Islet cells share promoter hypomethylation independently of expression, but exhibit cell-type–specific methylation in enhancers

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DNA methylation at promoters is an important determinant of gene expression. Earlier studies suggested that the insulin gene promoter is uniquely unmethylated in insulin-expressing pancreatic β-cells, providing a classic example of this paradigm. Here we show that islet cells expressing insulin, glucagon, or somatostatin share a lack of methylation at the promoters of the insulin and glucagon genes. This is achieved by rapid demethylation of the insulin and glucagon gene promoters during differentiation of Neurogenin3^{+} embryonic endocrine progenitors, regardless of the specific endocrine cell-type chosen. Similar methylation dynamics were observed in transgenic mice containing a human insulin promoter fragment, pointing to the responsible cis element. Whole-methylome comparison of human α- and β-cells revealed generality of the findings: genes active in one cell type and silent in the other tend to share demethylated promoters, while methylation differences between α- and β-cells are concentrated in enhancers. These findings suggest an epigenetic basis for the observed plastic identity of islet cell types, and have implications for β-cell reprogramming in diabetes and diagnosis of β-cell death using methylation patterns of circulating DNA.

DNA methylation | islets | β-cells | epigenetic | development

DNA methylation at CpG sites in gene promoters is a fundamental epigenetic mark, ensuring cell-type–specific gene-expression patterns. Following genome-wide demethylation before implantation of the embryo and global de novo methylation postimplantation, specific gene promoters are demethylated in cells that will express these genes (1). The stable inheritance of methylation patterns throughout life is tightly linked to the stability of cell identity, and poses a barrier to cell-type conversions (1). This arrangement has important ramifications for the field of regenerative medicine, as it implies that forced conversion of cells to a desired new identity must involve the reprogramming of their methylation patterns.

The methylation pattern of pancreatic β-cells provides a classic example of the relationship between promoter methylation and cell identity. β-Cells residing in the islets of Langerhans are the only source of insulin in the body, and their deficiency is a hallmark of diabetes. An early study by Gilbert and colleagues (2) has shown that the promoters of the two rat insulin genes are unmethylated in insulin-expressing cell lines, and are methylated in multiple nonexpressing tissues. More recently, methylated reporter constructs were used to demonstrate that promoter methylation can prevent insulin expression in β-cell lines (3). Thus, it is assumed that lack of methylation at the insulin gene promoter is a unique feature of β-cell identity, similar to the situation in other cell types expressing unique genes. In the context of cell therapy for diabetes, the reprogramming of different cell types to transplantable β-cells will likely require demethylation of the insulin gene promoter; alternatively, cell types in which the insulin promoter is unmethylated from the start (none known so far) might be attractive permissive candidates for reprogramming efforts.

Here we investigate the dynamics of promoter methylation in islet cell genes, using FACS-purified cells from mice and humans. Surprisingly, we find that the promoters of most α- and β-cell–specific genes are not hypomethylated only in the expressing cell type. Rather, these gene promoters undergo demethylation in both α- and β-cells, reflecting cell lineage and a potential for activation, but not actual expression. Finally, using global methylome analysis, we find that differential methylation in α- and β-cells is concentrated mostly in enhancer regions involved in the regulation of cell-type–specific gene expression. These findings may explain recent reports on spontaneous conversion of β-cells in diabetes to other islet cell types (but not to other lineages) (4), and on reprogramming of α- and δ-cells (but not other endoderm-derived cells) to β-cells (5, 6).

Results

Methylation of Insulin and Glucagon Gene Promoters. To study methylation patterns in a pure population of primary islet cells, we sorted cells from dissociated mouse and human islets following intracellular immunostaining for specific hormones (Fig. S1). We extracted genomic DNA from sorted cells and examined the methylation status of CpG sites at the promoters of hormone genes by bisulfite sequencing (7) and by pyrosequencing (8). As expected, the ins2 gene promoter was unmethylated in mouse β-cells as well as in the mouse β-cell line Min6, and was almost fully methylated in

Significance

We have studied the dynamics of DNA methylation in pancreatic α- and β-cells and reached surprising insights into the establishment of islet cell identity. Different islet cell types share lack of methylation in cell-type–specific gene promoters, while DNA methylation differences between islet cell types are concentrated in enhancer regions. The findings support the fundamental role of enhancer methylation in determining cell identity, and have implications for the understanding of islet cell plasticity in diabetes.

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DNA from mouse exocrine pancreas and tail fibroblasts (Fig. 1 *A* and *B*). A similar pattern was observed in human cells: the insulin gene promoter was unmethylated in β-cells, and was heavily methylated in DNA from pancreatic ducts, pancreatic exocrine tissue, and leukocytes (Fig. 1 *C*–*E*). Surprisingly, the insulin gene promoter was unmethylated in sorted glucagon$^+$ and somatostatin$^+$ cells from human islets (Fig. 1 *C*–*E*). Mouse α-cells and the mouse α-cell line αTC2 showed a low level of methylation at the insulin promoter, consistent with the results in human α-cells (Fig. 1 *A* and *B*). Thus, the insulin gene promoter is unexpectedly unmethylated in both α- and β-cells of mice and humans.

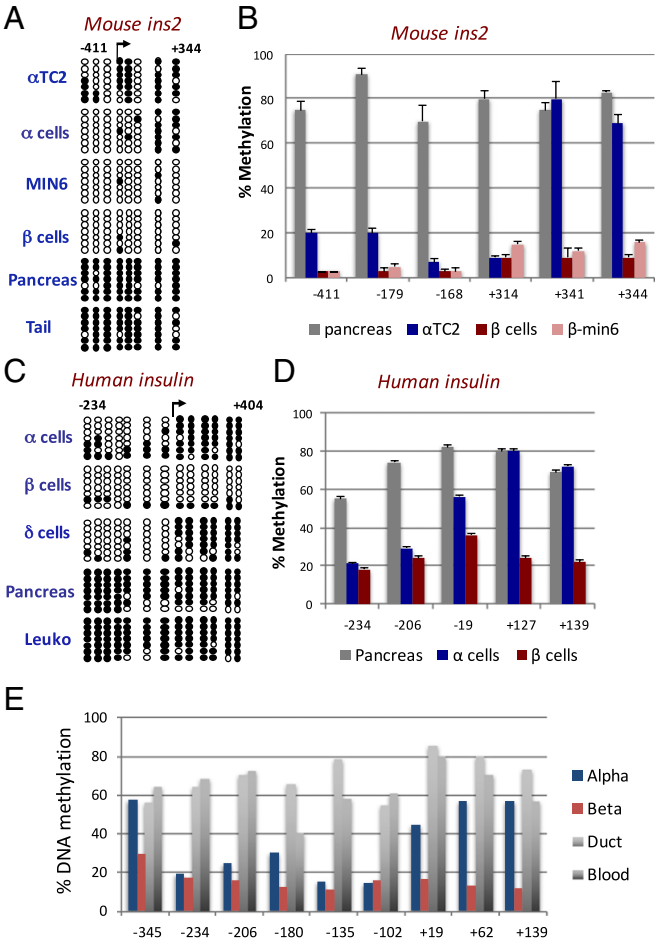

Fig. 1. DNA methylation at the insulin gene promoter. (*A*) Bisulfite sequencing of the mouse ins2 gene promoter, containing eight CpGs from positions −411 to +344 relative to the transcription start site (arrow). αTC2, mouse α-cell line; α-cells, sorted glucagon$^+$ cells from adult mice; MIN6, mouse β-cell line; β-cells, sorted insulin$^+$ cells from adult mice; pancreas, largely exocrine pancreas; tail, fibroblasts from a mouse tail biopsy. Each horizontal line represents a single DNA molecule. Empty circles, unmethylated CpGs; filled circles, methylated CpGs. (*B*) DNA methylation status of individual CpG sites in the mouse ins2 gene promoter, quantified using pyrosequencing on DNA extracted from the indicated sources. Bars represent average and SE of three independent experiments. Mouse α-cells differed significantly ($P < 0.005$) from total pancreas in their methylation level at the −411, −179, −168, and +314 sites, but not in the +341 and +344 sites. (*C*) Bisulfite sequencing of the human insulin gene promoter, containing 13 CpG dinucleotides from positions −234 to +404 relative to the transcription start site (arrow). α-, β-, and δ-cells are glucagon$^+$, insulin$^+$, and somatostatin$^+$ cells from human islets, sorted after intracellular staining for hormones. Leuko, leukocytes. (*D*) DNA methylation status of individual CpG sites in the human insulin promoter using pyrosequencing. Bars represent the average and SE from three independent experiments. Human α-cells differed significantly ($P < 0.005$) from total human pancreas in their methylation level at the −234, −206 and −19 sites, but not in the +127 and +139 sites. (*E*) DNA methylation status of individual CpG sites in the human insulin promoter using illumina 450k methylation array. Values represent the average methylation level per site from two to three arrays.

To examine whether the similarity in methylation among different islet cell types extends beyond the insulin promoter, we determined the status of methylation at the glucagon gene promoter. Consistent with observations at the insulin promoter, the glucagon promoter was unmethylated not only in the mouse α-cell line, but also in primary mouse α- and β-cells, while showing complete methylation in the murine exocrine pancreas and in tail fibroblasts (Fig. 2 *A* and *B*). Human cells showed a similar pattern: the glucagon gene promoter was unmethylated in α-cells, as well as in β- and δ-cells; the exocrine pancreas and leukocytes were heavily methylated (Fig. 2 *C* and *D*).

Interestingly, CpG sites downstream to the transcription start sites of the glucagon and insulin gene promoters showed a methylation pattern that did reflect expression: β-cells lacked methylation at these sites in the insulin promoter, while insulin$^-$ islet cells were methylated (Fig. 1). Similarly α-cells lacked methylation at the sites downstream to the transcription start site of glucagon promoter, while glucagon$^-$ islet cells were fully methylated (Fig. 2 *A*, *C*, and *D*). Thus, pancreatic islet cells share lack of methylation upstream to the transcription start site of insulin and glucagon gene promoters, regardless of actual expression. However, the methylation of CpG sites downstream to the transcription start site of glucagon and insulin genes does correlate with expression. These patterns contrast with the complete methylation of hormone gene promoters in nonislet tissues.

Dynamics of Methylation During Development. If hormone gene promoters are unmethylated in all islet cell types and not only in cells that express a particular hormone, it is likely that the underlying mechanism operates at the level of the pancreatic endocrine lineage, which is established during late embryogenesis. To understand the dynamics of methylation at hormone gene promoters during development, we analyzed DNA methylation in multiple developmental stages. As shown in Fig. 3 and Fig. S3, the insulin and glucagon promoters were heavily methylated in murine embryonic stem cells (ES cells), in endoderm derived from ES cells, and in Pdx1$^+$ progenitor cells isolated from the pancreas of embryonic day 12.5 (E12.5) mice. Neurogenin3$^+$ (NeuroG3$^+$, NGN3$^+$) endocrine progenitor cells in E16.5 embryos were also fully methylated at the insulin and glucagon promoters. Strikingly, the immediate progeny of Neurog3$^+$ cells, namely insulin$^+$ and glucagon$^+$ cells at E16.5 and in newborn mice (NB, postnatal day 1), showed a complete lack of methylation at the insulin and glucagon gene promoters upstream to the transcription start site (Fig. 3 *A*–*C*). Thus, hormone gene promoters undergo rapid demethylation as progenitor cells shut off Neurog3 expression and turn on hormone gene expression, regardless of the specific hormone selected for expression. Endocrine differentiation of Neurog3$^+$ cells occurs with minimal cell division (9, 10), suggesting an active demethylation mechanism. In support of a rapid process of demethylation, immunostaining showed a dichotomy between Neurog3$^+$ cells and hormone-expressing cells in the embryonic pancreas, with no evidence of intermediate cell types coexpressing Neurog3 and hormones (Fig. 3*D*).

Interestingly, methylation patterns in the upstream regions of hormone gene promoters became more correlated to expression with age (Fig. 3*C*). β-Cells maintained a very low level of methylation at the insulin promoter (~5% of CpG sites methylated), but the glucagon promoter in β-cells gradually gained methylation (from 5% at E16.5 to ~20% of the sites in adult β-cells). Similarly, adult α-cells had low methylation at the glucagon promoter (5% of site methylated), but their insulin promoter became more methylated with age (from 5% at E16.5 to ~25% in adult α-cells). Nonetheless, methylation of nonexpressed hormone gene promoters remained always low compared with nonendocrine cells (Fig. 3 *A*–*C*).

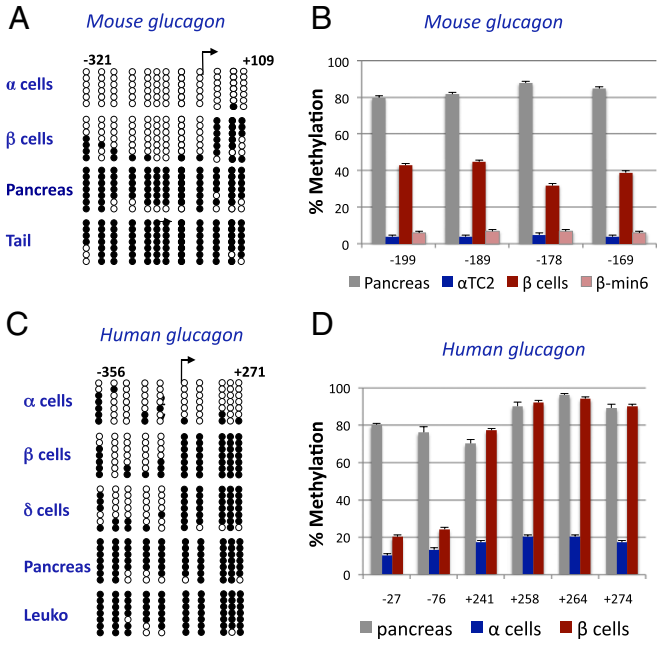

Fig. 2. DNA methylation at the glucagon gene promoter. (*A*) Bisulfite sequencing of nine CpG dinucleotides in the mouse glucagon promoter, from positions −320 to +109. (*B*) Methylation status of individual CpG sites in the mouse glucagon promoter, quantified using pyrosequencing. Bars represent the average and SE from three independent experiments. Mouse β-cells differed significantly ($P < 0.005$) from total pancreas in their methylation level at all sites examined. (*C*) Bisulfite sequencing of CpG sites in the human glucagon promoter, from positions −356 to +271. (*D*) Methylation status of individual CpG sites in the human glucagon promoter, quantified using pyrosequencing. Bars represent the average and SE from three independent experiments. Human β-cells differed significantly ($P < 0.005$) from total pancreas in their methylation level at the −27 and −76 sites.

In summary, hormone gene promoters are promptly demethylated in progeny of Neurog3$^+$ endocrine progenitor cells, via a mechanism that is likely active, and does not depend on expression from the particular promoter (model in Fig. 3*E*). With time, the upstream promoter region of unexpressed hormones accumulate low levels of methylation. In contrast, methylation downstream of transcription start sites does reflect expression.

Human Insulin Promoter Demethylation in Transgenic Mice. To define the *cis* elements responsible for the pan-islet demethylation of hormone gene promoters, we generated transgenic mice in which a short fragment of the human insulin gene promoter (−366 to +42) drives EGFP expression (Fig. 4*A*). As expected, EGFP was expressed specifically in β-cells of transgenic mice (Fig. 4*B*). Strikingly, sorting experiments revealed that the human promoter fragment was unmethylated in both β- and α-cells, despite undetectable expression in the latter cells. The transgene was completely methylated in nonendocrine cells found in islets and in tail tissue (Fig. 4*C*). These results are consistent with our observations that the endogenous mouse and human insulin promoters are unmethylated in both α- and β-cells. Importantly, they point to a short sequence at the insulin gene promoter (11), as the responsible *cis* regulatory element mediating lineage-specific, expression-independent demethylation. Despite the unmethylated state of the transgene in α-cells, no EGFP was observed in this cell type, suggesting that cell-type–specific transcription factors are likely responsible for the differential expression (12).

Global Comparison of DNA Methylation Profiles in Human α- and β-Cells. To assess the relationship between DNA methylation and gene expression in α- and β-cells on a global level, we performed a whole methylome analysis of isolated pancreatic cell types. We used immunostaining and FACS to isolate β-cells (n = 3 donors), α-cells (n = 2 donors), duct cells (n = 1), acinar cells (n = 1), and leukocytes (n = 2), and extracted genomic DNA. We then obtained the methylomes of these samples using the Illumina Infinium HumanMethylation450 BeadChip array, which reports on the methylation levels of over 450,000 CpG sites in the genome. Hierarchical clustering analysis showed that α-cells and β-cells cluster together (Fig. 5*A*), implying that they have similar global DNA methylation profiles. However, the β-cell samples and the α-cell samples can be separated into two independent clusters, indicating differences in the DNA methylation between the two cell types. As two of the β-cell samples (β1 and β2) came from the same donors as the two α-cell samples (α1 and α2, respectively), it appears that cellular identity

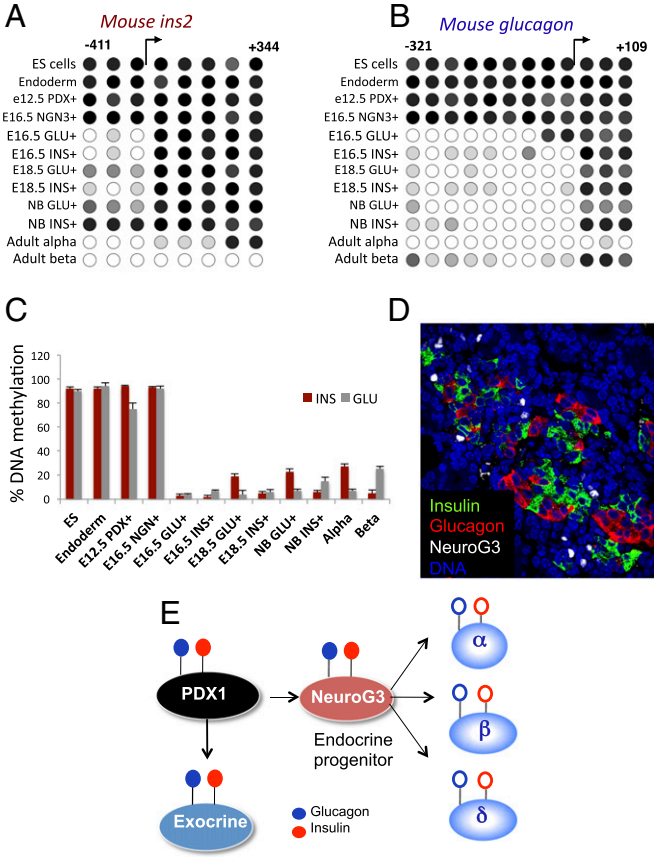

Fig. 3. Methylation dynamics of insulin and glucagon gene promoters during mouse development. (*A*) DNA methylation status of CpG sites in the mouse insulin gene promoter determined by bisulfite sequencing. ES cells, undifferentiated mouse ES cells; endoderm, mouse ES cells directed to differentiate to endoderm as previously described (33); other samples describe embryonic date of material and marker according to which cells were sorted (*Materials and Methods*). Shades of circles represent methylation level (0% methylated white, 100% methylated black). (*B*) DNA methylation of the mouse glucagon gene promoter, using the same samples as in *A*. (*C*) Quantification of DNA methylation in CpG sites upstream to the transcription start site of the insulin and glucagon promoters during mouse development. (*D*) Staining for insulin (green), glucagon (red), NeuroG3 (white), and DNA in (DAPI, blue) in E16.5 mouse pancreas. (Magnification: 40×.) There is no overlap between NeuroG3 and hormone expression, indicating a rapid switch from progenitor cells to hormone-producing cells. (*E*) Model for lineage-specific methylation dynamics of hormone gene promoters. The immediate progeny of Neurog3$^+$ endocrine progenitor cells undergo active demethylation at the promoters of hormone genes. Demethylation of hormone gene promoters is not associated with expression, but with a potential for expression in the particular tissue. Filled and empty lollipops represent methylated and unmethylated promoters, respectively.

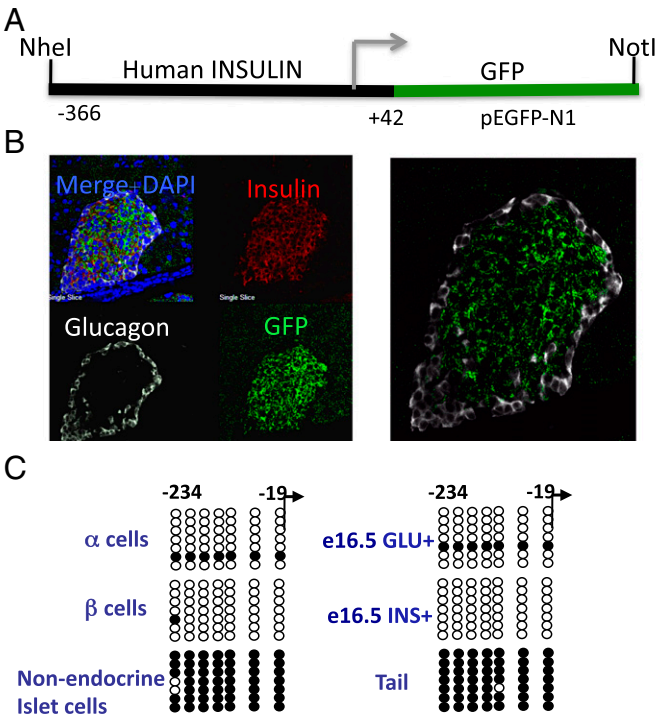

Fig. 4. DNA methylation in transgenic mice carrying a human insulin promoter fragment. (*A*) Schematic of the transgene. Arrow represents transcription start site of the human insulin gene. (*B*) Immunofluorescence analysis of insulin, glucagon, and GFP expression in pancreatic sections from transgenic mice. (Magnification: 40×.) GFP is expressed exclusively in β-cells. (*C*) Methylation status of the human insulin promoter in transgenic mice. Dissociated islet cells were sorted using intracellular hormone immunostaining into α-cells, β-cells, and hormone-negative cells. In addition, tail fibroblasts were used. Similar results were obtained from a second, independent founder transgenic line.

contributes more to the global methylation profiles than genetic identity.

We initially examined the methylation status of CpG sites in the promoter regions of 1,447 genes (10,877 CpGs) that are expressed in β-cells and silent in α-cells (13) (Fig. 5*B*). Most of these promoters were not differentially methylated between β-cells and the exocrine pancreas. Fig. 5*B* shows the 40 gene promoters (73 CpGs) that were methylated in exocrine pancreas and hypomethylated in β-cells. Of these, the majority (31 gene promoters containing 61 CpGs) were also hypomethylated in α-cells, while only nine promoters (containing 12 CpGs) were methylated in α-cells (that is, were uniquely hypomethylated in β-cells). In other words, genes expressed only in β-cells that are differentially methylated in β-cells and the exocrine pancreas are usually unmethylated in α-cells, similarly to the insulin gene promoter. Fig. S2 shows validation of the methylation status of the β-cell–specific gene SLC2A2 (Glut2), one of the few genes whose promoter methylation does reflect its expression in β-cells (and liver) and not in α-cells or the exocrine pancreas.

We carried out a similar analysis of the promoter regions of 1,184 genes (8,608 CpGs) expressed in α-cells but not in β-cells (Fig. 5*C*). Of the 37 gene promoters that were unmethylated in α-cells and methylated in the exocrine pancreas, 31 promoters (containing 51 CpGs) were hypomethylated in both β-cells and α-cells, while only 6 gene promoters (containing 10 CpGs) were uniquely hypomethylated in α-cells. Thus, most promoter regions of cell-type–specific genes are unmethyalted in α- and β-cells regardless of their expression profile, similarly to our findings with the insulin and glucagon gene promoters.

We then performed a global methylation analysis to identify the sites that are hypomethylated in α-cells, β-cells, or in both cell types relative to the exocrine pancreas, regardless of association with genes and expression patterns (Fig. 6*A*). Among over 450,000 sites analyzed from all autosomal chromosomes, we found 745 sites uniquely hypomethylated in β-cells, 353 sites uniquely hypomethylated in α-cells, and 3,753 sites commonly hypomethylated in both cell types. Thus, similar to the situation in promoters of cell-type–specific genes, islet-specific hypomethylation is more common than islet cell-type–specific hypomethylation.

We investigated the nature of the genomic regions that contain differentially methylated CpG sites in α- and β-cells. The majority of differentially methylated regions (DMRs, 75%) were located in gene bodies or in intergenic regions, while only 50% of the sites analyzed in the array are located in gene bodies or intergenic regions (Fig. 6*B* and Dataset S1). Since in mammals enhancers are distributed in both gene bodies and intergenic regions (14), we propose that the DMRs of α- and β-cells are located in distal regulatory regions rather than in promoter regions.

Since active enhancers are specifically labeled with histone H3K4me1 and H3K27Ac, while poised enhancers are labeled with H3K4me1 (14), we compared methylation patterns to the published distribution of these chromatin marks in human pancreatic islets (15). The α- and β-DMRs were highly enriched in histone H3K4me1 and H3K27Ac ($P < 3.00e-08$ and 8.89e-30, respectively) (Dataset S1), supporting the idea that an important part of islet cell-type identity is based on differential methylation in enhancer elements rather than in promoters (Fig. 6*B* and Dataset S1).

To further examine the correlation between methylation and enhancer activity in β-cells, we analyzed DNA methylation and H3K27ac levels at enhancer regions, which are marked with H3K4me1. We found that DNA methylation in β-cells and H3K27ac in pancreatic islets are negatively correlated ($P < 2.2e-16$) (Fig. 6 *C* and *D* and Fig. S4), suggesting that hypomethylation of enhancer regions is related to their activity.

Furthermore, we found that differential methylation of enhancers is associated with differential gene expression in α- and β-cells: we examined the methylation of CpG sites within enhancers

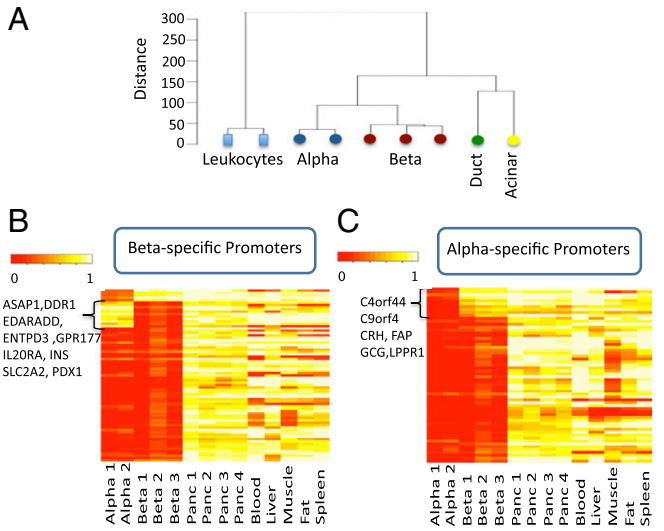

Fig. 5. Global analysis of DNA methylation in promoters of cell-type–specific genes. (*A*) Dendrogram showing hierarchical clustering (Ward's method) of α-cells, β-cells, pancreatic duct and acinar cells, and leukocytes. The *y* axis shows Euclidian distance between samples. (*B*) Methylation heatmap of promoters expressed in β-cells and silent in α-cells. Shown are only the promoters that are hypomethylated in α- or β-cells compared with whole pancreas. Methylation of these sites is also shown for blood, liver, muscle, fat, and spleen. INS refers to one CpG site (different from the cluster of sites in the promoter discussed earlier). (*C*) Heatmap of CpG sites hypomethylated in α-cells, β-cells, or in both cell types compared with whole pancreas, in the promoters of genes expressed in α-cells and silent in β-cells. Methylation of these sites is also shown for blood, liver, muscle, fat, and spleen. GCG refers to one CpG site (different from the cluster of sites in the promoter discussed earlier).

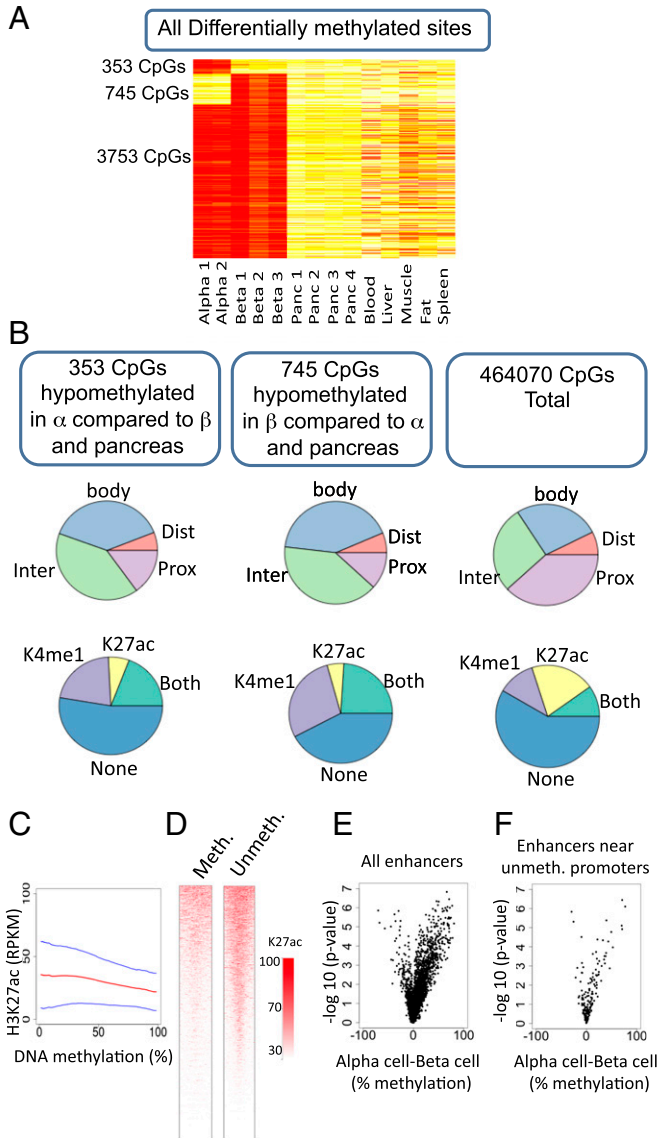

Fig. 6. Characterization of CpG sites differentially methylated in human α- and β-cells. (*A*) Heatmap of CpG sites hypomethylated in α-cells, β-cells, or in both cell types compared with whole pancreas. (*B*) Genome-wide distribution of CpG sites that are differentially methylated in α- and β-cells compared with the exocrine pancreas. (*Upper*) Association of differentially methylated sites with distal and proximal promoters, gene bodies, and intergenic regions. (*Lower*) Association of differentially methylated sites with enhancer regions in α- and β-cells, as defined by the presence of histone H3K4me1, H3K27Ac, or both marks. Body, gene body; Dist, distal promoter; Inter, intergenic region; Prox, proximal promoter. (*C*) Negative correlation of DNA methylation and enhancer activity. DNA methylation of β-cells and H3K27ac levels of islets were analyzed at enhancer regions (regions marked with H3K4me1 in islets). Locally smoothed means (red) and SDs (blue) of DNA methylation and H3K27ac at enhancer regions are plotted. DNA methylation and H3K27ac are negatively correlated ($R = -0.2300397$, $P < 2.2e\text{-}16$). (*D*) Levels of H3K27ac (reads per kilobase and million mapped reads) plotted in 10-bp blocks 3,000-bp downstream and upstream of H3K4me1 peaks either methylated (met, 4,527 peaks, >80% DNA methylation) or unmethylated (unmet, 8,022 peaks, <20% DNA methylation) in β-cells. Regions surrounding unmethylated peaks appear to be highly marked with H3K27ac. (*E*) A volcano plot showing differential methylation of α and β-cells at enhancers whose nearest gene is expressed specifically in β-cells. The 396 sites related to 192 different genes are hypomethylated specifically in β-cells [false-discovery rate (FDR) < 0. 05]. In contrast, only 46 sites are specifically hypomethylated in α-cells ($P < 2.2e\text{-}26$, binomial test). (*F*) Methylation of enhancers near genes with hypomethylated promoters in both α- and β-cells is associated with differential expression. A volcano plot showing differential methylation in α- and β-cells at enhancers whose nearest gene is expressed specifically in β-cells and is hypomethylated in both α- and β-cells. Among 29 candidate genes, 10 have enhancers with CpGs hypomethylated in β-cells (23 different CpG sites), while only 3 genes have enhancers with CpGs hypomethylated in α-cells (6 CpG sites) ($P = 0.001887$, binomial test).

whose nearest gene is expressed specifically in β-cells, and found that many CpGs are in these regions are uniquely hypomethylated in β-cells ($P < 2.2e\text{-}26$) (Fig. 6*E*).

We also found differentially methylated enhancers near genes that are expressed specifically in β-cells and show promoter hypomethylation in both α- and β-cells relative to the exocrine pancreas (Fig. 6*F*). This indicates that cell-type–specific gene expression relies on differentially methylated enhancers rather than on differential methylation in promoters.

Discussion

We show here that α- and β-cells in the islets of Langerhans share a similar DNA methylation pattern in gene promoters, which extends beyond the genes that these cells commonly express. Genes uniquely expressed in just one of these cell types—insulin and glucagon being prime examples—tend to have their promoters hypomethylated in both cell types, while methylated in other tissues. Thus, promoter hypomethylation in α- and β-cells is associated with the islet lineage, not with actual gene expression as classically described (1). We note that lineage-specific, expression-independent methylation patterns have been described in other organ systems; for example, certain promoters are unmethylated in both T and B cells, despite being expressed in only one of these sublineages (16).

The differences between α- and β-cell methylation are found more in enhancers, consistent with evidence from other organ systems. Indeed, analysis of tissue-specific DMRs showed that they are predominantly regulatory elements located intergenically, that their DNA methylation state is variable among different cell types (17), and that they are enriched for the histone modifications H3K4me1 and H3K27ac (18).

Furthermore, the observation that differential methylation of enhancers is associated with differential expression of pancreatic α- and β-cells supports the idea that an important part of islet cell-type identity is based on differential methylation in enhancer elements, rather than in promoters.

The molecular mechanisms responsible for lineage-specific promoter demethylation remain to be defined, but certain important properties are already clear. First, since NeuroG3$^+$ cells are only minimally dividing (19) and since newly formed hormone-expressing cells already lack methylation (Fig. 3), demethylation in this case is likely an active and rapid process. Second, the possible recruitment of demethylase activity to hormone gene promoters is unlikely to rely on cell-type–specific transcription factors, since the same promoters undergo demethylation in both α- and β-cells. We therefore predict that demethylation in the pancreas is mediated by sequence-specific factors which are direct targets of NeuroG3, and are present at least transiently in all progeny of endocrine progenitors, for example Nkx2.2 or NeuroD1. Our identification of a 400-bp fragment of the human insulin promoter that is sufficient to drive demethylation in both β-cells and α-cells will aid in the search for the responsible factors.

What is the physiological significance of hormone gene promoter demethylation? Lack of methylation at the insulin gene promoter is important for insulin expression in β-cells (3). However, β-cells will normally never express glucagon nor will α-cells express insulin. It appears therefore that promoter demethylation of islet genes is part of the generic specification program of the islet lineage. While the status of promoter methylation does not dictate gene expression, we propose that it does define a potential for gene expression. Such a situation may explain in part recent evidence on the plasticity of islet cell-type

identity. β-Cell reprogramming to alternative islet cell fates was suggested to account for β-cell failure in type 2 diabetes (4, 20–22). In the opposite direction, α- and δ-cells spontaneously reprogram to β-cells after near-total ablation of β-cells in mice (5, 6). Importantly, and unexplained so far, plasticity in both scenarios is confined to islet cells (23). We thus propose that hardwired promoter methylation status plays a part in restricting plasticity to the islet lineage.

Moreover, the common status of promoter methylation in islet cells may have implications for attempts to reprogram other cell types to β-cells, which have proven challenging so far. Our results suggest that extraislet cells, such as hepatocytes, neurons, and exocrine pancreatic cells have a higher barrier for reprogramming into β-cells, as demethylation of the insulin and other gene promoters will be required, on top of supplying the transacting transcriptional machinery of β-cells. We propose that non–β-cells in islets, such as α- and δ-cells, represent a preferred starting material for reprogramming toward β-cells, given their a priori lack of methylation at the insulin promoter. This view is consistent with the finding that the overall chromatin make-up and transcriptome of β-cells and α-cells are highly similar (15). How enhancer methylation status, which shows more cell-type specificity, interphases with cell reprogramming is an interesting topic that requires further investigation.

The findings also have implications for the emerging field of β-cell death diagnosis based on detection of methylation patterns in circulating DNA (24–28). The cytosines in the insulin gene that were previously used to identify β-cell–derived DNA circulating in plasma are indeed β-cell–specific (methylated in all other tissues examined, including α-cells; positions +304 to +404). However, when searching for additional methylation sites that can serve as markers of β- or α-cell death, the similarity and differences in the methylomes of β-cells and other islet cells must be taken into account.

In conclusion, promoters of islet cell genes, including those encoding hormones, are rapidly demethylated during the differentiation of Neurog3^{+} endocrine progenitor cells. Demethylation is independent of future gene expression, and marks the differentiated islet cell lineage. Methylation differences between islet cell types are concentrated in enhancer elements. Lineage-specific epigenetic patterns may indicate the potential capacity of cells for reprogramming toward β-cells and other desired fates.

Materials and Methods

Cadaveric human islets, cell lines, and mouse tissues were processed for DNA extraction and bisulfite treatment, as detailed in *SI Materials and Methods*.

Methylation was assessed using sequencing of bacterial clones, pyrosequencing or Illumina 450k arrays. For the 450k arrays, we processed raw data using RnBeads (29), substracted background using the noob method from the methylumi package (30), and normalized using BMIQ (31). Hierarchical clustering was performed using Ward's minimum variance method with Euclidian distances. Differential methylation was performed using the RnBeads package. Chip-seq data for H3K4me1 and H3K27ac in pancreatic islets was downloaded from Array Express (https://www.ebi.ac.uk/arrayexpress/), accession no. E-MTAB-1919. Peaks were called using SICER (32).

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Hebrew University. The Hebrew University is an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited institute.

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