Integration-Free Human Induced Pluripotent Stem Cells From Type 1 Diabetes Patient Skin Fibroblasts Show Increased Abundance of Pancreas-Specific microRNAs

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Type 1 diabetes (T1D) is a disease that is typically associated with multigenetic changes as well as environmental triggers. Disease-specific induced pluripotent stem cells (iPSCs) are preferable cell sources to study T1D, as they are derived from patient cells and therefore capture the disease genotype in a stem cell line. The purpose of this study was to generate integration-free iPSCs from adult skin fibroblasts with T1D. iPSCs were generated by transfection of synthetic mRNAs encoding transcription factors OCT4, SOX2, KLF4, c-MYC, and LIN28. Phase-contrast microscopy, immunocytochemistry, karyotyping, bisulfite genomic sequencing, reverse transcription-polymerase chain reaction, and teratoma formation assay were used to determine reprogramming efficiency, pluripotency, and differentiation potential. Following 18 consecutive days of synthetic mRNA transfections, the T1D patient skin fibroblasts underwent morphological changes, and the aggregated clumps exhibited a human embryonic stem cell (ESC)-like morphology with a high nucleus/cytoplasm ratio. Highly efficient generation of iPSCs was achieved using the mRNA reprogramming approach. The disease-specific iPSCs expressed pluripotency markers, maintained a normal karyotype, and formed teratomas containing tissues representative of the three germ layers when injected into immune-deficient mice. Of interest, the iPSCs showed upregulations of pancreas-specific microRNAs, compared with parental fibroblasts. These data indicate that T1D patient skin fibroblasts can be reprogrammed to pluripotency using a synthetic mRNA approach. These cells can serve as a useful tool for the identification of genes that are involved in autoimmune reactions as well as generating patient-matched β -cells for cell-based therapy.

Key words: Type 1 diabetes (T1D); Induced pluripotent stem cells (iPSCs); Pluripotency reprogramming; microRNA

INTRODUCTION

A cure for type 1 diabetes (T1D) patients could be achieved by β -cell transplantation therapy combined with induction of tolerance to the transplanted cells (24). Currently, tissue/cell replacement is severely limited by the need for allogenic donors with inadequate pancreas donation, high surgical morbidity, high rejection rates, poor long-term success (35–50% insulin independence at 5 years), and significant complications from immunosuppressive therapy posttransplant. Pluripotent stem cells provide an alternative source of β -cells, as they can be differentiated into any cell type of the body. Patient-specific induced pluripotent stem cells (iPSCs) constitute a

preferable pluripotent source over human embryonic stem cells (ESCs), as they are autologous to the patient and capture the disease genotype. Moreover, T1D-iPSCs could be induced to differentiate into immune cells as well as β -cells, for interaction studies or differentiated β -cells that are resistant to autoimmune destruction through genetic modification.

Reprogramming of somatic cells, such as skin fibroblasts, to pluripotency was first achieved by forced expression of four transcription factors using integrating retroviral or lentiviral vectors (11,20,22,26), which presents a formidable barrier to therapeutic application of iPSCs (13). Recently, iPSCs have been generated from fibroblasts from

Received February 20, 2014; final acceptance April 29, 2014. Online prepub date: May 2, 2014.

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nonobese diabetic (NOD) mouse and T1D using viral-based methods for studying the disease (10,12). The objectives of the present study were to derive and characterize integration-free iPSCs from adult T1D patient skin fibroblasts using synthetic mRNAs encoding reprogramming factors.

MATERIALS AND METHODS

Ethics Statement

Informed written consent was obtained from the participant before collection of skin samples. The Human Research Ethics Committee of the Southern Health Monash Medical Centre at Clayton approved the study.

Cell Culture

All reagents were obtained from Life Technologies (Mulgrave, VIC, Australia) unless otherwise indicated. Skin fibroblasts from a T1D patient (34 years old, male) were derived from a dermal punch biopsy. Briefly, ~8-mm-diameter skin biopsies were sliced into smaller pieces and grown in a six-well dish (Corning, Mornington, VIC, Australia). Fibroblast medium [Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin] was added to just immerse the tissue fragments. Fibroblasts that grew out of the tissue were propagated (designated as MMCF1). Human BJ foreskin fibroblasts (Stemgent, Cambridge, MA, USA) were used as a disease-free control. Commercial Pluriton medium (Stemgent) was used for induction of pluripotency. The generated iPSCs and a human male ESC line MEL-1 (Australia Stem Cell Centre, Monash University, Clayton, VIC, Australia) were cultured in human iPSC medium, consisting of knockout DMEM/F-12, 20% knockout serum replacement, 1% non-essential amino acids, 2 mM L-glutamine, 8 ng/ml basic fibroblast growth factor (bFGF, Millipore, Macquarie Park, NSW, Australia), 0.1 mM 2-mercaptoethanol, and 1% penicillin/streptomycin (25 U/ml penicillin and 25 µg/ml streptomycin). All of the iPSCs and ESCs were maintained on inactivated mouse embryonic fibroblast (MEF) feeder cells and passaged enzymatically using 4 mg/ml dispase.

Conditioned Medium Preparation

Mitomycin-C (4×10^6)-treated newborn human foreskin fibroblast (NuFF) cells (GlobalStem, Rockville, MD, USA) were plated in a T-75 flask (Corning) and cultured in 25 ml Pluriton medium with 4 ng/ml bFGF. After 24-h incubation, the medium in the T-75 flask can be collected as conditioned Pluriton medium. All of the conditioned medium from six collections was pooled together, filtered using 0.22 µm low protein-binding filters (Corning), and frozen at -20°C until use.

mRNA Reprogramming

MMCF1 and BJ cells were seeded 1 day prior to the mRNA transfection at the density of 2.5×10^4 cells/well

and 1×10^4 cells/well, respectively, in a six-well cell culture plate. The mRNA reprogramming cocktail (mRNA reprogramming kit, Stemgent) constituted a mixture of individual 100 ng/µl RNA stocks at a molar stoichiometry of 3:1:1:1:1:1 for the octamer-binding transcription factor 4 (*OCT4*), sex-determining region Y box 2 (*SOX2*), Kruppel-like factor 4 (*KLF4*), *c-MYC*, *LIN28*, and green fluorescent protein (*GFP*) mRNAs, respectively, and transfected to MMCF1 and BJ cells according to manufacturer's instructions. Cells were transfected with the mRNA mixture every day for 18 days. The GFP expression was monitored as an indicator for mRNA transduction. To establish iPSC lines, each colony exhibiting typical human ESC morphology (compact cells within a colony and defined colony edge) was manually cut into small pieces, and all the pieces were transferred to a well of a 12-well plate (Corning) with fresh plated MEF feeder layers. The iPSCs were passaged manually for three to four passages to build robust growth and dense colonies followed by routine enzymatic passages in T-75 flasks (Corning) using dispase.

Gene Expression Analysis

RNA was isolated from cells using RNeasy kit (Qiagen, Chadstone Centre, VIC, Australia), and cDNA synthesis was performed using SuperScript III Reverse Transcriptase and Oligo (dT) primers. Primers used for amplification are listed in Table 1. Quantitative real-time PCR (qPCR) was carried out as described previously (3). Briefly, RNA was isolated using Trizol and was reverse transcribed using cDNA archival kit and analyzed after PCR using TaqMan "Assay-on-Demand" primers/probes (Table 2). All mRNA qPCR results were normalized to 18S ribosomal RNA. MicroRNA-specific stem-loop primers listed in Table 2 were used for reverse transcription. Data are presented as fold over detectable, where a Ct value of 39 is considered undetectable. Fold over this limit was calculated using the $\Delta\Delta\text{Ct}$ method as described earlier (3). For whole-genome expression analysis, hybridization was carried out using an Agilent 4×44k Whole Human Genome microarray (Agilent Technologies, Santa Clara, CA, USA; #G4112F). Data from all samples were loaded into GeneSpring software (Agilent Technologies) for data analysis. All samples were prepared in duplicates. Data were scaled using default settings for Agilent technologies that included normalizing to the 75th percentile and log₂ transformation.

Immunofluorescence and Alkaline Phosphatase Staining

Cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100 (Sigma-Aldrich, Sydney, Australia). Immunofluorescence staining was performed using primary antibodies (1:100) against OCT4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; #sc-5279), NANOG (Abcam, Cambridge, MA, USA; #ab21624), stage-specific embryonic antigen 3 (SSEA3; Millipore,

Table 1. Gene-Specific Primer Sequences Used for RT-PCR and Bisulfate Sequencing PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
For RT-PCR		
<i>OCT4</i>	TATGCACAACGAGAGGATTTTG	TGTGTCCCAGGCTTCTTTATTT
<i>SOX2</i>	CACCTACAGCATGTCCTACTCG	GGTTTTCTCCATGCTGTTTCTT
<i>NANOG</i>	TCAATGATAGATTTTCAGAGACAG	GGGTAGGTAGGTGCTGAGGC
<i>REX1</i>	GGAAATAGCAGAGTGCTTCGC	CTCTTCTGTTCTGTTTCACACAG
<i>β-ACT</i>	CACCACACCTTCTACAATGAGC	TCGTAGATGGGCACAGTGTGGG
For bisulfate sequencing PCR		
<i>OCT4 (1)</i>	GGATGTTATTAAGATGAAGATAGTTGG	CCTAAACTCCCCTTCAAAATCTATT
<i>OCT4 (2)</i>	TAGTTGGGATGTGTAGAGTTTGAGA	TAAACCAAAACAATCCTTCTACTCC
<i>NANOG</i>	GAGTTAAAGAGTTTTGTTTTTAAAAATTAT	TCCCAAATCTAATAATTTATCATATCTTTC

OCT4, octamer-binding transcription factor 4; SOX2, sex-determining region Y box 2; REX1, reduced expression protein 1; β-ACT, β-actin (control).

#MAB4303), SSEA4 (Millipore, #MAB4304), TRA-1-60 (Millipore, #MAB4360), and TRA-1-81 (Millipore, #MAB4381). Appropriate Alexa Flour® 488 or 594 secondary antibodies (1:400) were used for visualization. Alkaline phosphatase staining was performed using the Alkaline Phosphatase Detection Kit (Millipore), according to the manufacturer's instructions.

DNA Fingerprinting

Genomic DNA was isolated from iPSCs and fibroblasts using DNAeasy kit (Qiagen). DNA fingerprinting of one MMCF1-iPSC line and MMCF1 fibroblasts was analyzed using AmpFISTR Identifiler® Applied Biosystems (Foster City, CA, USA) kit to amplify 15 polymorphic short tandem repeat (STR) loci and amelogenin for sex chromosomes. Analysis was performed by the Medical Genetics Facility, Monash Health Translation Precinct (Clayton, VIC, Australia).

Karyotyping

Karyotype analysis was based on G-band staining of the iPSCs according to a standard protocol described

elsewhere (11). Twenty metaphase spreads were evaluated for each cell line.

Bisulfite Genomic Sequencing

Genomic DNA from the patient iPSCs, their parental fibroblasts, and MEL-1 ESCs were processed for bisulfite modification using the CpGenome DNA modification kit (Millipore). Two conserved cytosine-phosphate-guanosine (CpG)-enriched regions in the OCT4 promoter and one in the NANOG promoter were selected for amplification by PCR as described elsewhere (1), and the primers are listed in Table 1. The PCR products were subcloned into pGEMT-easy vector (Promega, Alexandria, NSW, Australia). Ten clones of each sample were verified by sequencing with Sp6 primer, and methylation status was determined using BiQ Analyzer software (Max Planck Institut Informatik, Saarbrücken, Germany).

Spontaneous Differentiation

For each line analyzed, $\sim 2 \times 10^6$ iPSCs were injected into the hindleg muscle of 6-week-old male nonobese diabetic severe combined immunodeficient (NOD-SCID) mice (Monash Animal Research Platform, Clayton, VIC, Australia) for teratoma formation under Animal Ethics Committee, Monash University-approved ethics. Teratomas were collected 6–8 weeks after injection, fixed in HistoChoice (Amresco, Solon, OH, USA), embedded in paraffin, and stained with hematoxylin and eosin (Sigma-Aldrich) for histological examination.

RESULTS

Generation of Integration-Free T1D-iPSCs From Patient Skin Fibroblasts

We obtained an 8 mm in diameter skin biopsy sample from a 34-year-old Caucasian male patient with T1D from 3 years of age. Body mass index, human leukocyte antigen (HLA) haplotype, and other clinical data are provided in Table 3. Fibroblasts isolated from the patient skin sample were transfected, along with control BJ fibroblasts, using

Table 2. TaqMan qRT-PCR Gene Expression Assay ID and miRNA Assay ID

No.	Name of the gene	Assay ID
1	Insulin (INS)	Hs00355773_m1
2	Glucagon (GCG)	Hs00174967_m1
3	Somatostatin (SST)	Hs00174949_m1
4	Pancreas and duodenal homeobox 1 (PDX1)	Hs00236830_m1
5	Neurogenin 3 (NGN3)	Hs00360700_g1
6	Hepatocyte nuclear factor 3 β (HNF3b)	Hs00232764_m1
7	18S ribosomal RNA	Hs99999901_s1
8	microRNA 7 (miR-7)	000386
9	miR-9	000583
10	miR-30c	000419
11	miR-30d	000420
12	miR-375	000564

Table 3. Patient Information

Patient	Race	Age/Sex	Diagnosed at Age of	Family History	History of Ketoacidosis	Body Mass Index	Medication	Hemoglobin A1C, %	HLA
Type 1 diabetes	Caucasian	34/male	3	Grandmother and cousin with type 1 diabetes	Yes	30	Insulin 45 U/day	7.3%	DR0301/0404

A1C, glycated hemoglobin; HLA, human leukocyte antigen.

the mRNA mixtures (Fig. 1A). GFP-positive cells could be observed under the fluorescent microscope 15 h after the first transfection, and more cells fluoresced following repeat transfections in both cell types (Fig. 1B and C). Human iPSC colonies were recognizable by day 11 of

transfection in BJ cell cultures, and by day 18 in MMCF1 cell cultures (Fig. 1B and C). A total of 56 and 11 iPSC colonies were picked from transfected BJ and MMCF1 cells, respectively (Fig. 1D). The efficiency of iPSC derivation was therefore 0.56% and 0.044% in BJ and

Figure 1. Five factor mRNA reprogram type 1 diabetes (T1D) patient fibroblasts to pluripotency. (A) Schematic of mRNA reprogramming protocol using Pluriton medium and conditioned Pluriton medium. Day 0 is the start of mRNA transfection. (B) Representative images of MMCF1 fibroblasts (from patient), reprogrammed cells (colony formation), and green fluorescent protein (GFP) expression at 15 h and 18 days of mRNA transfection. Scale bar: 200 μ m. (C) Representative images of BJ fibroblasts, reprogrammed cells (colony formation), and GFP expression at 15 h and 11 days of mRNA transfection. (D) Numbers of BJ-induced pluripotent stem cell (iPSC) colonies (blue bar) and MMCF1-iPSC colonies (green bar) per well at day 18 of transfection. (E) Alkaline phosphatase activity staining and immunostaining for pluripotency markers NANOG, octamer-binding transcription factor 4 (OCT4), stage-specific embryonic antigen 3 (SSEA3), Tra-1-60, and Tra-1-81 in the MMCF1-iPSC1. Scale bar: 100 μ m. (F) Expression of endogenous genes *OCT4*, sex-determining region Y box 2 (*SOX2*), *NANOG*, and reduced expression protein 1 (*REX1*) in the all iPSC lines, but not in the parental fibroblast lines, was assessed by RT-PCR. β -Actin was used as a loading control.

MMCF1 fibroblasts, respectively. Colonies were picked and expanded in six-well plates and mechanically split for the first three to four passages. Then these cells were expanded following enzymatic digestion with dispase in T-75 flasks. Three iPSC lines from the T1D patient fibroblasts (MMCF1-iPSC1, MMCF1-iPSC2, and MMCF1-iPSC4) and two lines from BJ cells (BJ-iPSC1 and BJ-iPSC7) were selected for further characterization.

Pluripotency Characterization of T1D-iPSCs

The T1D-iPSCs were positive for alkaline phosphatase activity (Fig. 1E) and expressed transcription factors *OCT4*, *NANOG*, *SOX2*, reduced expression protein 1 (*REX1*), and cell surface antigens SSEA3, TRA-1-60, and TRA-1-81, as analyzed by immunofluorescence staining (Fig. 1E) and RT-PCR (Fig. 1F). DNA fingerprinting confirmed that the parental origin of the MMCF1-iPSCs was from the T1D patient fibroblasts (Table 4). Bisulfite sequencing analysis revealed that promoter regions examined for both *OCT4* and *NANOG* were demethylated in MMCF1-iPSCs in a manner similar to the MEL-1 ESCs, compared with the heavily methylated patterns observed in the parental fibroblasts (Fig. 2A). G-banding analysis demonstrated a normal chromosome number (46, XY) karyotype (Fig. 2B). Global gene expression profiles of the MMCF1-iPSCs, parental MMCF1 fibroblasts, BJ cells, BJ-iPSCs, and MEL-1 ESCs were obtained using DNA microarrays. Hierarchical clustering analyses confirmed that genome-wide expression profiles of MMCF1-iPSC lines were similar to and cluster with MEL-1 ESC and BJ-iPSC lines rather than MMCF1 or BJ fibroblasts (Fig. 2C). Next, the patient iPSCs demonstrated

differentiation potential by teratoma formation assays. MMCF1-iPSCs generated well-differentiated teratomas, which showed tissues representing three germ layers including gland-epithelium (endoderm), cartilage, muscles, and hepatocyte-like cells (mesoderm), and neuron rosettes (ectoderm) (Fig. 3).

Characterizations of Pancreatic-Specific mRNAs and microRNAs

Similarities in expression profiles of pancreatic transcription factors [pancreatic and duodenal homeobox 1 (*PDX1*), neurogenin 3 (*NGN3*), and hepatocyte nuclear factor 3b (*HNF3B*) or forkhead box A2 (*FOXA2*)] and prohormones [insulin (*INS*), glucagon (*GCG*), and somatostatin (*SST*)] in MMCF1 as well as BJ fibroblasts and the iPSC lines compared with MEL-1 ESCs implies an open chromatin conformation at these gene promoters in all iPSC lines and MEL-1 ESCs (Fig. 4A). Pancreas-specific microRNA 7 (miR-7), miR-9, and miR-375 are five- to 80-fold abundant in the iPSCs compared to the parental fibroblasts, while miR-30c and miR-30d that are involved in maintaining β -cell phenotype as well as insulin transcription remain unchanged in iPSCs compared to parental fibroblasts (Fig. 4B and C).

DISCUSSION

The study shows that integration-free iPSCs can be obtained from adult T1D patient skin fibroblasts by transfection of synthetic mRNA encoding the five transcription factors. The disease-specific iPSCs demonstrate normal karyotypes, expression of key pluripotent genes, and differentiation potential to the three germ layer tissues. The T1D patient-specific iPSCs also show upregulation of pancreas-specific microRNAs.

The advent of iPSC technology has significant implications for research and clinical application in T1D. In T1D, insulin-producing islet β -cells are destroyed by the autoimmune activities; moreover, any transplanted pancreas, purified islets, or β -cells will be rapidly destroyed by the body's own established anti-islet memory T-lymphocytes in the absence of any efficacious immunosuppression (19). Disease iPSCs capturing the T1D genotype have multiple advantages. These can be differentiated into β -cells for therapeutic use or into immune cells to provide an in vitro tool to study cellular interactions in β -cell death. A critical goal in advancing research and future therapies in T1D is to make patient-specific iPSCs that are resistant to autoimmune attack. The generation of such integration-free T1D human iPSCs delivers a unique tool for studying T1D progression and autoimmune mechanisms and ultimately providing cells that can advance current limited options for effective transplantation.

To translate the promises of iPSCs to T1D clinical application, the first step is to develop a reprogramming

Table 4. DNA Fingerprint of Parental Fibroblast Line and MMCF1-iPSC1 Cell Line

Loci	MMCF1 Fibroblasts		MMCF1-iPSC1	
D8S1179	13	–	13	–
D21S11	28	29	28	29
D7S820	9	10	9	10
CSF1P0	10	11	10	11
D3S1358	16	17	16	17
THO1	9	9.3	9	9.3
D13S317	13	14	13	14
D16S539	11	12	11	12
D2S1338	23	24	23	24
D19S433	12	13	12	13
VWA	15	18	15	18
TOPX	9	–	9	–
D18S51	15	–	15	–
Amelogenin	X	Y	X	Y
D5S818	9	13	9	13
FGA	20	22	20	22

MMCF1, fibroblasts from T1D patient; iPSC, induced pluripotent stem cell.

Figure 3. T1D patient iPSCs differentiated to three-germ layer tissue. Hematoxylin and eosin staining of teratomas derived from type 1 diabetes patient three iPSC clones showing endoderm (gland epithelium, arrows)-, mesoderm (muscle, arrow; cartilage, *; hepatocyte-like cells, circled area)-, and ectoderm (neural rosette, Δ)-like structures. Scale bar: 500 μ m.

process without the risk of introducing genetic changes in the resulting iPSCs. To generate integration-free iPSCs, several reprogramming techniques have been explored. iPSCs have been derived with excisable lentiviral and transposon vectors, transient plasmids and episomal systems, or nonintegrated adenoviral vectors (18). However, DNA-based approaches remain the possibility of genomic integration of introduced sequences. Nonintegrating RNA genome Sendai virus-mediated reprogramming requires stringent steps to purge iPSCs of replicating viruses (2,9,17). Reprogramming using protein transduction has been reported, but the recombinant proteins are technically challenging to generate and purify in the quantities required (7,29). Double-stranded microRNAs have been shown to have the ability to induce reprogramming in mouse and human but are limited to adipose stromal cells with very low derivation efficiency so far (14). Modified synthetic mRNAs that encode reprogramming factors have been demonstrated to efficiently reprogram fetal fibroblasts into integration-free iPSCs (25), and this was

extended to disease-specific adult T1D patient skin fibroblasts in the present study.

Several studies have demonstrated that microRNAs (miRNAs) are involved in pancreas development, B-cell proliferation, and insulin secretion (4). Our studies demonstrate that miR-30 family miRNAs, which are involved in maintaining a mature β -cell phenotype (5) and proinsulin gene transcription (21,28), are expressed in iPSCs at levels that are not different from the parental fibroblasts. Of interest, islet-specific miRNAs (miR-7, miR-9, and miR-376) that are expressed in developing (immature) pancreatic endocrine cells are expressed at significantly higher levels in the iPSCs than in the parental fibroblasts. The differential expressions of miRNAs during the cell pluripotent reprogramming may reflect regulatory steps in development of human pancreatic islets.

With major clinical challenges presented by current allogeneic pancreas organ and islet cell transplants, the potential not only to use these cells to understand disease etiology but also to work toward autologous pancreas

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Figure 2. Characterization of the MMCF1-iPSC. (A) Methylation analysis of *OCT4* and *NANOG* promoter regions in MEL-1 ESCs, three MMCF1-iPSC, and MMCF1 fibroblasts. Top numbers indicate the cytosine-phosphate-guanosine (CpG) position relative to the transcription start site. Global percentages of methylated cytosines (% Me) are shown. Each row of circles for a given amplicon represents the methylation status of each CpG in one bacterial clone for the region. Ten clones are shown. Open and filled circles indicate unmethylated and methylated CpG dinucleotides, respectively. (B) Karyotype of the MMCF1-iPSC1 line. (C) Hierarchical cluster analysis of different iPSC, MEL-1 ESC, and fibroblast lines.

Figure 4. Gene and microRNA expression analysis. Pancreatic hormones [insulin (*INS*), glucagon (*GCG*), and somatostatin (*SST*)] and transcription factors [pancreatic and duodenal homeobox 1 (*PDX1*), neurogenin 3 (*NGN3*), and hepatocyte nuclear factor 3b (*HNF3B*)] were analyzed using real-time PCR (A). Pancreas-specific microRNA 7 (*miR-7*), *miR-9*, and *miR-375*, as well as *miR-30c* and *miR-30d* (involved in pancreatic epithelial to mesenchymal transition) were analyzed using real-time PCR in patient MMCF1-iPSCs (B) and BJ-iPSCs (C). The miRNA expression profiles in MMCF1-iPSCs and BJ-iPSCs were calculated for fold over parental MMCF1 and BJ fibroblasts, respectively.

transplants with cells derived from dermal biopsy is potentially transformative in diabetes. Although clonal variations were observed upon directed differentiation of iPSCs into islet-like cells, derivation and differentiation of T1D-specific iPSCs into disease-relevant cell types paves the way for novel individualized medicine application for the disease (23). The efficiency of an in vitro differentiation process, which directs pluripotent ESCs into insulin-producing, glucose-responsive cells, has improved. One of the strategies is to recapitulate the stepwise process of embryonic development, including

definitive endoderm, pancreatic progenitors, and endocrine progenitors (16). Endocrine progenitors could be further matured in vivo into insulin-expressing cells after transplantation (6,8) or differentiated in vitro into glucagon-secreting α -cells or insulin-secreting β -cells using small molecules that promote directed differentiation (15,27). Without exception, human ESC-directed differentiation protocols can be applied to human iPSCs (27). Moreover, T1D-iPSCs generated by viral reprogramming from two patients were shown to have the ability to differentiate to insulin-producing cells in a previous study

(12). Generation of integration-free T1D patient iPSCs represents an important incremental advance in the field of stem cell research. Clearly many steps remain before this is feasible, but the work presented here moves one step closer to this aspiration. In conclusion, the synthetic mRNA reprogramming system provides an ideal method to generate integration-free iPSCs from adult somatic cells of a patient with T1D. These findings demonstrate the potential utility of the integration-free iPSCs, which are more amenable for transplantation research as they carry no transgene footprint for stem cell-based cell therapy.

ACKNOWLEDGMENTS: *We thank the patient for his participation, the Monash Health Translation Precinct Medical Genomics Facility for reagents and assistance for microarray and data analysis. The authors declare no conflict of interest.*

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