Intrapatient Variations in Type 1 Diabetes-specific iPS Cell Differentiation Into Insulin-producing Cells

Tayaramma Thatava[1], Yogish C Kudva[2], Ramakrishna Edukulla[3], Karen Squillace[1], Josep Genebriera De Lamo[4], Yulia Krotova Khan[4], Toshie Sakuma[1], Seiga Ohmine[1], Andre Terzic[5–7] and Yasuhiro Ikeda[1]

[1]Department of Molecular Medicine, College of Medicine, Mayo Clinic, Rochester, Minnesota, USA; [2]Division of Endocrinology, College of Medicine, Mayo Clinic, Rochester, Minnesota, USA; [3]Nephrology and Hypertension Research, College of Medicine, Mayo Clinic, Rochester, Minnesota, USA; [4]Dermatology, College of Medicine, Mayo Clinic, Rochester, Minnesota, USA; [5]Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Department of Medicine, College of Medicine, Mayo Clinic, Rochester, Minnesota, USA; [6]Department of Molecular Pharmacology and Experimental Therapeutics, College of Medicine, Mayo Clinic, Rochester, Minnesota, USA; [7]Department of Medical Genetics, College of Medicine, Mayo Clinic, Rochester, Minnesota, USA

Nuclear reprogramming of adult somatic tissue enables embryo-independent generation of autologous, patient-specific induced pluripotent stem (iPS) cells. Exploiting this emergent regenerative platform for individualized medicine applications requires the establishment of bioequivalence criteria across derived pluripotent lines and lineage-specified derivatives. Here, from individual patients with type 1 diabetes (T1D) multiple human iPS clones were produced and prospectively screened using a battery of developmental markers to assess respective differentiation propensity and proficiency in yielding functional insulin (INS)-producing progeny. Global gene expression profiles, pluripotency expression patterns, and the capacity to differentiate into SOX17- and FOXA2-positive definitive endoderm (DE)-like cells were comparable among individual iPS clones. However, notable intrapatient variation was evident upon further guided differentiation into HNF4α- and HNF1β-expressing primitive gut tube, and INS- and glucagon (GCG)-expressing islet-like cells. Differential dynamics of pluripotency-associated genes and pancreatic lineage-specifying genes underlined clonal variance. Successful generation of glucose-responsive INS-producing cells required silencing of stemness programs as well as the induction of stage-specific pancreatic transcription factors. Thus, comprehensive fingerprinting of individual clones is mandatory to secure homogenous pools amenable for diagnostic and therapeutic applications of iPS cells from patients with T1D.

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INTRODUCTION

Ectopic expression of a set of defined stemness factors triggers reprogramming of adult somatic tissue and generation of induced pluripotent stem (iPS) cells that deploy comprehensive traits of embryonic stem (ES) cells.[1–3] Embryo-independent iPS cell-based technology allows derivation of autologous patient-specific PS cells,[4–8] with ensuing specification of disease-relevant lineages offering, in principle, authentic cell-based platforms for personalized diagnostic and therapeutic applications. In fact, iPS clones have been established from diverse human disease conditions.[9–13] Recapitulation of disease phenotypes through redifferentiation of patient-specific iPS cells underscores the utility of nuclear reprogramming and bioengineered lineage specification for individualized disease modeling and drug screening.[14–16]

Type 1 diabetes (T1D) is caused by immune-mediated destruction of insulin (INS)-producing pancreatic β-cells. T1D pathobiology implicates multiple genetic loci and environmental influences in disease susceptibility.[17] Several genes located in the major histocompatibility complex on chromosome 6p21.3 have been identified as major genetic factors, while other genes, such as INS, CTLA4, and PTPN22, make additional contributions to the disease pathogenesis.[17] Extensive studies in spontaneous T1D rodent models, including the nonobese diabetic mouse strain, have also identified combinations of major histocompatibility complex and other susceptibility genes responsible in the development of T1D.[18] Notably, the nonobese diabetic mouse strain is nonpermissive for derivation of ES cells,[19,20] while iPS cells from nonobese diabetic mice demonstrate an unstable pluripotent state,[21] suggesting possible influence of the T1D-predispositing genetic background on achieving and maintaining pluripotency.

For diagnostic and therapeutic applications it is critical to ensure reliable and efficient differentiation of T1D-derived somatic tissue into iPS cells proficient to yield INS-producing islet-like cells. Based on protocols which facilitate conversion of human ES cells into INS-producing islet-like progeny,[22–26] human iPS clones have been recently differentiated into INS-producing cells.[27–29] Generation of INS-producing cells from three iPS clones from two T1D patients (one clone from a 30-year-old and two clones from a 32-year-old)

Correspondence: *Yasuhiro Ikeda, Department of Molecular Medicine, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, USA. E-mail: ikeda.yasuhiro@mayo.edu*

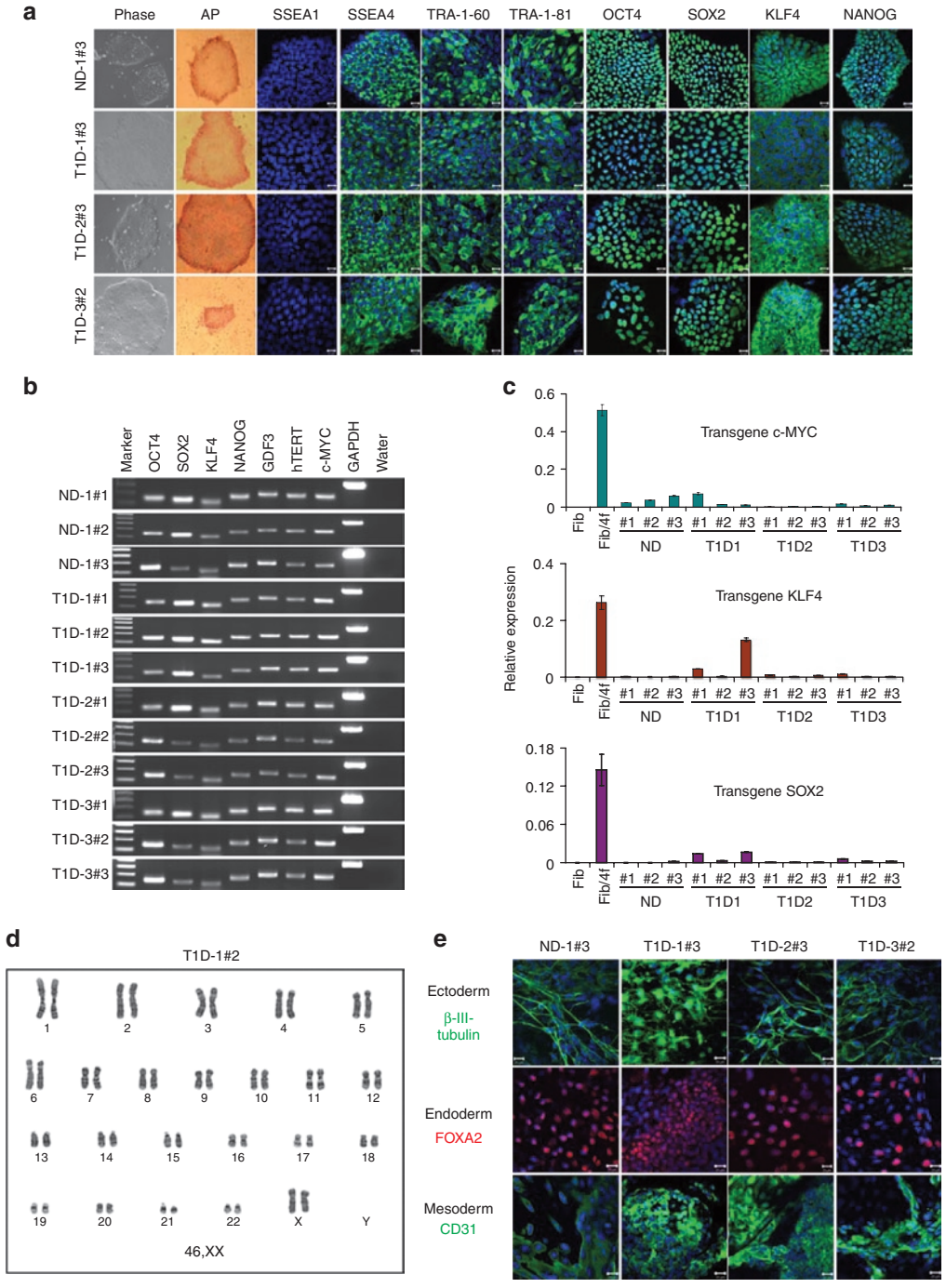


Figure 1 Generation of iPS cells from patients with type 1 diabetes (T1D). (**a**) Skin cells from nondiabetic (ND) and type 1 diabetic (T1D) donors were reprogrammed with lentiviral vectors expressing pluripotency-associated factors, *OCT4*, *SOX2*, *KLF4*, and *c-MYC*. Patient-specific iPS clones cultured under serum- and feeder-free conditions were examined for expression of a series of human pluripotent stem cell markers, including alkaline phosphatase (AP), SSEA1, SSEA4, TRA-1-60, TRA-1-81, OCT4, SOX2, KLF4, and NANOG. (**b**) RT-PCR analysis was performed to examine expression of key pluripotency-associated genes using total cellular RNA from ND- and T1D-iPS clones. Three clones from ND (ND-1#1, #2, and #3) and three clones each from T1D patients (T1D-1, T1D-2, and T1D-3) were characterized. No cDNA template was used as control (water). (**c**) Efficient silencing of lentiviral transcripts in induced pluripotent cells. Transgene-specific quantitative real-time PCR demonstrates that the expression of viral transcripts (*c-MYC*, *KLF4*, and *SOX2*) was downregulated in iPS clones. Shown are (Fib) uninfected fibroblasts, (Fib/4f) fibroblasts 3 days after infection with all four viruses, ND iPS clones (ND-1#1, #2, and #3), and T1D-specific iPS clones (T1D1#1, #2, #3; T1D2#1, #2, #3; and T1D3#1, #2, #3). (**d**) G-banding chromosome analysis of T1D-iPS clone T1D1#2. (**e**) iPS clones were spontaneously differentiated through embryoid body formation. Pluripotency of derived iPS clones was verified by generation of cells of ectoderm (β-III tubulin, green), endoderm (FOXA2, red), and mesoderm (CD31, green) upon spontaneous differentiation. Nuclei were counterstained by DAPI (blue). Bars = 20 µmol/l. DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iPS, induced pluripotent stem cell; RT-PCR, reverse transcription-PCR.

has further verified the utility of T1D-specific iPS cells for experimental and clinical studies.[30] Of note, T1D-specific iPS clones exhibited marked clonal differences in differentiation propensity,[30] mandating evaluation of suspected intrapatient variation.

Here, to address the influence of the T1D milieu, we recruited T1D patients, generated multiple iPS clones from each individual, and systematically determined respective differentiation propensities. While distinct from healthy controls, derived T1D iPS clones demonstrated similar pluripotency gene expression profiles and comparable differentiation proficiency for definitive endoderm (DE) conversion. Yet, clonal variations became increasingly prominent upon further guided differentiation of iPS progeny into primitive gut tube- and islet-like cells, and differential regulation of pluripotency-associated genes and pancreatic lineage-specifying genes was associated with inconsistent lineage specification. The observed intrapatient variance in differentiation potential within derived T1D-specific pluripotent clones points to the importance of quality control screening for selection of proficient patient-specific lines.

Table 1 Information of nondiabetic and diabetic individuals

Sample	Age/sex	Age at diagnosis	Family history of T1D	Medication	Hemoglobin A1c (%)
ND-1[a]	31/Male	NA	No	NA	NA
T1D-1[b]	38/Female	29	No	Insulin	6.8
T1D-2[c]	47/Male	15	No	Insulin	6.6
T1D-3[d]	21/Male	14	Yes	Insulin	9

Abbreviation: NA, not applicable.
[a]Nondiabetic individual-1. [b]Type 1 diabetic patient-1. [c]Type 1 diabetic patient-2. [d]Type 1 diabetic patient-3.

RESULTS

Reprogramming T1D patient-derived cells

Lentiviral vectors encoding *OCT4*, *SOX2*, *KLF4*, and *c-MYC* were added to transduce epidermal cells from three T1D patients (T1D-1, T1D-2, and T1D-3; 21–38 years old), and one nondiabetic (ND) donor (31-year-old) (**Table 1**). Under feeder-free conditions, small iPS-like colonies with a sharp-edged, flat, and tightly packed morphology appeared within 2 weeks after vector transduction. T1D- and ND-iPS clones, cultured under feeder-free conditions, exhibited morphology similar to those typical of human PS cells. These T1D- and ND-iPS clones were positive for alkaline phosphatase activity (**Figure 1a**, **Supplementary Figure S1**), and expressed human pluripotency-associated markers; SSEA-4, TRA-1-60, TRA-1-81,

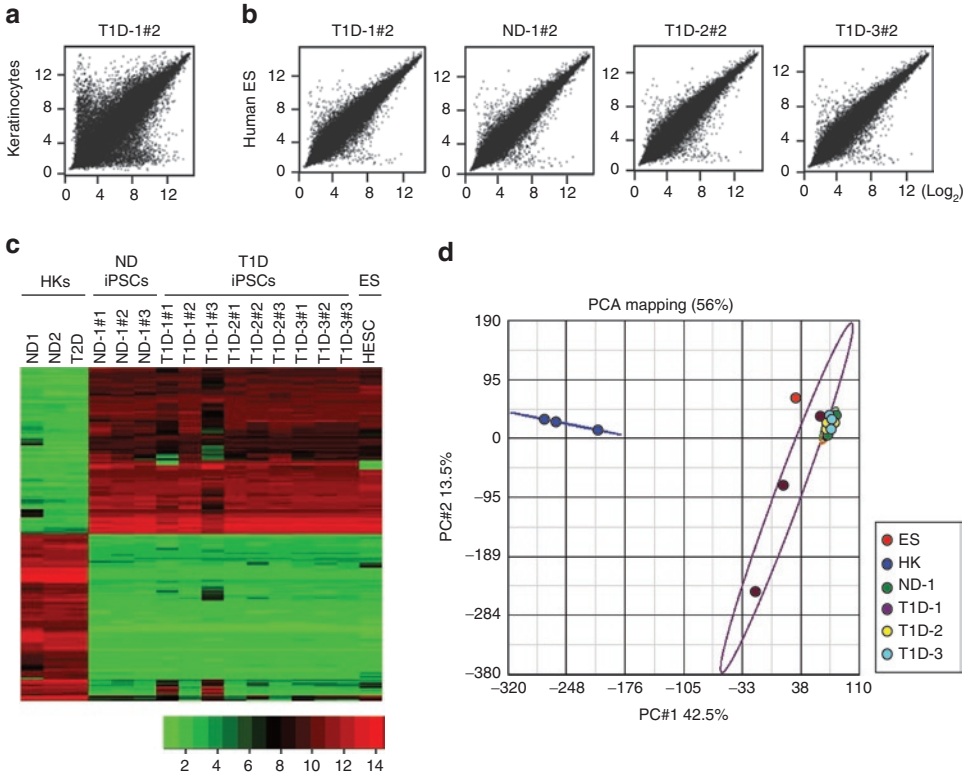
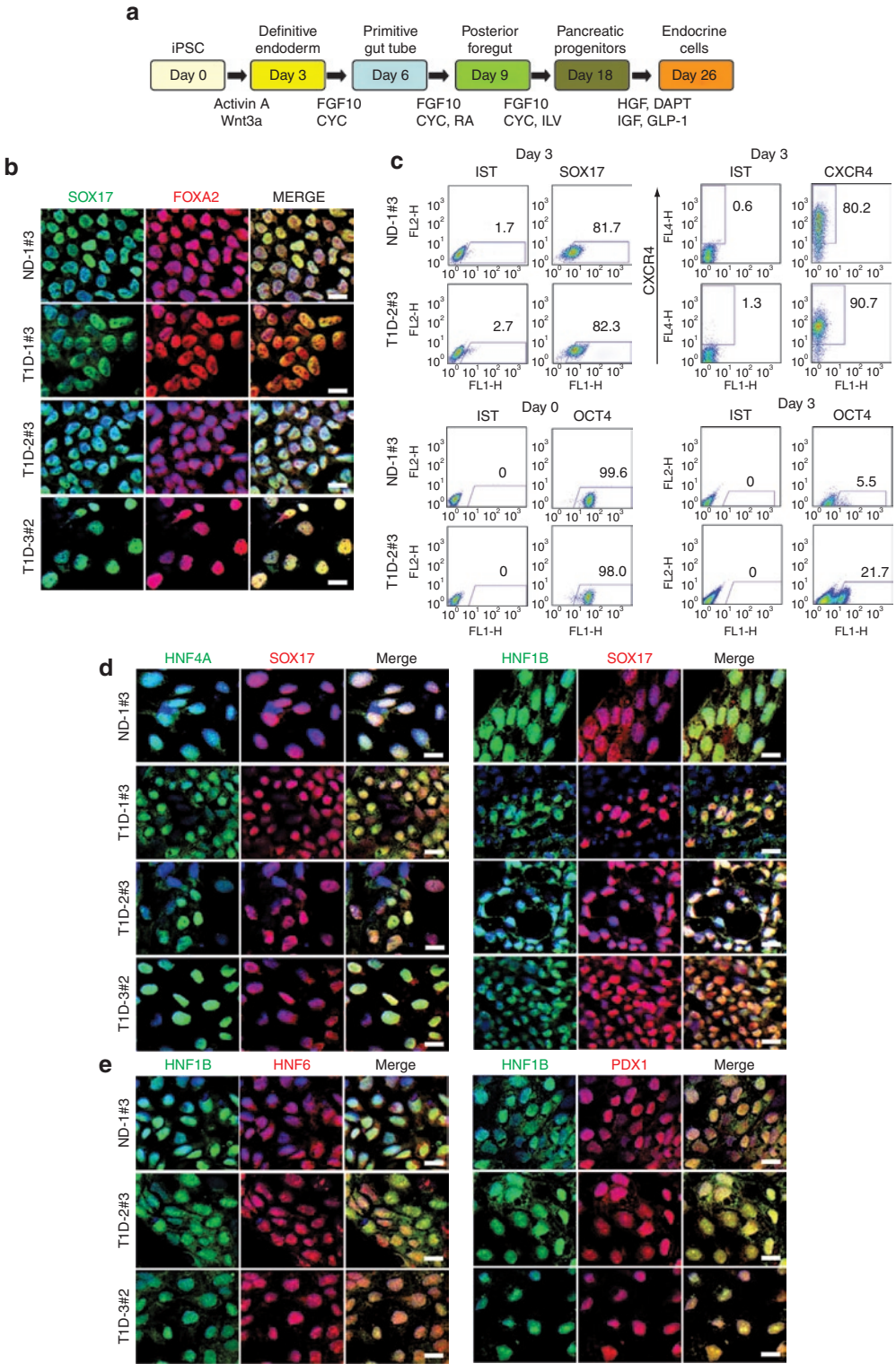


Figure 2 Microarray-based gene expression profiles of nondiabetic (ND)- and type 1 diabetic (T1D)-induced pluripotent stem (iPS) cells. (**a**) Global gene expression patterns were compared between primary human keratinocytes (HKs) and T1D iPS clone (T1D-1#2), or between (**b**) human embryonic stem (ES) cells (H9) and ND iPS clone (ND-1#2) or T1D-iPS clones (T1D-1#2, T1D-2#2, and T1D-3#2). (**c**) Heat map analysis of HKs (ND1, ND2, and T2D), iPS cell clones (ND iPS clones, ND-1#1, #2, #3; T1D iPS clones, T1D-1#1, #2, #3; T1D-2#1, #2, #3; and T1D-3#1, #2, #3), and human ES cells (HESC). Expression of genes that are differentially expressed between HK and iPS clones. (Top) Hundred human iPS cell—enriched probe sets; (bottom) top hundred keratinocytes enriched probe sets. The color key (below) indicates the color code gene expression in $\log_2$ scale. (**d**) Principal component analysis (PCA) of the ND- and T1D-iPS clone's microarray data. The PCA plot illustrates the principal components of three ND-, nine T1D-iPS clones, three keratinocytes, and HESC. iPS clones derived from different individuals were grouped together, and keratinocytes were clearly separated from iPS clones.

OCT4, SOX2, KLF4, and NANOG (**Figure 1a**, **Supplementary Figure S2**). All tested iPS clones were negative for SSEA-1 expression (**Figure 1a**, **Supplementary Figure S2**). Semiquantitative reverse transcription-PCR analysis confirmed induction of endogenous pluripotency-associated genes including *OCT4*, *SOX2*, *GDF3*, *TERT*, *KLF4*, *c-MYC*, and *NANOG* (**Figure 1b**). Real-time PCR assays demonstrated the silencing of lentiviral transgene expression in derived iPS clones (**Figure 1c**, with an exception, clone T1D1#3 maintaining relatively high exogenous *KLF4* expression), indicating that derived iPS clones do not depend on exogenous reprogramming factors for sustained pluripotency. Due to technical constraints, the exogenous OCT4 transgene levels were not determined by real-time PCR. Since the four reprogramming factors were expressed from lentiviral vectors of the same vector background, it



is likely that vector-derived OCT4 transgene expression was similarly silenced in derived iPS cells. Karyotyping analysis of derived iPS clones revealed cells with normal diploid karyotypes (**Figure 1d** and **Supplementary Figure S3**). Only one abnormal karyotype (trisomy 8) was found in iPS T1D-3#3 clone at passage 27 (1 out of 20 cells; **Supplementary Figure S3a,b**). As the majority of observed cells had normal karyotypes and frequent trisomy 8 has been noted in human iPS cells and ES cells after prolonged passages,[31] we conclude that the observed chromosomal abnormality in clone T1D-3#3 is due to continuous *in vitro* passage.

Derived patient-specific iPS clones were further assayed for the ability to spontaneously differentiate into cells of three germinal layers *in vitro*. When iPS cells were cultured in suspension, all T1D- and ND-iPS clones formed embryoid bodies (EBs). Immunostaining of EB-derived adherent cells revealed generation of cells prototypic of the ectoderm (β-III tubulin), endoderm (FOXA2) or mesoderm (CD31), (**Figure 1e**, **Supplementary Figure S4a**). Moreover, upon subcutaneous injection into immunodeficient mice, T1D- and ND-iPS clones formed tumors within 1 month. Tumors, reaching 1–2 cm in diameter, were harvested for histological analysis. Hematoxylin and eosin staining of tumor sections demonstrated heterogeneous tissues representing ectodermal, endodermal, and mesodermal lineages (**Supplementary Figure S4b**), verifying the pluripotent differentiation propensity *in vivo*. These results demonstrate that T1D- and ND-iPS clones are pluripotent, and are able to form cells of three germ layers both *in vitro* and *in vivo*.

Global gene expression profiles of derived iPS clones

To further assess the similarity of derived iPS clones, global gene expression profiles of ND- and T1D-iPS clones were determined and compared against primary human keratinocytes[32] and H9 human ES cells (GSM551202; GEO DataSets, Boston, MA). Scatter plot analysis demonstrated that the transcriptome of ND- and T1D-iPS clones showed higher similarity to those of H9 human ES cells than primary keratinocytes (**Figure 2a,b**). Heat map analysis of differentially expressed genes further confirmed that gene expression patterns of derived iPS clones were similar to those of human ES cells, but highly divergent from basal human keratinocytes (**Figure 2c**). The transcriptome of derived iPS clones were strikingly similar to each other. Two iPS clones, T1D-1#1 and T1D-1#3, showed some variations in gene expression patterns (**Figure 2c**). We then performed principal component analysis (PCA) of the array data with a 37,788 probe set. Except for the two iPS clones T1D-1#1 and T1D-1#3, 10 iPS clones from four subjects grouped tightly together, illustrating the strong intrapatient and interpatient homogeneity among patient-derived iPS clones. The data set from a human ES cell line clusters closely with the iPS cluster, while primary human keratinocytes form a distinct cluster (**Figure 2d**). From this we conclude that global gene expression profiles support a high degree of similarity in transcriptome between ND-, T1D-iPS cells, and human ES cells. The diversity noted between human ES cells and our 10 iPS clones (excluding T1D-1#1 and T1D-1#3) could be due to differences in culture conditions. The ES cells used to derive comparative microarray data were cultured with feeder cells, while our experimental clones were derived and maintained under feeder-free conditions.

Guided differentiation of T1D-specific iPS cells into pancreatic progenitors

Next, we employed an established step-wise differentiation protocol[28] to test the proficiency of derived iPS cells in generating pancreatic progenitor cells *in vitro*. Since DE formation is a prerequisite for pancreatic lineage development, we first evaluated the efficiency of DE induction from T1D- and ND-iPS cells using SOX17 and FOXA2 expression as DE markers. Treatment with Activin A and Wnt3a facilitated induction of DE cells with strong SOX17 and FOXA2 expression (**Figure 3b**, **Supplementary Figure S5**). Fluorescence-activated cell sorting analysis revealed that 71–95% of iPS progeny were positive for SOX17, similarly 80–99% of cells were positive for CXCR4 (**Figure 3c**, **Supplementary Figure S6**), in line with a robust DE induction from derived iPS clones through Activin A and Wnt3a treatment. Pluripotency marker OCT4 expression level was decreased from 99 to 5% after DE induction (**Figure 3c**, **Supplementary Figure S6**).

DE cells were then differentiated into gut tube endoderm cells through activation with FGF10 and CYC. iPS progeny upon FGF10 and CYC treatment expressed high levels of primitive gut tube markers HNF4A (hepatocyte nuclear factor 4α) and HNF1B (hepatocyte nuclear factor 1 homeobox B) (**Figure 3d**, **Supplementary Figure S7**). Of note, signals for HNF4A and HNF1B colocalized with SOX17, confirming that primitive gut tube cells were derived from SOX17-positive DE cells (**Figure 3d**, **Supplementary Figure S7**). Colocalization of HNF1B and HNF4A signals was also observed (**Figure 3d**). Upon further differentiation with FGF10, CYC, and retinoic acid, iPS-derived gut tube cells began to express high levels of posterior foregut markers, PDX1 (pancreatic and duodenal homeobox 1) and HNF6 (hepatocyte nuclear factor 6) (**Figure 3e**, **Supplementary Figure S8**).

Figure 3 Differentiation of nondiabetic (ND)- and type 1 diabetic (T1D)-induced pluripotent stem (iPS) clones into definitive endoderm and foregut endoderm cells. (**a**) Schematic diagram of the differentiation process. iPS clones were differentiated into insulin-producing endocrine cells through definitive endoderm, primitive gut tube, posterior foregut, and pancreatic progenitor stages. (**b**) iPS cells were treated with activin A and Wnt 3a for 3 days. iPS-derived cells were immunostained with antibodies against SOX17 and FOXA2 to verify induction of definitive endoderm cells. SOX17 (green) expressing cells coexpressed FOXA2 (red). Nuclei were counterstained with DAPI (blue). (**c**) Flow cytometric analyses were performed to determine SOX17, CXCR4, and OCT4-positive cell populations. iPS-derived definitive endoderm cells were dissociated and stained with anti-SOX17, anti-CXCR4, and anti-OCT4 antibodies. Cells stained with an IgG isotype control were used as negative control. Over 80% of iPS progeny expressed definitive endoderm marker SOX17, 80–90% of cells were CXCR4-positive and 5–20% of cells expressed OCT4. More than 98% of undifferentiated iPS clones were OCT4 positive. (**d**) iPS progeny on differentiation at day 5 were stained with primitive gut tube markers HNF4A and HNF1B. Induction of primitive gut tube markers was verified by high levels of HNF4A (green) and HNF1B (green) expression. Of note, HNF4A-positive cells were also positive for SOX17 (red) and HNF1B. Nuclei were counterstained with DAPI (blue). (**e**) iPS progeny differentiated for 9 days were stained for foregut endoderm markers. Detection of pancreatic transcription factors HNF1B (green), HNF6 (red), and PDX1 (red) verified the induction of foregut endoderm cells. Nuclei were counterstained with DAPI (blue). Bars = 20 µmol/l. One clone from ND (ND-1#3) and three clones from T1D patients (T1D-1#3, T1D-2#3, and T1D-3#2) were analyzed. CYC, KAAD-cyclopamine; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; ILV, indolactam V; RA, all-trans retinoic acid.

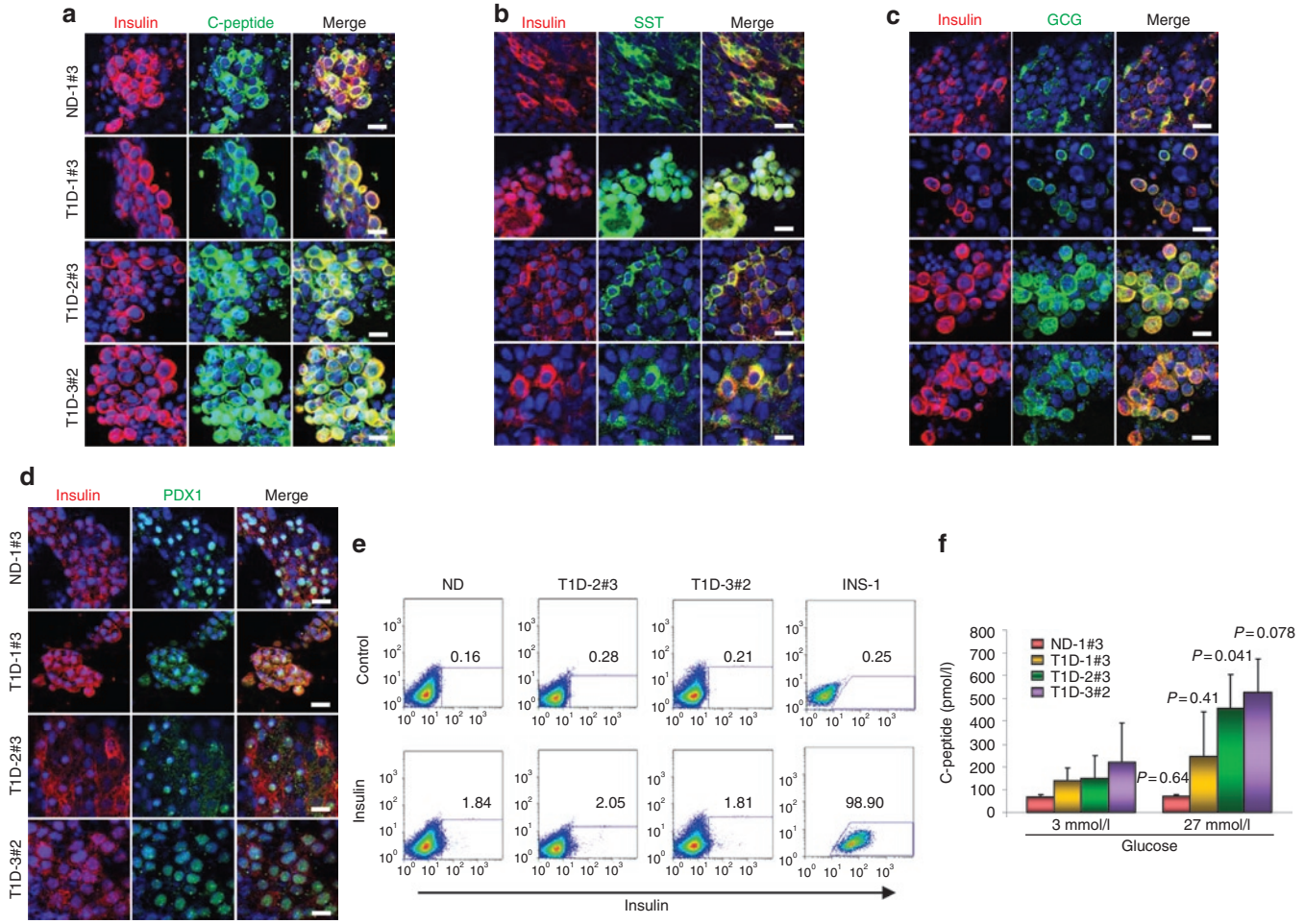


Figure 4 Successful differentiation of type 1 diabetes (T1D)-specific induced pluripotent stem (iPS) cells into pancreatic hormones-expressing cells. (**a**) Expression of pancreatic hormones insulin, C-peptide, glucagon (GCG), somatostatin (SST), and PDX1 were detected in differentiated cells at day 26. Insulin (red) expression colocalized with C-peptide (green) expression. (**b**,**c**) Some of the insulin-positive cells were also positive for GCG (green) or SST (green). PDX1 (green) expression was also detected in insulin-positive cells. Nuclei were counterstained with DAPI (blue). Bars = 20 µmol/l. (**d**) Flow cytometry analysis demonstrated ~2% of iPS progeny positive for insulin. Staining with the secondary antibody alone was used as a control. Rat insulinoma cell line INS-1 was used as positive control for insulin staining. (**e**) Generation of glucose-responsive, insulin-producing cells was confirmed by human C-peptide secretion after high glucose stimulation. (**f**) The amounts of C-peptide secretion upon high glucose stimulation was measured by C-peptide ELISA assay. Error bars indicate SD. Statistical significance tested by Student's *t*-test. DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; ND, nondiabetic.

PDX1- and HNF6-expressing cells were also positive for HNF1B, suggesting derivation of posterior foregut from primitive gut tube cells. A subset of iPS progeny were negative for PDX1 or HNF6, indicating the generation of heterogeneous cell populations (**Figure 3e**, **Supplementary Figure S8**). Posterior foregut cells upon guided differentiation in the presence of FGF10, indolactam V, and GLP-1 generated PDX1-positive pancreatic progenitors (**Supplementary Figure S9a**). PDX1-positive cells were found in all iPS clones tested, indicating a reproducible pancreatic differentiation potential of T1D-specific iPS clones.

Generation of pancreatic hormone-producing islet-like cells from T1D-specific iPS cells

Further guided differentiation generated pancreatic islet-like cells through a pancreatic endoderm stage. iPS-derived islet-like cells, on immunocytochemistry, expressed INS. In islet-like clusters, INS signals colocalized with those of C-peptide, confirming *de novo* INS synthesis, rather than INS uptake from the medium (**Figure 4a**). Glucagon (GCG)- and somatostatin (SST)-positive cells were also found predominantly in islet-like clusters (**Figures 4b** and **3c**). Islet-like clusters appeared to be a mixture of immature and mature β-cells; a subset of INS-producing cells coexpressed GCG or SST, characteristics of immature islet cells, whereas others coexpressed the mature β-cell marker, PDX1 (**Figure 4d**). Quantification, by flow cytometry, of INS-positive cell populations within differentiated patient-specific iPS-derived islet-like clusters demonstrated that 2–3% of the cell population was INS-positive (**Figure 4e**). Rat insulinoma cell line INS-1 was used as a positive control for staining (**Figure 4e**) and undifferentiated iPS clones as negative control (**Supplementary Figure S10b**). OCT4 expression was downregulated after differentiation to 0.04–2.0% (**Supplementary Figure S10c**). When T1D patient-specific iPS progeny was tested for the capability of glucose-responsive C-peptide secretion, T1D- and ND-iPS–derived cells secreted more C-peptide in response to high glucose stimulation

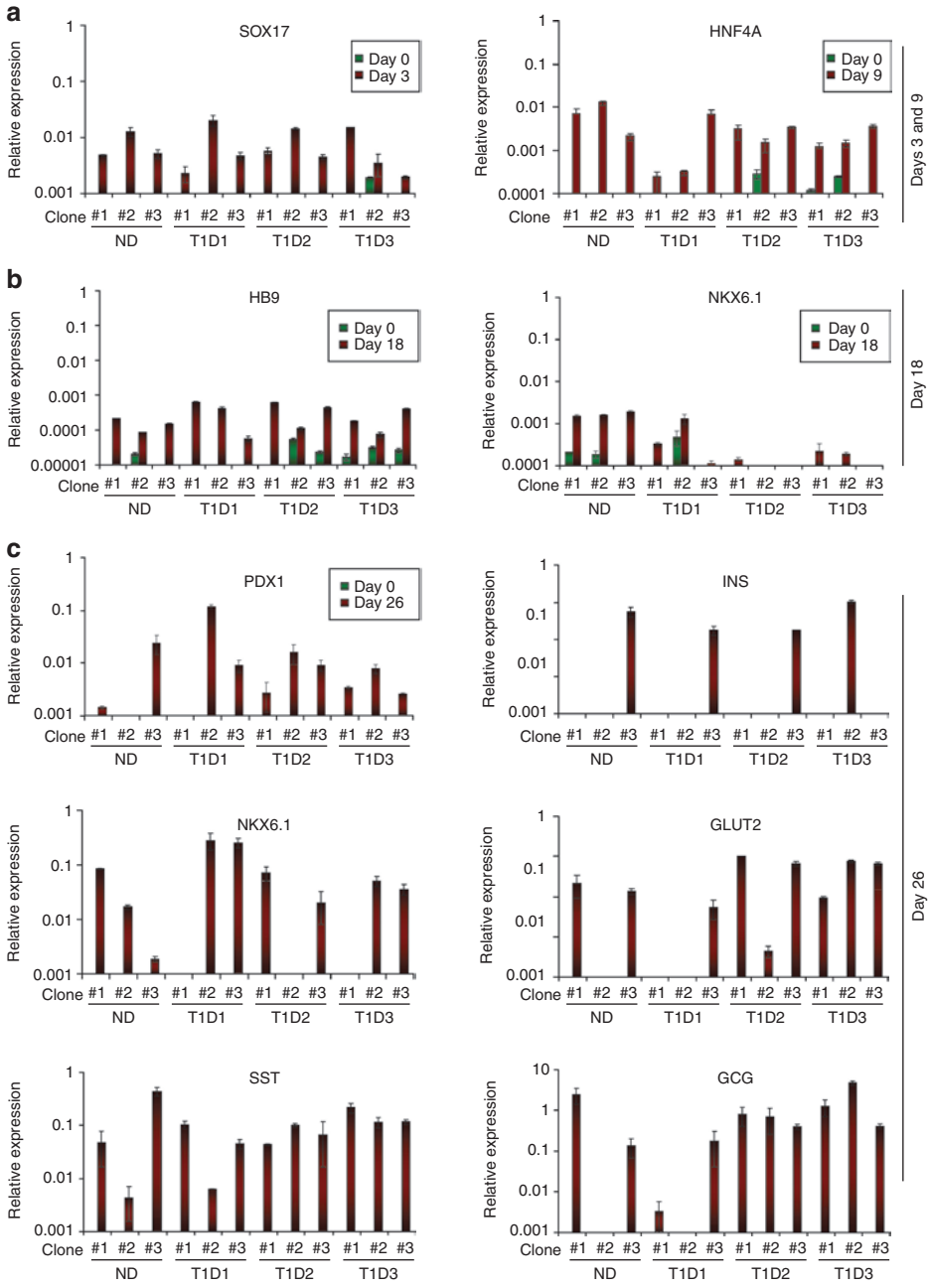


Figure 5 Differential regulation of stage-specific pancreas developmental genes in differentiated type 1 diabetes (T1D)-specific induced pluripotent stem (iPS) cells. RNA was isolated from differentiated iPS progeny at each differentiation stage and quantitative PCR analysis was performed to detect the induction of key stage-specific pancreatic factors. (**a**) Specific primers were used to detect expression of definitive endoderm marker (SOX17), posterior foregut markers (HNF4A), (**b**) pancreatic progenitors (HB9 and NKX6.1), and (**c**) pancreatic endocrine cells (PDX1, INS, NKX6.1, GLUT-2, SST, and GCG). The expression levels were normalized to those of GAPDH. Green bars represent before differentiation, red bars after differentiation. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCG, glucagon; ND, nondiabetic; SST, somatostatin.

(Figure 4f). These data demonstrate the proficiency of T1D-specific iPS cells to yield INS-producing cell types. Importantly, however, a remarkable intrapatient clonal variation was evident in the generation of INS-positive cells. Most iPS clones could be differentiated into SST- and GCG-producing cells (**Supplementary Figure S9b**), but failed to generate INS-positive cells. Indeed, among 12 iPS clones tested, only four clones (ND-1#3, T1D-1#3, T2D-2#3, and T1D-3#2) were reproducibly guided into INS-producing cells.

Differential regulation of pancreatic lineage-specifying and pluripotency-associated factors in individual iPS clones

To understand the molecular mechanisms of the observed intrapatient variations in differentiation propensities of iPS cells, we analyzed the expression of stage-specific transcription factors at each differentiation step. In line with immunostaining, *FOXA2* and *SOX17* transcripts were detected by quantitative PCR in

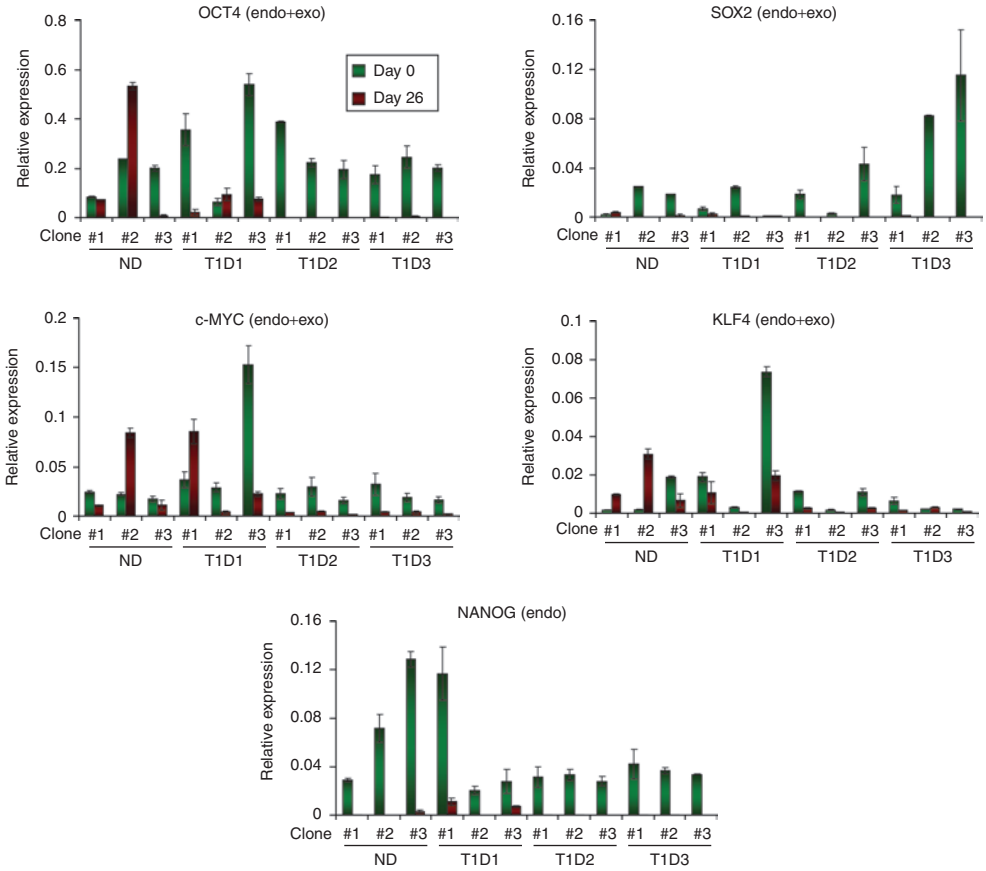


Figure 6 Expression of pluripotency-associated genes after step-wise differentiation to insulin-producing cells. The levels of *OCT4*, *SOX2*, *c-MYC*, *KLF4*, and *NANOG* transcripts in type 1 diabetes (T1D)-specific induced pluripotent stem (iPS) cells before (green bars) and after (red bars) differentiation was examined by real-time PCR analysis. The expression levels were normalized to those of *GAPDH*. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ND, nondiabetic.

all iPS clones treated with Activin A and Wnt3a (**Figure 5a**, **Supplementary Figure S10a**), although expression levels of *FOXA2* were relatively low (**Supplementary Figure S10a**). Similarly, at day 9 of differentiation, transcripts of primitive gut tube marker *HNF4A* were detected in all clones, but the expression levels varied depending on clones (**Figure 5a**). Clonal variations were more prominent at day 18. T1D-2#2, T1D-2#3, and T1D-3#3 clones did not express some of the key pancreatic endoderm markers including *HNF6* and *NKX6.1* in this timepoint, while ND-1–derived clones demonstrated higher levels of *NKX6.1* induction (**Figure 5b**). *HB9* expression was detected in all the clones differentiated. Most striking differences were observed in iPS progeny in the final stage of differentiation. Reverse transcription-PCR analysis demonstrated variable pancreatic gene expression patterns in individual clones at day 26. In accordance with immunostaining, four clones (ND-1#3, T1D-1#3, T1D-2#3, and T1D-3#2) expressed *INS* gene as well as other pancreatic genes tested. Three clones (ND-1#1, T1D-2#1, and T1D-3#3) expressed all other genes, except *INS*. Three clones (T1D-1#2, T1D-2#2, and T1D-3#1) did not express two genes including *INS* and *NKX6.1* or *GLUT-2*, while two clones (ND-1#2 and T1D-1#1) failed to express three genes (*PDX1/GCG/INS* and *PDX1/NKX6.1/INS*). Thus, lack of *INS* gene induction was frequently associated with absence of *NKX6.1* and *PDX1* transcripts in fully differentiated cells. Next, we assessed the influence of guided differentiation on the levels of pluripotency-associated factors, *OCT4*, SOX2, *KLF4*, *NANOG*, and *c-MYC*, in T1D-specific iPS clones. Most clones demonstrated marked downregulation of *OCT4* transcripts upon differentiation, while four clones, ND-1#1, ND-1#2, T1D-1#2, and T1D-1#3, showed sustained *OCT4* expression even after 26 days of differentiation (**Figure 6**). *NANOG* expression levels were downregulated after step-wise differentiation (**Figure 6**), while *SOX2* expression was downregulated in all the clones except ND-1#1 (**Figure 6**). In contrast to those pluripotent genes, most iPS clones showed sustained expression of *c-MYC* upon differentiation (**Figure 6**). Among them, ND-1#2 and T1D-1#1 showed over twofold increase in *c-MYC* transcripts. Similarly, *KLF4* expression generally persisted after differentiation and three clones (ND-1#1, ND-1#2, and T1D-3#2) showed upregulation of *KLF4* upon guided differentiation (**Figure 6**).

DISCUSSION

Present study provides a systematic blueprint for generation and characterization of multiple iPS clones from individuals with or without T1D, and determines patient- and clone-specific differentiation propensity and proficiency to yield functional INS-producing progeny. Derivation of iPS cells from T1D patients was reproducible and T1D-specific iPS clones maintained pluripotency

under feeder-free conditions. All iPS clones tested demonstrated the capacity to differentiate into SOX17- and FOXA2-positive DE cells. Although clonal variations became increasingly prominent upon further guided differentiation of iPS progeny into primitive gut tube- and islet-like cells, one iPS clone from each patient were capable of differentiating into INS-producing islet-like cells. Derivation and differentiation of T1D-specific iPS cells into disease-relevant cell types paves the way for novel individualized medicine applications for T1D.

Disease-specific iPS cells allow, in principle, generation of large numbers of disease-relevant, genetically matched cells which could provide a unique platform for diagnostic and therapeutic applications. Successful derivation of disease-specific iPS cells and their differentiation into disease-relevant cell types have been reported from patients with various human diseases.[9–13,30,33] Importantly, several studies demonstrated that patient-specific iPS cells recapitulate disease phenotypes,[14–16] verifying the potential applications of patient-specific iPS cells for disease modeling. T1D-specific iPS cells have been reported from three patients to date,[11,30] and three iPS clones from two patients have been successfully differentiated into INS-producing cells.[30] However, as only few disease-specific iPS clones and an ES cell line, rather than iPS clones from a ND individual, was used as a control,[30] the reproducibility of the differentiation proficiencies among T1D-specific iPS clones remain to be determined. The present study demonstrates the feasibility and reproducibility of multiple iPS clones derivation from T1D patients. Selected T1D-specific iPS clones were proficient in yielding glucose-responsive, INS-producing cells upon guided differentiation, in turn providing a platform whereby to model disease and eventually lead to novel cell replacement therapy. In modeling T1D, a complex genetic trait involving immunological reaction, both iPS-derived β-like cells and the reconstruction of the autologous immune response would be required to recapitulate patient-specific T1D progression. Nevertheless, an iPS-based system would enable detailed analysis of patient-specific immune-mediated destruction of β cells at a cellular level.

Despite the promise of iPS technologies, recent studies with standard mouse and human iPS lines have demonstrated potential barriers to diagnostic and therapeutic applications of patient-specific iPS cells. For instance, the reprogramming process and subsequent expansion of iPS cells can induce genetic and epigenetic abnormalities,[34–37] whereas atypical gene expression patterns in iPS progeny can induce T-cell–dependent immune response in syngeneic recipients.[38] In addition, substantial differences in spontaneous differentiation propensities have been demonstrated among human ES cell lines and standard iPS lines.[39,40] Variations in differentiation propensities for INS-producing cells have also been noted among human iPS lines.[27–29] However, these studies focused on few standard iPS lines and, therefore, the variations among patient-specific iPS clones, especially for their pancreatic differentiation propensities, remain to be determined. Here, immunostaining and quantitative PCR analysis of differentiating iPS cells at various timepoints revealed differential induction of stage-specific pancreatic genes among iPS clones. Almost all clones were able to induce key pancreatic differentiation factors, such as *PDX1* and *NKX6.1*, at some stage, but strong variations were evident in the degree of expression and the timing of induction. For instance, *NKX6.1* transcript was present on differentiation at day 26, but not on day 18, in iPS clone T1D-2#3, while other clones showed induction of *NKX6.1* on day 18. Although PDX1 protein expression was detected on day 9 in all iPS clones by immunostaining, *PDX1* expression was not detected in iPS clones ND-1#2 and T1D-1#1 on day 26. Notable correlation was observed between the lack of *PDX1* and/or *NXK6.1* induction on day 26 and the failure to induce INS-producing cells. In addition, notable variations in regulating pluripotency gene expression were observed before and after step-wise differentiation. Successfully differentiated clones also downregulated pluripotency genes, except one clone, T1D-3#2 where *KLF4* expression was upregulated. Thus, differential regulation of pancreatic lineage-specifying genes and pluripotency-associated genes underlined the clonal variability of T1D-specific iPS clones.

In contrast to the clear intrapatient variability, interpatient differences were not prominent. This is in part due to the strong intrapatient variations which could mask variations between T1D patients or between ND and T1D subjects. Nevertheless, common trends in iPS clones were consistent from the same donors. Six iPS clones from T1D-2 and T1D-3 showed consistent and high levels of induction of most islet markers; *ISL1*, *NEUROD1*, *MAFA*, *PDX1*, *GLUT-2*, *SST*, and *GCG*. Further analysis using additional iPS clones from individual donors may reveal interpatient or inter-disease variations.

Individualized iPS applications require the selection of representative iPS clones that reproducibly and reliably differentiate into disease-relevant cell types from each patient. The observed intrapatient variations will impose an additional translational challenge, especially for diagnostic or disease-modeling purposes. In this context, reducing clonal variations would accelerate iPS applications in practice for T1D research. Studies have demonstrated that continuous passaging of the iPS cells diminishes the differences between iPS cells and ES cells, implying that iPS cells lose the epigenetic memory inherited from parental cells upon prolonged passaging.[41] This notion suggests increased bioequivalence of individual iPS clones at later passage, and calls for quality assessments after long passage. On the other hand, we also need to pay attention to avoid prolonged *in vitro* passage, which could lead to accumulation of chromosomal abnormalities. Here, we used relatively mature iPS cells at passage 12–34, with the majority exhibiting normal karyotypes. Importantly, PCA of global gene expression profiles of derived iPS clones demonstrated the homogeneity of 10 out of 12 iPS clones and their similarity to human ES cell data. This observation indicates that our iPS clones have lost their epigenetic memories of parental cells by passage 12–34. Other markers for proper reprogramming, such as silencing of transgene expression[42] and *in vitro* spontaneous differentiation through EB formation, further confirmed consistent reprogramming of patient-derived cells. Nevertheless, these commonly used quality assessments were insufficient to achieve consistent pancreatic differentiation of each iPS clone. Of note, successful induction of DE through spontaneous differentiation of human PS cells has been used as one of the key criteria to evaluate the pluripotency of iPS cells.[40] Our data demonstrate that successful DE formation does not guarantee proficiency for the ensuing differentiation process, highlighting the complexity of the problem of clonal variations.

Two of the three iPS clones from patient T1D-1 (T1D-1#1 and T1D-1#3) showed sustained exogenous KLF4 and SOX2 expression (**Figure 1c**) and did not cluster with other iPS clones in the PCA (**Figure 2d**). These data suggest that the two clones were not fully reprogrammed. Stringent quality control would eliminate such partially reprogrammed clones as inappropriate for diagnostic and therapeutic applications. Intriguingly, clone T1D-1#3 was the only clone among the three T1D-1–derived clones, able to differentiate into INS-producing cells. This discrepancy not only underscores the challenge in selecting patient-specific iPS clones for clinical applications, but also offers an attractive potential of generating INS-producing cells from partially reprogrammed cells. Previous studies have demonstrated that human iPS cells retain a residual epigenetic memory of tissue of origin and tend to differentiate preferentially into the lineage from which they were originally derived.[43,44] Here, iPS clones were isolated from ectodermal lineage, skin-derived keratinocytes. It is plausible that deriving iPS cells from endodermal origin cells, such as heptaocytes, may improve the iPS differentiation propensity into INS-producing cells.

As clonal variability arises from differential regulation of stage-specific pancreatic factors and pluripotency-associated genes, the variations could be minimized by increasing lineage specification and omission of the possible reactivation of integrated, pluripotent transgenes. Significant improvement in β-cell differentiation protocols, which currently achieves only few percent of iPS progeny-producing INS[27–30] would result in a more robust, consistent, and orchestrated induction of pancreatic stage-specifying factors. Efficient pancreatic differentiation would also lead to consistent silencing of pluripotency genes. In addition, reprogramming through non-integrating vector system[45–48] would avoid/minimize sustained expression or reactivation of reprogramming factors, including oncogenic *c-MYC*, in differentiating iPS cells. We have recently established transgene-free iPS clones from T1D and T2D patients using Sendai viral vectors.[32] Investigation of their pancreatic differentiation potential will elucidate the role of reprogramming vector integration in clonal variations of iPS cells.

To secure homogenous pools amenable for diagnostic and therapeutic applications of iPS cells for T1D, it would be necessary to derive multiple patient-specific iPS clones and carry out comprehensive fingerprinting of individual clones for their pluripotency and differentiation propensity. As initial screening for the integrity of derived iPS clones, the pluripotency and spontaneous differentiation propensities can be assessed by the high-throughput characterization system, recently established by Bock *et al.*[40] We propose to include additional assessments, induction of key stage-specific markers, such as *SOX17*, *PDX1*, and *NKX6.1*, as well as suppression of pluripotency factors across lineage-specified iPS progeny, in the bioequivalence criteria. This would allow selection of representative iPS clones for diagnostic and disease-modeling applications based on their "central tendency" of differentiation propensity.

In summary, the presented results demonstrate reproducible generation of T1D-specific iPS cells and the requirement of appropriate regulation of pluripotency-associated genes and stage-specific pancreatic factors for successful generation of glucose-responsive INS-producing cells. Our study also highlights the intrapatient variations of patient-specific iPS clones, and the challenges for diagnostic and therapeutic applications of iPS cells. Comprehensive fingerprinting of multiple patient-specific clones and improved differentiation protocols would enable diagnostic and therapeutic applications of iPS cells for basic and translational T1D research.

MATERIALS AND METHODS

Protocols were approved by Mayo Clinic Institutional Review Board and Institutional Animal Care and Use Committee. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Derivation of skin cells from healthy and T1D donors. Three T1D patients and one healthy volunteer were recruited for the study. Patient informations are shown in **Table 1**. Skin cells were isolated from 8 mm dermal biopsies as reported previously.[49] Resulting fibroblasts and keratinocytes are herein referred as T1D patients #1 (T1D-1), #2 (T1D-2), #3 (T1D-3) and subject without diabetes (ND; ND-1).

Reprogramming of patient-specific fibroblasts and keratinocytes. Reprogramming of patient-derived somatic cells was performed as described.[28,50] Briefly, skin cells were transduced with lentiviral vectors, pSIN-OCT4, pSIN-SOX2, pSIN-KLF4, and pSIN-cMYC[50] at multiplicity of infection of 5 each. Three days after infection, cells were replated on Matrigel (#354277; BD Biosciences, San Jose, CA)-coated plates and fed with the serum-free human iPS media[28] consisting of HEScGro medium (Millipore, Billerica, MA) supplemented with 20% (vol/vol) mTeSR-1 maintenance media (Stemcell Technologies, Vancouver, British Columbia, Canada). One to two weeks after vector infection, reprogrammed cells began to form iPS-like colonies and at 3–6 weeks, colonies were picked based on size and morphology. T1D and ND iPS clones were cultured under feeder-free conditions on Matrigel, and expanded using cell dissociation buffer (cat. no. 13151014; Invitrogen, Carlsbad, CA). Three clones from each individual were characterized, expanded, and iPS clones at passage 12–25 were analyzed for their biological properties, including the differentiation propensity for INS-producing cells.

Differentiation of iPS cells into INS-producing cells. *In vitro* differentiation of patient-specific iPS cells was carried out as described.[28] Pancreatic differentiation was initiated by treating iPS clones with 100 ng/ml activin A (Peprotech, Rocky Hill, NJ) and 25 ng/ml Wnt3a (R&D Systems, Minneapolis, MN) in advanced RPMI (A-RPMI; Invitrogen) for 1 day, followed by treatment with 100 ng/ml activin A in A-RPMI supplemented with 0.2% fetal bovine serum (FBS) (Invitrogen) for 2 days. Differentiated cells were then cultured in A-RPMI medium containing 50 ng/ml FGF10 (R&D Systems), 0.25 µmol/l KAAD-cyclopamine (CYC), and 2% FBS for 2 days. Next, cells were treated with 50 ng/ml FGF10, 0.25 µmol/l CYC, and 2 µmol/l all-*trans* retinoic acid (Sigma-Aldrich, St Louis, MO) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 1× B27 supplement (Invitrogen) for 4 days. Cells were then cultured in the presence of 50 ng/ml FGF10, 300 nmol/l IndolactamV (Axxora, San Diego, CA), and 55 nmol/l GLP-1 (Sigma-Aldrich) in DMEM with 1× B27 for 4 days. Differentiation medium including 10 µmol/l DAPT (Sigma-Aldrich) and 55 nmol/l GLP-1 in DMEM with 1× B27 was used to culture cells for the next 6 days. Finally cells were cultured in 50 ng/ml hepatocyte growth factor (R&D Systems), 50 ng/ml INS-like growth factor 1 (R&D Systems) and 55 nmol/l GLP-1 in CMRL-1066 medium (Invitrogen) with 1× B27 for 6 days. All media were supplemented with antibiotics penicillin/streptomycin. All experiments were validated three or more times.

Immunostaining. Immunofluorescence analysis was performed for characterization of undifferentiated and differentiated cells at various stages of differentiation. Cells were fixed for 20 minutes at room temperature in 4%

paraformaldehyde in phosphate-buffered saline (PBS), washed three times in PBS and blocked for 30 minutes in PBST (PBS with 0.1% Tween-20 and 5% FBS). Cells were stained with primary antibodies overnight at 4 °C and with relevant secondary antibodies for 1 hour at room temperature. Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) and analyzed by confocal laser-scanning microscopy (LSM 510; Zeiss, Thornwood, NY). The primary and secondary antibodies are listed in **Supplementary Table S1**. Alkaline phosphate staining was performed with an Alkaline Phosphatase Detection Kit (Millipore).

Spontaneous differentiation and teratoma formation assay. For spontaneous differentiation, T1D and ND iPS clones were dissociated using collagenase IV and cultured on low adhesion plates in basal HEScGRO medium (#SCM 021; Millipore) for EB formation. EBs were cultured as suspension for 10 days and then allowed to adhere in knockout DMEM with 20% FBS and further cultured for 10–14 days. Differentiated cells were analyzed for markers of three germ layers. Primary and secondary antibodies used are listed in **Supplementary Table S1**. Undifferentiated iPS cells dissociated with collagenase were injected subcutaneously into severe combined immunodeficiency Beige mice. Tumors were detected after 1 month of injection, and dissected out after 2–5 months. Tumor sections were analyzed by hematoxylin and eosin staining.

Karyotyping. Conventional cytogenetic analysis was performed on 20 metaphase cells of representative six iPS clones. Chromosomes were banded following standard methods for high-resolution G-banding. Cells were captured and karyotyped using a CytoVision Karyotyping System (Genetix, New Milton, UK).

Microarray. Total RNA was isolated from iPS clones using Trizol (Invitrogen). RNA probes for microarray hybridization were prepared and hybridized to HG-U133 Plus 2 (Affymetrix, Santa Clara, CA) oligonucleotide microarrays according to the manufacturer's protocols. Microarrays were scanned and data were analyzed using standard in-house MicroArray PreProcessing software. The microarray data for H9 human ES cells (GSM551202) was obtained from GEO DataSets. The fold changes were calculated for all genes in keratinocytes over the corresponding average in iPS clones. The top 100 probe sets most specifically expressed in the keratinocytes and human ES cells were selected for the heatmap generation. Partek software (Partek, St Louis, MO) was used to generate heatmap and PCA plot.

Gene expression. Total RNA was isolated with the Trizol reagent (Invitrogen) was used for semiquantitative reverse transcription-PCR and real-time PCR analysis. Reverse transcription was performed using the RNA to cDNA EcoDry TM Premix (Oligo dT) kit (cat. no. 639543; Clontech, Mountain View, CA). Platinum Taq DNA polymerase (Invitrogen) was used for PCR reactions. Primers used to determine expression of pluripotency genes (*OCT4*, *SOX2*, *KLF4*, *NANOG*, *GDF3*, *hTERT*, and *c-MYC)* are listed in **Supplementary Table S2**. Real-time PCR was performed using pancreatic genes (*FOXA2*, *SOX17*, *HNF4α*, *HB9*, *HNF6*, *PDX1*, *ISL-1*, *NGN3*, *NKX 6.1*, *NKX2.2*, *GLUT-2*, *INS*, *SST*, and *GCG*), and control *GAPDH* using Roche PCR probes (Roche, Indianapolis, IN). Values calculated using the comparative threshold cycle (ΔCt) method and normalized to *GAPDH* values. Real-time PCR primers and probes are listed in **Supplementary Table S3**. Quantitative real-time PCR analysis was performed for lentiviral transgenes using IDT probes, **Supplementary Table S4**.

Measuring glucose-stimulated C-peptide secretion from iPS-derived pancreatic hormone-expressing cells. T1D- and ND-iPS–derived islet-like cells were tested for C-peptide secretion upon glucose stimulation. Krebs-Ringer solution with bicarbonate and HEPES (KRBH; 129 mmol/l NaCl, 4.8 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 5 mmol/l $NaHCO_3$, 10 mmol/l HEPES, 0.1% (wt/vol) BSA) was used for the assay. Cell were initially incubated in KRBH buffer containing 3 mmol/l D-glucose for 1 hour at 37 °C, followed by glucose stimulation conditions containing 27 mmol/l D-glucose for 1 hour at 37 °C. C-peptide levels were determined using an ultrasensitive C-peptide/proinsulin ELISA kit (Alpco Diagnostics, Salem, NH).

Flow cytometry. T1D- and ND-iPS–derived cells were dissociated into single cells using TrypLE (#12605; Invitrogen) at 37 °C. Intracellular antibody staining was performed using BD Cytofix/Cytoperm and BD Perm/Wash buffer (BD, San Diego, CA). The primary and secondary antibodies are listed in **Supplementary Table S1**. Flow cytometry data were acquired on a Becton Dickinson FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed using Flowjo software.

SUPPLEMENTARY MATERIAL

Figure S1. ND and T1D iPS clones exhibit ES-like cell morphology and express alkaline phosphatase.
Figure S2. T1D-iPS clones express pluripotency markers.
Figure S3. ND- and T1D-iPS clones maintain normal karyotype.
Figure S4. Spontaneous differentiation and teratoma formation of T1D-iPS clones.
Figure S5. Efficient induction of definitive endoderm after Activin A and Wnt 3a treatment.
Figure S6. Quantification of iPS-derived definitive endoderm progeny.
Figure S7. Generation of primitive gut tube cells.
Figure S8. Successful generation of posterior foregut cells from iPS-derived primitive gut tube cells.
Figure S9. Guided differentiation of iPS clones generated pancreatic progenitors and somatostatin- and glucagon-expressing cells.
Figure S10. Pancreas development–stage-specific gene expression in differentiated T1D-specific iPS cells.
Table S1. Antibodies used in this study.
Table S2. Primers for characterization of human iPS cells.
Table S3. Primers and probes used in this study.
Table S4. Primers and probes used to detect the exogenous genes.

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