T1D at 8 weeks	T1D at 1 year	Lean adult control	Obese adult control	Adult T2D	Adult autoimmune hepatitis
Age (years)	9.5 ± 3.6	10.8 ± 3.0	10.7 ± 3.1	11.6 ± 3.1	51.3 ± 9.0	49.3 ± 5.6	48.6 ± 7.5	47 ± 14
Female/male	14/13	14/18	11/13	1/7	12/3	11/10	5/12	12/2
BMI *z* score	−0.08 ± 0.7	0.07 ± 1.4	0.54 ± 0.9	0.16 ± 1.0				
BMI					22.9 ± 1.2	32.7 ± 3.5	35.9 ± 7.4	30.5 ± 7.7
HbA_{1c} (%)		11.3 ± 1.7	7.6 ± 0.8	8.3 ± 1.4			8.4 ± 1.6	
C-peptide (pmol/L)		150 ± 167	303 ± 166					

Data are presented as mean ± SEM.

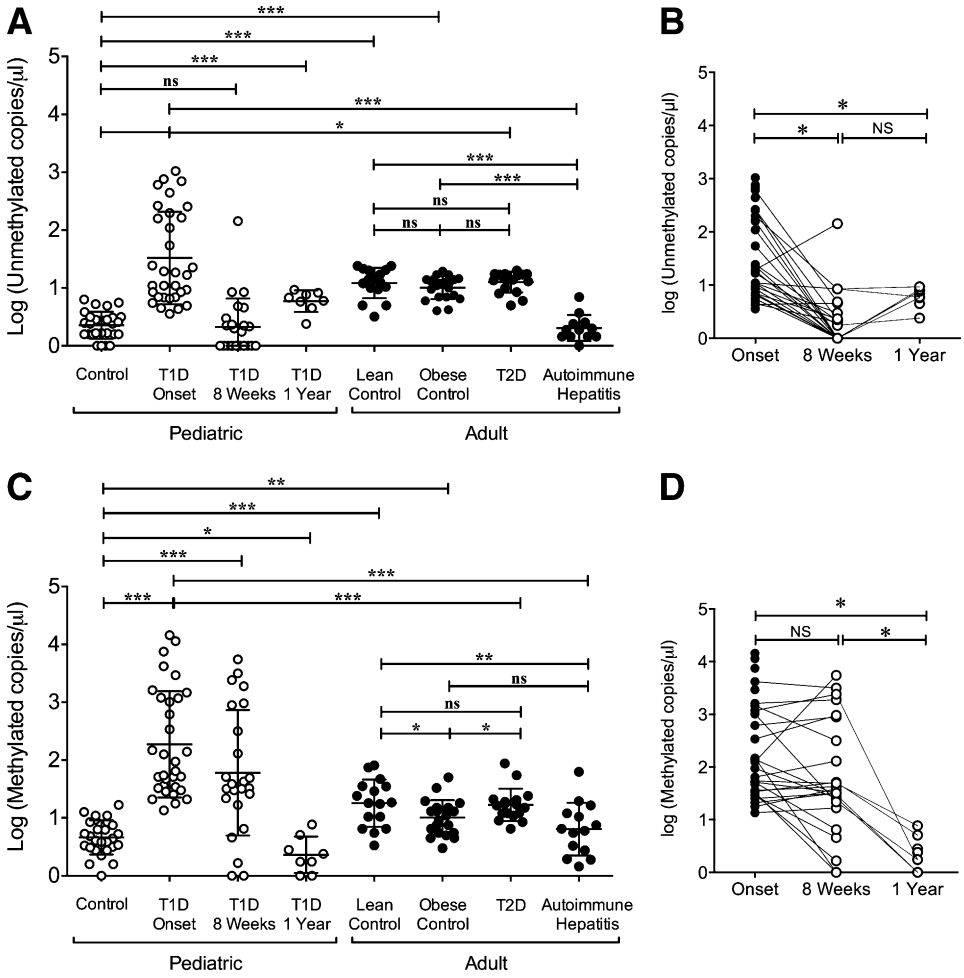

Figure 3—Circulating unmethylated and methylated *INS* DNA levels in human cohorts. *A*: Circulating unmethylated *INS* DNA levels in human cohorts depicted as Log(copies/μL). *B*: Longitudinal change in circulating unmethylated *INS* DNA levels in pediatric subjects with T1D at onset, 8 weeks after onset, and 1 year after onset. *C*: Circulating methylated *INS* DNA levels in human cohorts depicted as Log(copies/μL). *D*: Longitudinal change in circulating methylated *INS* DNA levels in pediatric subjects with T1D at onset, 8 weeks after onset, and 1 year after onset. $^{*}P < 0.05$, $^{***}P < 0.0001$, $^{**}P < 0.001$. ns, not significant ($P > 0.05$).

methylated *INS* DNA are not observed in T2D or autoimmune disease in general.

DISCUSSION

Elevations of unmethylated *INS* DNA have been shown to correlate with dying β-cells in both mice and humans (4–6,10). Whereas the ratio of unmethylated to methylated *INS* was used in prior studies, we used ddPCR to determine absolute copy numbers of both DNA species in several human cohorts. Our data reveal three important new findings *1*) unmethylated *INS* is increased at T1D onset and falls to control levels by 8 weeks post-onset, *2*) methylated *INS* is elevated at T1D onset and 8 weeks post-onset but falls by 1 year, and *3*) elevations in both methylated and unmethylated *INS* DNA appear to be specific for new-onset T1D, since concomitant elevation of both species is observed neither at any subsequent time point in T1D nor in other disorders of immunity or glycemia.

Whereas elevations in unmethylated *INS* are thought to arise primarily from islet β-cells, an unexpected finding in our study was the elevation of methylated *INS* in T1D, which could arise from any cell type (6). A previous study examined the correlation between cell-free plasma DNA (human β-globin DNA) and severity of illness (sepsis) in humans (16); the authors observed increasing levels of cell-free DNA with sepsis severity. In this regard, our cohort with new-onset T1D did not present with underlying infections or ketoacidosis that could have led to elevated methylated *INS*. Moreover, other concurrent stresses (hyperglycemia and autoimmunity) are unlikely to contribute, since individuals with T2D and autoimmune hepatitis did not present with elevations relative to control populations.

Several scenarios might account for the elevated unmethylated and methylated *INS* in both human and mouse new-onset T1D. First, it is possible that both species of DNA arise from different cells within the islet affected

by T1D immunopathogenesis (β-cells, α-cells, T cells, and macrophages). However, our results from the mouse transplant studies suggest the source of methylated *INS* DNA is extraislet, since xenorejection of transplanted human islets did not produce a significant increase in methylated *INS* DNA. Second, it is possible that the species arise not exclusively from islets but, rather, from some other cell type specific for T1D, such as thymic cells or T cells. In blood and spleen cells, up to 10–20% of clones exhibit unmethylated *INS* DNA (6), a finding that is consistent with the relative levels of circulating unmethylated and methylated *INS* in our studies. Finally, it is possible that the elevation in unmethylated *INS* DNA arises only from β-cells, while the elevation in methylated *INS* arises from other cell types related to T1D autoimmunity; this explanation seems most convincing, as xenorejection of human islets produced a significant increase in unmethylated *INS*.

Several differences between our study and those previously published should be emphasized. Ours is the first to study a population with new-onset T1D. Prior studies have primarily tested individuals with recent-onset T1D (within 4–18 months of onset) (5,8), in whom insulin administration might have impacted β-cell survival. Also, our assay interrogates methylation at a site different than those of prior studies. It is possible that different sites exhibit different methylation patterns as disease evolves (12), making comparisons between different site-specific assays difficult. Finally, ours is the first study to examine index cases of T1D longitudinally and to compare cases to other relevant populations to exclude confounding effects of concurrent pathophysiologic phenomena. Our study suggests that unique patterns of circulating methylated and unmethylated *INS* DNA may be specific for new-onset T1D and thereby emphasizes the need to consider absolute levels of differentially methylated DNA species as potential biomarkers of disease. Examination of circulating levels of these DNA species in populations with prediabetes are needed to determine their utility in predicting eventual disease onset.

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