Cell Stem Cell
Brief Report

Epigenetic Memory and Preferential Lineage-Specific Differentiation in Induced Pluripotent Stem Cells Derived from Human Pancreatic Islet Beta Cells

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SUMMARY

Human induced pluripotent stem cells (HiPSCs) appear to be highly similar to human embryonic stem cells (HESCs). Using two genetic lineage-tracing systems, we demonstrate the generation of iPSC lines from human pancreatic islet beta cells. These reprogrammed cells acquired markers of pluripotent cells and differentiated into the three embryonic germ layers. However, the beta cell-derived iPSCs (BiPSCs) maintained open chromatin structure at key beta-cell genes, together with a unique DNA methylation signature that distinguishes them from other PSCs. BiPSCs also demonstrated an increased ability to differentiate into insulin-producing cells both in vitro and in vivo, compared with ESCs and isogenic non-beta iPSCs. Our results suggest that the epigenetic memory may predispose BiPSCs to differentiate more readily into insulin producing cells. These findings demonstrate that HiPSC phenotype may be influenced by their cells of origin, and suggest that their skewed differentiation potential may be advantageous for cell replacement therapy.

Human embryonic stem cells (HESCs) are capable of unlimited proliferation in vitro while maintaining their potential to differentiate into cells from the three embryonic germ layers (Itskovitz-Eldor et al., 2000; Thomson et al., 1998). Human induced pluripotent stem cells (HiPSCs) have been generated from human somatic fibroblasts (Lowry et al., 2008; Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007) and other human cell types, including $CD34^+$ cells from peripheral blood (Loh et al., 2009), keratinocytes (Aasen et al., 2008), and neural stem cells (Kim et al., 2009). Although HiPSCs were shown to be similar to HESCs, as judged by expression of pluripotency genes and their ability to generate embryoid bodies (EBs) and teratomas, mounting evidence suggested that iPSCs differed from ESCs in gene expression profiles (Chin et al., 2009), persistence of donor-cell gene expression (Ghosh et al., 2010; Marchetto et al., 2009), differentiation abilities (Feng et al., 2010; Hu et al., 2010), genetic stability (Mayshar et al., 2010), stability of imprinted gene expression (Pick et al., 2009), and disease modeling (Urbach et al., 2010). Recently, it has been shown that following the reprogramming of mouse iPSCs, an epigenetic memory is inherited from the parental cell (Kim et al., 2010; Polo et al., 2010). Some emerging works also show a unique DNA methylation signature that is inherited from a parental human cell following reprogramming (Doi et al., 2009; Lister et al., 2011; Ohi et al., 2011).

Differentiation of HESCs and HiPSCs into insulin-producing cells represents an attractive approach for cell replacement therapy of diabetes (McKnight et al., 2010; Kroon et al., 2008). We wished to evaluate the differentiation potential of iPSCs derived from human beta cells. To this end, we utilized two recently developed genetic lineage-tracing approaches (Russ et al., 2008, 2009) (Figure 1A) to specifically label human beta cells within cultures of pancreatic islet cells containing multiple cell types (Figure 1B). Both labeling approaches are based on the Cre/lox system, routinely used in transgenic animals (Dor et al., 2004). In our procedure, the human islet cells are infected with a mixture of two lentiviral vectors. In the first system, Cre recombinase is expressed under control of the insulin promoter, while the second system is improved by fusing Cre recombinase to the estrogen receptor (ER), rendering DNA recombination inducible by tamoxifen. Since the insulin promoter is expressed only in beta cells, the Cre enzyme excises the floxed DNA region of the reporter constructs, thereby activating constitutive GFP expression specifically in beta cells (Figures 1A and 1B). The genetic label is stable in all beta cell-derived (BCD) progeny, making it possible to determine the cellular origin of reprogrammed cells derived from beta cells. BCD cells undergo dedifferentiation and epithelial to mesenchymal transition following their growth in culture, as was described previously (Russ et al., 2008, 2009). Human islet cell cultures containing about 20% lineage-traced BCD cells following 3–9 weeks in culture were transduced with retroviral vectors encoding the four pluripotency-inducing factors OCT4, SOX2, KLF4, and cMYC. ES-like colonies were detected 3–6 weeks later with an extremely low efficiency (0.0001%) (Figure S1A). A number of these colonies were isolated and expanded for further analysis. To demonstrate the beta cell origin of the iPSCs, DNA PCR analysis of the integrated recombined reporter cassettes was performed. Out of dozens of iPSC lines established from four islet donors, four cell lines containing only the shorter PCR product corresponding to the recombined reporter gene (Figure 1C)

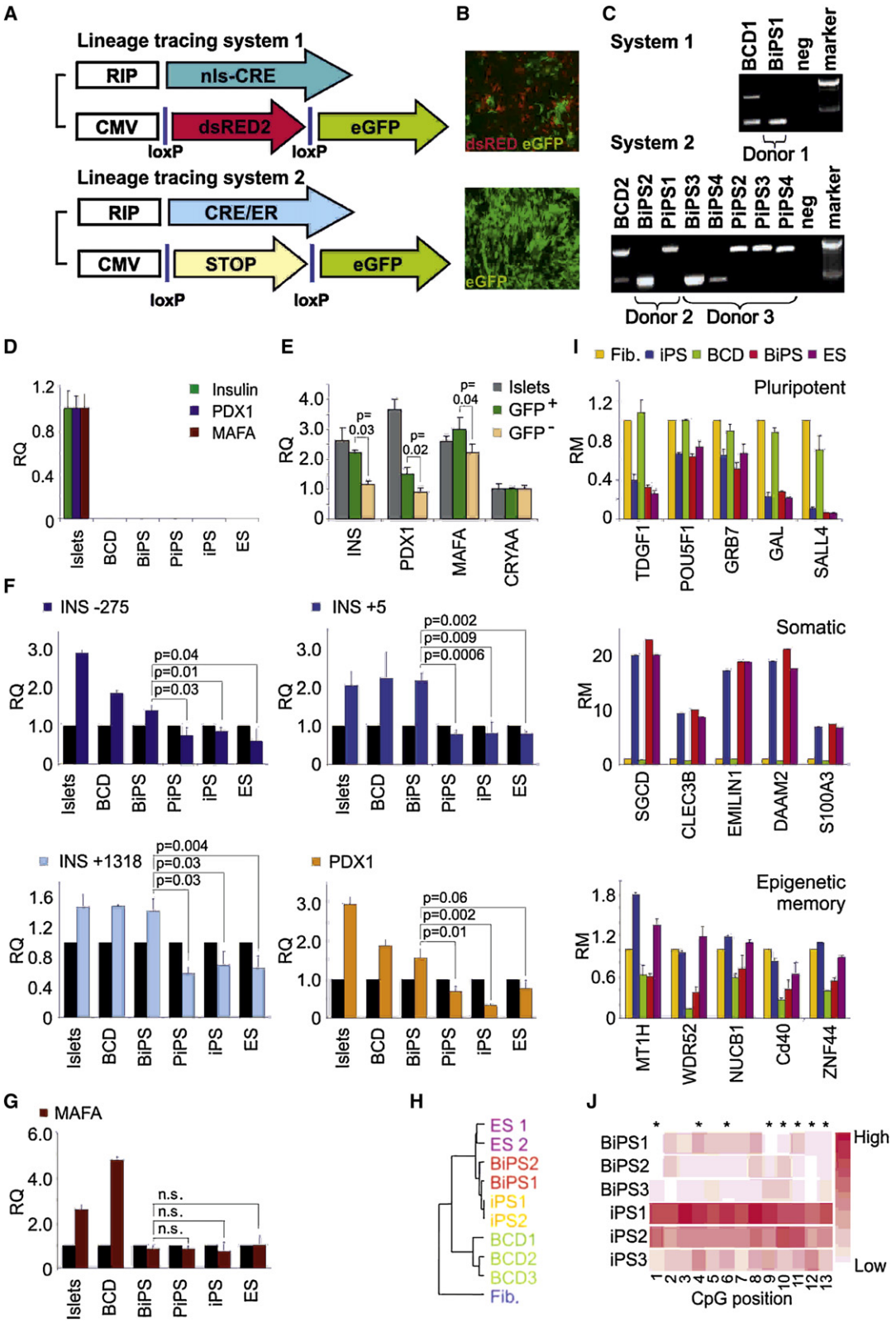

Figure 1. Genetic Labeling of Human Beta Cells, Generation of BiPSC Clones, and Epigenetic Memory in BiPSCs

(A) Schematic representation of two dual lentiviral lineage-tracing systems for specific labeling of human beta cells.

(B) Representative images of GFP$^+$ BCD cells at passage 3 generated with each labeling system. Note that cells labeled with system 2 were enriched by FACS sorting.

were identified as true BCD iPSCs (BiPSCs). We could also obtain four isogenic non-beta pancreatic iPSC (PiPSC) lines derived from non-beta cells of the same donors, as judged by the absence of a recombined reporter gene (Figure 1C and Figure S1B) (in spite of the presence of DNA of the Cre gene from the two labeling vectors). When foreskin fibroblasts were infected with the genetic lineage-tracing system, no DNA recombination of the reporter vector or GFP expression was observed, further indicating the specificity of the labeling system (Figures S1C and S1D). Transduction of the lineage-traced fibroblasts with the pluripotency-inducing factors resulted in establishment of iPSC colonies at a normal efficiency. However, no GFP expression or recombination event could be detected following reprogramming, indicating that the labeling system was not affected by the reprogramming process (Figures S1C and S1D).

The BiPSC lines contained all four retroviral transgenes, as judged by genomic DNA PCR analyses (Figure S1E). The relatively low efficiency of human BCD cell reprogramming is in accordance with a report describing low reprogramming rate of mouse beta cells (Stadtfeld et al., 2008). This low efficiency, compared to that of fibroblasts (0.0025%), may be attributed to a more differentiated state of the BCD cells, which has been shown to affect the efficiency of reprogramming (Eminli et al., 2009). BiPSC lines showed a typical ES-like morphology (Figure S1A) and could be maintained for at least 30 passages. We have thoroughly characterized the BiPSC clones by the most stringent criteria for pluripotency of HiPSCs (Daley et al., 2009; Maherali and Hochedlinger, 2008). Thus, BiPSCs were positive for many pluripotency markers at both RNA and protein levels, silenced the retroviral transgenes, and maintained a normal diploid karyotype (Figures S1F–S1I). A recent work (Chan et al., 2009) suggested that bona fide iPSCs can be distinguished from partially reprogrammed cells by the expression of *REX1*, *DNMT3B*, and Tra-1-60. BiPSCs express these markers in comparable levels to other HiPSCs and HESCs, confirming their full reprogramming (Figures S1G, S1H, and S2A). In addition, microarray analysis of gene expression showed that BiPSCs were globally similar to HiPSCs or HESCs, as shown by scatter plot and hierarchical cluster analyses, while differing markedly from the parental BCD cells (Figures S2B and S2C). Of note, the linear correlation coefficient between BiPS1 and ESCs was 0.94, similar to the correlation coefficient between two different ESC lines (R^2 = 0.94), and different from the correlation coefficient between BiPS1 and its original somatic cell lines (R^2 = 0.65). BiPSCs gave rise to differentiated cells from the three embryonic germ layers in vitro, as judged by EB formation assay, and in vivo, as evidenced by teratoma formation assay (Figures S2D–S2F). Taken together, these results demonstrate that BiPSCs are truly reprogrammed PSCs.

Epigenetic memory of active gene transcription or persistent chromatin structure has been suggested to explain the clinical anomalies observed in cloned animals generated by somatic cell nuclear transfer (Gao et al., 2003; Ng and Gurdon, 2005, 2008; Santos et al., 2003; Wee et al., 2006). This epigenetic memory was shown to manifest in donor cell-specific gene transcription patterns in most cells of the cloned animal (Gao et al., 2003; Ng and Gurdon, 2005), or in histone modifications persisting from the donor cell (Ng and Gurdon, 2008; Santos et al., 2003; Wee et al., 2006). We thus evaluated the levels of histone H3 acetylation, a hallmark of open chromatin structure, by chromatin immunoprecipitation (ChIP) in fresh human islets, BCD cells, BiPSCs, PiPSCs, iPSCs derived from fibroblasts, and ESCs (see Table S1 for cell lines used in this study, and the relevant passage number). Although BCD cells do not express *INSULIN* or the beta-cell transcription factors *PDX1* and *MAFA* (Figure 1D), they maintain a partially open chromatin structure on the promoter regions of these genes, as judged by histone H3 acetylation (Figure 1E). This epigenetic imprint was also maintained in the *INSULIN* and *PDX1* gene promoters in three BiPSC lines in levels comparable to those of BCD cells, while not detected in PiPSCs, iPSCs, or ESCs of similar passage numbers (Figure 1F and Table S1). In contrast, the histone H3 on the *MAFA* promoter underwent deacetylation during reprogramming of BCD cells into BiPSCs (Figure 1G). To determine whether a similar preservation of epigenetic memory occurred at the DNA methylation level, we utilized Illumina's Infinium HumanMethylation27 BeadChiP arrays to compare the DNA methylation patterns of BCD cells, BiPSCs, fibroblasts, iPSCs, and ESCs. Hierarchical clustering revealed a unique DNA methylation pattern in BiPSCs that considerably distinguished them from BCD cells, and also separated them from other PSCs (Figure 1H). A closer look at the DNA methylation status of specific

(C) PCR analysis of DNA from lineage-traced islet cells containing labeled BCD cells, BiPSCs, and PiPSCs, using primers spanning the floxed DNA fragment of each reporter vector. Both the original and the recombined DNA fragments are seen in the parental cells, while the four PiPSCs show the original fragment, and the four BiPSC clones show only the recombined DNA fragment (lower band).

(D) qPCR analysis of *INSULIN*, *PDX1*, and *MAFA* expression (mean ± SE) in islet cells, BCD cells, and undifferentiated BiPSCs, PiPSCs, iPSCs, and ESCs.

(E) Chromatin immunoprecipitation (ChIP) analysis of histone H3-tail acetylation in fresh islet cells in comparison to sorted GFP^+ BCD cells and nonlabeled islet cells after 7–11 weeks in culture. Note that the unlabeled fraction contains unlabeled BCD cells. qPCR analysis was performed on bound and input DNA with primers recognizing the promoter regions of *INSULIN* (−275), *PDX1*, or *MAFA*. Values are mean ± SD (n = 3–5 donors) and are normalized to *CRYSTALLIN*.

(F and G) ChIP analysis of histone H3-tail acetylation in islet cells, BCD cells, BiPSCs, PiPSCs, iPSCs, and ESCs at promoters of key beta-cell genes. qPCR was performed on bound and input DNA with primers recognizing the insulin locus at positions −275, +5, and +1318 from the transcription start site, and *PDX1* or *MAFA* promoters. Values are mean ± SE (n = 3–4 cell lines) (Table S1) and are normalized to *CRYSTALLIN*. n.s., not significant.

(H) Hierarchical clustering of BiPSCs, iPSCs, ESCs, BCD cells, and fibroblast cells using Illumina's Infinium HumanMethylation27 BeadChiP arrays.

(I) DNA methylation analysis of specific genes in somatic cells and PSCs. The status of DNA methylation was determined using Illumina's Infinium HumanMethylation27 BeadChiP arrays. Shown are the following: I, pluripotency-related genes that underwent demethylation following reprogramming, in correlation with upregulation in their gene expression; II, somatic-related genes that underwent methylation following reprogramming, in correlation with downregulation in their gene expression; and III, islet-expressed genes that are hypomethylated in BCD cells and remain hypomethylated in BiPSCs, relative to other cell types. Values are mean ± SE.

(J) Pyrosequencing analysis of DNA methylation at the *PDX1* promoter on 13 CpG island positions in BiPSCs, compared with that in iPSCs. *$p < 0.05$ in BiPSCs versus iPSCs. The color bar label "High" refers to >68% methylation, while "Low" indicates no detectable methylation at the respective position.

See also Figures S1 and S2.

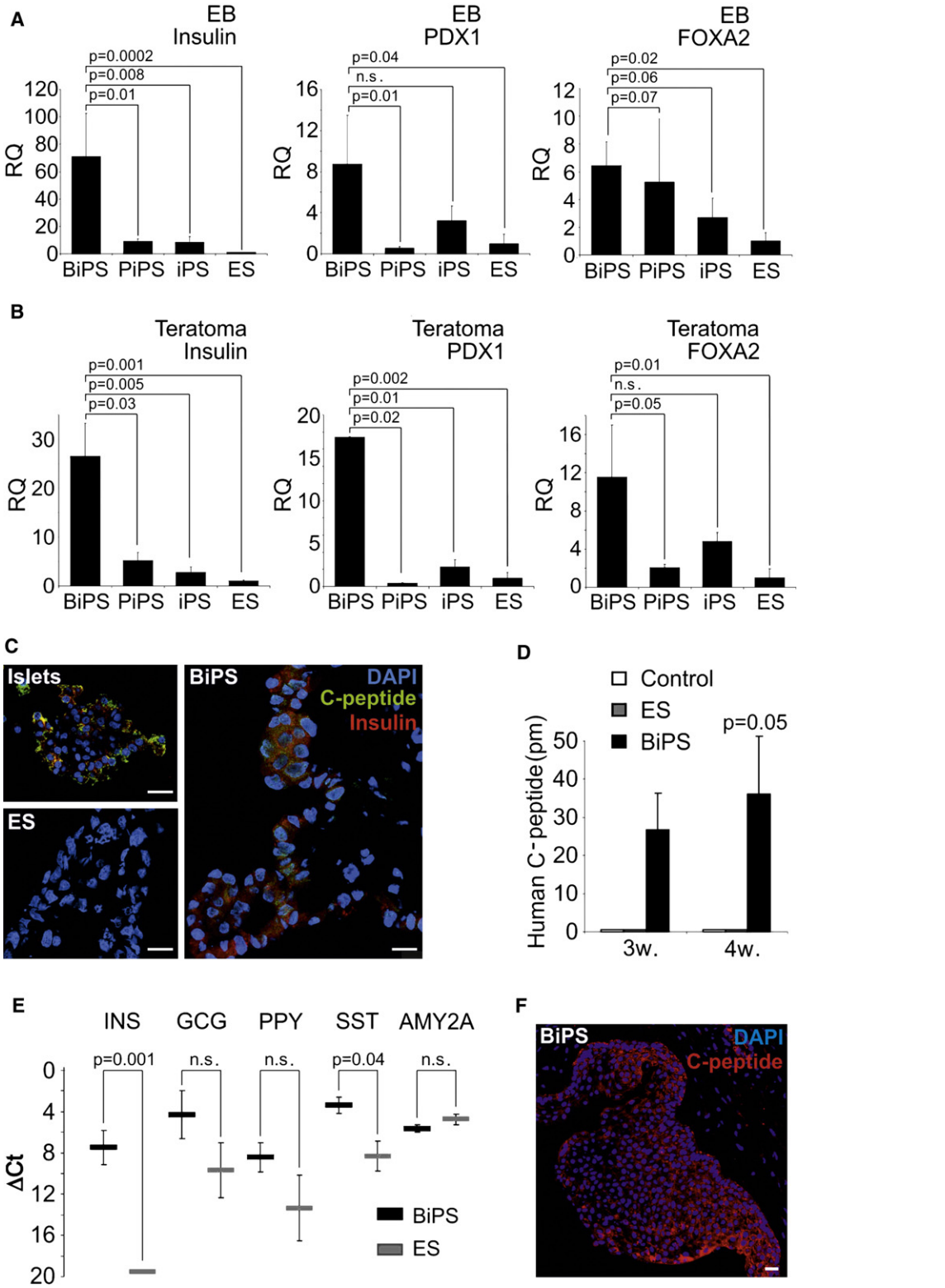

Figure 2. Skewed Differentiation of BiPSCs into Insulin-Producing Cells

(A) Expression levels of *INSULIN*, *PDX1*, and *FOXA2* transcripts in day 20 EBs analyzed by qPCR. Data represent mean of four BiPSC lines, three PiPSC lines, five iPSC lines, and three ESCI lines (Table S1). Values are mean ± SE, compared to EBs from ESCs (RQ = 1).

(B) qPCR analysis of *INSULIN*, *PDX1*, and *FOXA2* transcripts in day 30 teratomas derived from BiPSCs, PiPSCs, iPSCs, and ESCs. Data represent biological repeats of three BiPSC lines, three PiPSC lines, four iPSC lines, and three ESC lines (Table S1). Values are mean ± SE, compared to teratomas from ESCs (RQ = 1).

genes revealed that pluripotency related genes have undergone demethylation during reprogramming, while somatic-specific genes became more methylated (Figure 1I). We could also identify genes expressed in human islet cells that were hypomethylated in BCD cells, and remained hypomethylated in BiPSCs, while being methylated in fibroblasts, fibroblast-derived iPSCs, and ESCs (Figure 1I). These findings support the retention of an epigenetic memory in BiPSCs. Finally, methylation of the *PDX1* promoter was compared in BiPSCs and iPSCs by pyrosequencing analysis. BiPSCs exhibited significantly lower DNA methylation levels, characteristic of transcribed genes, in 8/13 positions analyzed, compared with iPSCs derived from fibroblasts cells (Figure 1J), and PiPSCs derived from non-beta pancreatic cells, which exhibited about 25% higher DNA methylation levels than BiPSCs. The higher DNA methylation levels in the *PDX1* promoter in iPSCs compared to those of BiPSCs may reflect a variable epigenetic memory in iPSCs derived from fibroblasts.

To address the possibility that the observed epigenetic memory rendered BiPSCs permissive to skewed differentiation into cells of the endodermal lineage, and possibly into insulin-producing cells, expression of beta-cell genes was analyzed in EBs and teratomas derived from BiPSC lines, in comparison to that in PiPSCs, iPSCs, and ESCs. Multiple differentiation experiments performed at similar passage numbers (Table S1) showed that *INSULIN*, *PDX1*, and *FOXA2* mRNA expression was significantly higher in both EBs and teratomas derived from BiPSCs, when compared to that in EBs and teratomas derived from PiPSCs, iPSCs, and ESCs (Figures 2A and 2B). Although both EBs and teratomas are composed of many different cell types, which may hinder the conclusions drawn from their analysis, our data does show preferred differentiation into endoderm in these complex differentiated structures. No significant differences were noted between the four BiPSC lines studied. Moreover, C-peptide protein could only be detected in EBs derived from BiPSCs (Figure 2C). However, C-peptide protein was not detectable in teratomas derived from either BiPSCs or ESCs (data not shown). In contrast to PDX1, MAFA expression in EBs from BiPSCs was not significantly different from that in iPSCs and ESCs (data not shown), in accordance with the lack of epigenetic memory in the *MAFA* promoter (Figure 1G). Unlike these beta-cell genes, no significant differences were found in genes of ectodermal, mesodermal, and endodermal tissues (data not shown).

Next we utilized a protocol for induced differentiation of HESCs into pancreatic endocrine progenitors (Kroon et al., 2008). BiPSCs could be differentiated into endocrine progenitor cells expressing the beta-cell transcription factors *PDX1*, *NKX6.1*, *FOXA2*, and *ISL1* by day 12 (Figures S2G and S2H). Previously, it was shown that pancreatic endocrine progenitors can further mature into beta-like cells when grafted into mice (Kroon et al., 2008). We thus transplanted 2.5–4 × 10^6 BiPSC- or ESC-derived pancreatic endocrine progenitors under the renal capsule of SCID mice and measured serum levels of human C-peptide following glucose challenge (Russ and Efrat, in press). Transplantation of BiPSC-derived pancreatic endocrine progenitor cells resulted in detectable serum levels of human C-peptide as early as 3 weeks posttransplantation, while no human C-peptide was detected in mice transplanted with human ESC-derived pancreatic endocrine progenitors during the first 4 weeks following transplantation (Figure 2D). Human C-peptide was detected in the ESC-derived progenitors 6 weeks after transplantation; however, these C-peptide levels were an order of magnitude lower than those in BiPSC-derived grafts. The levels of C-peptide detected in BiPSC-derived grafts after 3–4 weeks suggest that BiPSC-derived endocrine progenitor cells matured faster in vivo compared to ESC-derived endocrine progenitor cells. Graft analysis 6–7 weeks posttransplantation revealed insulin mRNA expression in all three BiPSC-derived grafts examined (Figure 2E). C-peptide staining was detected in 2/7 BiPSC-derived grafts at this time point (Figure 2F). These grafts also expressed glucagon, somatostatin, pancreatic polypeptide, and amylase transcripts (Figure 2E). In contrast, ESC-derived grafts expressed detectable levels of the other four pancreatic transcripts analyzed, but not insulin (Figure 2E).

The first reports of generation of HiPSCs focused primarily on their similarity to HESCs, although recent works have addressed the differences between the two PSC types (Chin et al., 2009; Doi et al., 2009; Feng et al., 2010; Ghosh et al., 2010; Hu et al., 2010; Kim et al., 2010; Lister et al., 2011; Marchetto et al., 2009; Mayshar et al., 2010; Ohi et al., 2011; Pick et al., 2009; Polo et al., 2010; Urbach et al., 2010). Here we report the reprogramming to pluripotency of human pancreatic beta cells, and demonstrate the utility of a lineage-tracing system in primary human cells for monitoring the origin of the reprogrammed cells. Our findings document the existence of an epigenetic memory in cells derived from human beta cells during their expansion in vitro. This memory is maintained during reprogramming into iPSCs in most of the markers analyzed. The epigenetic differences between BiPSCs and iPSCs/ESCs may be responsible for differences in their differentiation capabilities. BiPSCs may have a higher propensity to express PDX1 and insulin upon differentiation due to the relatively open chromatin structure of these loci. This skewed differentiation manifests as their increased capacity for spontaneous differentiation into insulin-producing cells.

Our results prescribe caution in utilizing HiPSCs derived from different cell types for disease modeling or for the study of early developmental events, since the cells may differ according to

(C) Immunofluorescence analysis of insulin and C-peptide in islet cells and day 20 EBs derived from ESCs and BiPSCs. The C-peptide antibody used is human specific. Nuclei were stained blue with DAPI. Bar = 20 μm. Only a small number of insulin-positive areas were detected in EBs derived from BiPSCs; however, none were seen in EBs derived from ESCs.

(D–F) Differentiation induced with the protocol of Kroon et al. (2008). (D) Serum human C-peptide levels following glucose challenge in mice transplanted with ESC- or BiPSC-derived endocrine progenitors at indicated time points posttransplantation, compared with those of sham controls. Values are mean ± SE (n = 8 mice in each group). The assay sensitivity is 1.5 pmol/L. (E) qPCR analysis of pancreatic gene expression in BiPS1 and BiPS2 cell-derived grafts, in comparison with ESC-derived grafts. ΔCt is normalized to *GAPDH*. Values are mean ± SE (n = 3 mice in each group). (F) Immunofluorescence analysis of C-peptide in a BiPSC-derived graft removed 6 weeks posttransplantation. Nuclei were stained blue with DAPI. Bar = 20 μm.

See also Figure S2.

their cellular origin. On the other hand, we propose that such differences may be harnessed for generation of PSCs with a skewed ability to differentiate into a specific cell type. This approach may prove useful in the generation of differentiated cell types that are currently hard to produce from existing PSCs for the treatment of human diseases.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE29880.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2011.06.007.

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