Transgene-Free Disease-Specific Induced Pluripotent Stem Cells from Patients with Type 1 and Type 2 Diabetes

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ABSTRACT
The induced pluripotent stem cell (iPSC) technology enables derivation of patient-specific pluripotent stem cells from adult somatic cells without using an embryonic cell source. Redifferentiation of iPSCs from diabetic patients into pancreatic islets will allow patient-specific disease modeling and autologous cell replacement therapy for failing islets. To date, diabetes-specific iPSCs have been generated from patients with type 1 diabetes using integrating retroviral vectors. However, vector integration into the host genome could compromise the biosafety and differentiation propensities of derived iPSCs. Although various integration-free reprogramming systems have been described, their utility to reprogram somatic cells from patients remains largely undetermined. Here, we used nonintegrating Sendai viral vectors to reprogram cells from patients with type 1 and type 2 diabetes (T2D). Sendai vector infection led to reproducible generation of genomic modification-free iPSCs (SV-iPSCs) from patients with diabetes, including an 85-year-old individual with T2D. SV-iPSCs lost the Sendai viral genome and antigens within 8–12 passages while maintaining pluripotency. Genome-wide transcriptome analysis of SV-iPSCs revealed induction of endogenous pluripotency genes and downregulation of genes involved in the oxidative stress response and the INK4/ARF pathways, including p16INK4a, p15INK4b, and p21CIP1. SV-iPSCs and iPSCs made with integrating lentiviral vectors demonstrated remarkable similarities in global gene expression profiles. Thus, the Sendai vector system facilitates reliable reprogramming of patient cells into transgene-free iPSCs, providing a pluripotent platform for personalized diagnostic and therapeutic approaches for diabetes and diabetes-associated complications.

INTRODUCTION
Because of the capability for multilineage differentiation, embryonic stem (ES) cells are regarded as a promising cell source for regenerative medicine applications. Successful differentiation of ES cells into transplantable tissues could lead to novel cell therapies for severe degenerative diseases, such as diabetes mellitus (DM). The first phase I clinical trials involving human ES cell-derived differentiated cells were recently initiated in the U.S. [1]. In preclinical studies, human ES cells have been differentiated into insulin-producing β cell-like cells [2–4], which can reverse diabetes in rodents [2]. However, ES cell use is associated with ethical issues and limited by immunosuppression, thus restricting widespread clinical applications.

The induced pluripotent stem cell (iPSC) technology allows the generation of autologous pluripotent stem cells from nonembryonic somatic cells, typically through introduction of pluripotency-associated factors OCT3/4, SOX2, KLF4, and c-MYC [5–11] or OCT-3/4, SOX2, NANOG, and LIN28 [12]. iPSCs behave similarly to ES cells with respect to their morphology, growth properties, expression of pluripotency-associated factors, self-renewal, and developmental potential [5–7, 12]. Similar to ES cells, iPSCs can be induced to differentiate into various cell types, including insulin-producing cells [13–15]. In principle, the differentiation of patient-derived iPSCs into disease-relevant cell types offers opportunities for novel personalized diagnostic and therapeutic approaches. Accordingly, disease-specific iPSCs have been derived from various conditions [13, 16–21]. It is notable that some disease-specific iPSCs have demonstrated recapitulation of the respective disease phenotypes upon redifferentiation [22, 23], underscoring the power of patient-specific iPSCs for disease modeling and molecular diagnostics.
Diabetes mellitus is increasing in an epidemic fashion worldwide. The prevalence of diabetes was 12.9% in the U.S. adult population in 2005 [24], and it is projected that by 2050, it will increase to 33% [25]. Globally, the number of affected adults is projected to increase from 300 million in 2010 to 440 million by 2030 [26]. Thus, it is critical to develop novel diagnostic and therapeutic approaches for diabetes. Type 1 diabetes (T1D)-specific iPSC derivation has been reported from 30-, 32-, and 42-year-old patients [13, 18]. Maehr et al. have also demonstrated successful differentiation of T1D-specific iPSCs into insulin-producing cells in vitro [13]. Recently, we have also demonstrated differentiation of T1D- and type 2 diabetes (T2D)-specific iPSCs from epidermal keratinocytes and their differentiation into glucose-responsive, insulin-producing cells [27] (T. Thatava, Y.C. Kudva, R. Edukulla et al., Differential regulation of pluripotency-associated genes and pancreatic lineage specifying genes results in in-patient variations in type 1 diabetes-specific iPSC differentiation into insulin-producing cells [manuscript in preparation]). These reports underscore the feasibility of autologous iPSC derivation from diabetic patients and their possible differentiation into disease-relevant insulin-producing cells. However, it should be noted that those studies used integrating retroviral [13, 18] or lentiviral vectors [27] (Thatava et al., manuscript in preparation) to induce pluripotency. Importantly, integration of reprogramming constructs into host genome inherently harbors the risks of insertional mutagenesis [28, 29] and increased tumorigenicity due to reactivation (or sustained expression) of the vector-encoded proto-oncogene c-MYC [30]. Reactivation of reprogramming factors from integrated vectors could also compromise iPSC differentiation [31] (Thatava et al., manuscript in preparation). Thus, for future applications of DM-specific iPSCs, it is critical to establish a robust and reproducible reprogramming system devoid of vector integration. To date, various nonintegrating vector-mediated reprogramming strategies have been reported, yet their utility in reprogramming clinical samples remains largely untested.

Sendai virus, a murine paramyxovirus, is an enveloped virus with a nonsegmented negative-strand RNA genome. Sendai virus with a deletion in one of the two envelope glycoproteins (F protein) has emerged as an efficient RNA-based gene delivery vehicle, which allows robust and sustained foreign gene expression. Since Sendai virus life cycle/RNA replication occurs in cytoplasm without a DNA phase, there is a minimum risk of vector genome (RNA) integrating into host genome [32, 33]. Recently, we demonstrated efficient, integration-free cellular reprogramming using Sendai viral vectors [34, 35]. Here, we examined the feasibility of iPSC derivation from patients with DM using the Sendai viral vectors and analyzed the biological properties of the cells. We found that the Sendai vectors offered efficient transgene-free reprogramming through the induction of endogenous pluripotent genes and the suppression of genes involved in senescence pathways.

**Materials and Methods**

Written informed consent was obtained from a T1D patient for skin biopsy. Deidentified surgical waste specimens were used to derive T2D-specific keratinocytes. All studies were approved by the Mayo Clinic Institutional Review Board and Biospecimen Review Board.

**Cells**

Normal human primary dermal fibroblasts (NHDFs) were purchased from American Type Culture Collection (Manassas, VA, http://www.atcc.org). For T1D-specific iPSC derivation, a 42-year-old male patient with T1D and end-stage renal disease (ESRD) was recruited for the study. This patient has had T1D for 37 years, and his ESRD is presumed to be DM-related. He has no endogenous insulin secretion (undetectable C-peptide levels). Skin specimens from 8-mm dermal biopsies were used to isolate keratinocytes for reprogramming. For T2D-specific somatic cells, clinical skin specimens from individuals with T2D (patient T2D1: female, 85 years old; T2D2: male, 67 years old) were used for derivation of human keratinocytes (HK cells). Briefly, skin samples were incubated for 3–4 hours at 37°C (for the skin biopsy sample) or overnight at 4°C (for surgical wastes) in a dispase solution (25 U/ml) to separate the epidermis from the dermis. The epidermal layer was then dissociated with a recombinant trypsin/EDTA solution (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) for 30 minutes at 37°C. Tryptsin was neutralized with a defined trypsin inhibitor (Invitrogen) and epidermal pieces were dissociated with pipetting. The suspended epidermal cells were then passed through a 70-μm cell strainer, seeded in a plate coated with an animal component-free coating matrix (Coating Matrix Kit; Invitrogen), and maintained in animal component-free (ACF) Epilife Medium (catalog no. M-EPI-500-CA; Invitrogen) with Supplement S7 (catalog no. S-017-5; Invitrogen). The primary culture initially contained both HK cell and fibroblast populations. Selective trypsinization removed fibroblast cells at approximately 6 minutes, whereas HK cells were dissociated only after 20 minutes of trypsinization. HK cells are readily differentiated when the cells are allowed to become fully confluent or stimulated with calcium. Since differentiated HK cells are resistant to reprogramming, it is critical to maintain HK cells semiconfluent and avoid any reagents containing high levels of calcium.

**Reprogramming Using Sendai and Lentiviral Vectors**

Green fluorescent protein (GFP)-expressing and pluripotency-associated factor-expressing Sendai viral vectors were described previously [34]. Sendai vectors encoding OCT3/4, SOX2, KLF4, and c-MYC were generated as described previously [36]. SeV18+OCT3/4/TsdF, SeV18+SOX2/TsdF, and SeV18+KLF4/TsdF are based on a modestly temperature sensitive Sendai viral vector SeV18+TsdF [36], whereas SeV-HNL-c-MYC/TsdF is highly temperature sensitive [35]. All transgenes in Sendai vectors are driven by this Sendai viral promoter (a 3’ leader sequence). Although we observed accelerated Sendai vector clearance through temperature up-shift, we did not use the temperature-controlled protocol in this study. Lentiviral vectors pSIN-OCT4, pSIN-SOX2, pSIN-KLF4, and pSIN-cMYC were produced as described previously [37]. To minimize calcium-mediated differentiation of HK cells during vector infection, lentiviral vectors were concentrated by ultracentrifugation and resuspended in phosphate-buffered saline (PBS) [38]. Lentiviral titers were determined by immunostaining [37] in primary cardiac fibroblasts [39].

**Generation of iPSCs**

NHDFs from neonatal foreskin were plated and cultured for 1 day in growth medium (Dulbecco’s modified Eagle’s medium...
[DMEM]-high glucose, 10% fetal bovine serum [FBS], nonessential amino acids [NEAA], l-glutamine] at a density of $1.25 \times 10^5$ cells per well of a six-well plate. On day 2, viral supernatant was added at a multiplicity of infection (MOI) of 3, 6, or 9 for each of the four Sendai viral vector constructs. The medium was changed the next day, and the transduced cells were cultured for 5 days. The cells were then trypsinized and plated on irradiated CF1 mouse embryonic fibroblasts in growth medium at density of $1.0 \times 10^5$ cells per well of a six-well plate. The next day the medium was switched to human ESC medium (Knockout DMEM [Invitrogen] with 20% Knockout Serum Replacement [Invitrogen], 0.1 mM β-mercaptoethanol [BME], 0.1 mM NEAA, 1 mM l-glutamine, and 20 ng/ml basic fibroblast growth factor [bFGF]). The medium was changed daily. After 2 weeks postinfection ESC-like colonies began appearing, and at 3 weeks colonies were picked for expansion. The colonies were manually picked using a round-ended pipette, one colony per well, onto irradiated mouse embryonic fibroblasts in a 12-well plate.

For patient-specific iPSC derivation, early passage human HK cells (passages 2–4) were plated at a density of $5 \times 10^5$ cells per well of a Coating Matrix (Invitrogen)-coated 24-well plate in ACF medium, and cells were transduced overnight with four pluripotency-associated factor-expressing Sendai viral vectors [34] or lentiviral vectors. The culture supernatants were replaced daily with ACF medium for 3 days. At 4 days after vector infection, the media were changed to the optimized serum-free/feeder-free iPSC medium (HEScGRO medium; Millipore, Billerica, MA, http://www.millipore.com) supplemented with 25% (vol/vol) mTeSR-1 medium (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) [15]. One to 2 weeks after vector infection, the reprogrammed cells formed small colonies with iPSC morphology. At 3–4 weeks after vector infection, IPS-like clones were picked up by a P200 pipette and plated at one colony per well in Matrigel (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com)-coated wells in a 96-well plate for further expansion.

For immunostaining, cells were fixed for 20 minutes at room temperature in 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 for 10 minutes, and blocked for 30 minutes in PBS supplemented with 5% FBS. Cells were then stained with primary antibodies overnight at 4°C, rinsed with PBS, incubated with secondary antibodies at 1:500 dilutions.

PCR Detection of Sendai and Lentiviral Sequences

Total DNA was isolated using the PureLink Genomic DNA Mini Kit (Invitrogen). ExTaQ DNA polymerase (Takara, Otsu, Japan, http://www.takara.co.jp) was used for the PCRs. Primer sequences for the corresponding amplifications were as follows: Sendai primer 1 ([Sendai virus hemagglutinin-neuraminidase [HN] protein sequence: forward, GCATCTCCTCGGTGCTAGCAGCCAACATAAC; reverse, GTCTGTATGTTGGGAGAAAG]), Sendai primer 2 ([Sendai virus protein sequence: forward, CATGGAAGCGGAGGAGGAAGCAG; reverse, TGGATGTGAGGATGTTGCTTG]), lentivirus primer 1 ([Lentivector spleen focus forming virus promoter sequence: forward, AGTTTTTGTGCTACATTCACTAAAAT; reverse, GTTTAACACAGGGTTGACATT]), lentivirus primer 2 ([Lentivector Rev response element sequence: forward, TAGTCCTTATCATTCCATTCA; reverse, TTCTGTTAAGTTCCATTCCAT]), and lentivirus primer 3 ([Lentivector sequence: forward, CATGGAAGCGGAGGAGGAAGCAG; reverse, TGGATGTGAGGATGTTGCTTG]). We analyzed four Sendai vector-iPS clones and four lentiviral vector-iPS clones for vector integration.
Cytogenetic Analysis

Conventional cytogenetic analysis was performed on 20 metaphase cells from each cell line. Briefly, chromosomes were banded following standard methods for G-banding using trypsin and Leishman (GTL banding) [40]. Cells were captured and karyotyped using a CytoVision Karyotyping System (Genetix, New Milton, U.K., http://www.genetix.com) and described according to the International System of Human Cytogenetic Nomenclature.

Amplified Fragment Length Polymorphism Analysis

Multiplex PCRs were performed using TaqGold (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) with fluorescent-labeled-primers. PCR products were detected in capillaries using an ABI 3730 Genetic Analyzer (Applied Biosystems), and alleles were determined using GeneMapper v4.0 software (Applied Biosystems). Fragment sizes were determined using the internal standard GeneScan 500 LIZ. Markers used in these studies are further outlined in the Short Tandem Repeat DNA Internet Database (National Institute of Standards and Technology, http://www.nist.gov).

Microarray

Microarray analysis was performed using the HG-U133 Plus2 GeneChip Array platform (Affymetrix, Santa Clara, CA, http://www.affymetrix.com), and data were analyzed as reported previously [27]. For comparison of transcriptive data between Sendai vector-reprogrammed iPSCs (SV-iPSCs) with primary keratinocytes and lentiviral vector-reprogrammed iPSCs (LV-iPSCs), the data set of the SV-iPSC clones (T1D SV#D, T2D1 SV#A, T2D2 SV#i) from patients T1D, T2D1, and T2D2; the LV-iPSC clone from the T1D patient (T1D LV2); and a verified fibroblast-derived iPSC clone HCF1 [15] were analyzed and compared with those of the transcriptome of primary keratinocytes (SW3 HK and SW4 HK, here indicated as nondiabetic patients 1 and 2: ND1 HK and ND2 HK; and SW8 HK from a 68-year-old female T2D patient, indicated as T2D3 HK) and SW3 HK- and SW4 HK-derived iPSCs (ND1 LV#b and ND2 LV#NI) [27]. A t test was performed to analyze the significance of the changes (p < 0.05) in the normalized gene expression levels between HK cells and HK-derived iPSC clones. Heatmap Builder software (kindly provided by Dr. Euan Ashley, Stanford School of Medicine) was used to generate the heat map for the transcriptome data set.

RESULTS

Reprogramming Fibroblasts into Transgene-Free iPSCs by Sendai Vectors

Experiments were conducted with human primary fibroblasts to establish the efficiency and effectiveness of the Sendai vector in comparison with lentiviral vectors. Fibroblasts were transduced with a replication-deficient, GFP-encoding Sendai vector at an MOI of 2. Approximately 20% of the cells became GFP-positive at 12 hours postinfection (p.i.), whereas most cells were strongly GFP-positive at 24 hours p.i. (Fig. 1A; also see the supplemental online data for video). We then analyzed the expression of the four reprogramming factors OCT4, SOX2, KLF4, and c-MYC. Fibroblasts were infected with the four reprogramming Sendai vectors at MOIs of 5 each. At 60 hours after infection, strong expression of each factor was verified by immunostaining with specific antibodies (Fig. 1B). When compared with our reprogramming lentiviral vectors, the signal intensities for OCT4, SOX2, and KLF4 were higher in cells transduced with Sendai vectors. Since we optimized the c-MYC-encoding Sendai vector to express low levels of c-MYC [34], c-MYC signals were higher in the cells infected with c-MYC-encoding lentivirus.

To establish efficiency of iPSC initiation, primary fibroblasts were transduced with various concentrations of the reprogramming Sendai vectors (Fig. 2A). Introduction of the four pluripotency factors led to NANOG-positive iPSC colonies, with slightly higher efficiencies observed at MOIs of 0.5, 1, and 2. These results are consistent with our previous studies [34], which showed that lower MOIs result in higher efficiencies. Immunostaining of SV-iPSC clones with anti-Sendai virus HN protein revealed that a subset of cells in SV-iPSC
colonies remained Sendai viral antigen-positive at passage 5 (Fig. 2B). In accordance with this, Sendai virus-mediated OCT4 transgene expression was detected by RT-PCR in the SV-iPSCs at passage 3, whereas no transgene expression was detected in the same clone at passage 8 (Fig. 2C). Immunostaining confirmed the expression of pluripotency markers NANOG and TRA-1–81 in the SV-iPSCs (Fig. 2D). RT-PCR analysis confirmed the upregulation of a series of genes characteristically expressed in human ES cells, including OCT4, SOX2, NANOG, GDF3, hTERT, NODAL, and DPPA4. Expression levels were found to be comparable to those of human embryonic stem cell clones (Fig. 2E).

Generation of Transgene-Free iPSCs from Patients with T1D and T2D

Skin samples from patients with DM were processed into epidermal keratinocytes (HK cells) and maintained in an animal component-free EpiLife medium. When maintained in appropriate conditions, HK cells could be expanded for at least 2 months (10 passages) irrespective of the patients’ age, sex, and diabetes status, although culturing HK cells in high density or exposure of HK cells to high Ca^{2+} resulted in rapid differentiation into terminally differentiated, keratin-rich HK cells. We infected early passage patient-derived HK cells with Sendai viral vectors encoding OCT4, SOX2, KLF4, and c-MYC at MOIs of 5 each, and the EpiLife medium was changed to the optimized feeder-free iPSC medium at 4 days after vector infection. Two weeks after Sendai vector transduction, small sharp-edged, flat, tightly packed iPSC-like colonies emerged, and individual colonies were picked on the basis of their size and morphology at 3–5 weeks p.i. for further expansion and characterization. The number of iPSC-like colonies, expanded without substantial spontaneous differentiation, was between 2 and 10 clones per 10^5 transduced cells, slightly lower than that seen for the fibroblasts. As a control, we also generated T1D-specific iPSCs from the same patients using reprogramming lentiviral vectors.

We first examined the expression of Sendai viral HN protein in derived iPSC clones. Similar to the SV-iPSCs made from fibroblast cells, HK-derived SV-iPSCs retained the SV antigen at passage 5, whereas no SV antigen was detected in lentiviral vector-reprogrammed iPSC clones (Fig. 3A, upper panels). SV-iPSCs lost SV antigens upon prolonged passages, and no HN protein was detected in the cells at passage 12 (Fig. 3A, lower panels). When total cellular DNA from the SV-iPSC clones was tested for possible vector integration, no Sendai viral vector-specific sequence was amplified. In contrast, HIV vector-specific sequences were readily detected in control LV-iPSCs (Fig. 3B). These data confirm the lack of genomic integration of the RNA virus-based Sendai vectors in SV-iPSCs. To exclude the possibility that iPSC clones arose from other human iPSC contamination in the laboratory, DNA fingerprinting analysis was performed and confirmed the genetic identity between the parental HK cells and derived iPSCs (Fig. 3C).

The HK-derived SV-iPSCs that were cultured under feeder-free conditions exhibited morphologies similar to those of human ES or fibroblast-derived iPSCs, and they expressed high levels of pluripotency-associated factors (TRA-1–60, SSEA-4, OCT4, SOX2, KLF4, and NANOG) comparable to those of LV-iPSCs (Fig. 3D). Upon spontaneous differentiation in vitro through embryoid body formation, all SV-iPSC clones
expressed markers of the three embryonic germ layers, including ectoderm (β-III tubulin), endoderm (FOXA2), and mesoderm (CD31) cells (Fig. 3E), verifying the pluripotency of the T1D- and T2D-specific SV-iPSCs.

HK-derived iPSC clones can be in continuous culture for at least 4 months after the initial vector infection (passage 40) without entering a period of replicative crisis. Karyotype analysis of the T1D-specific SV-iPSC clone at passage 37 found cells with normal karyotypes (Fig. 4A, 16 of 20 metaphase analyzed), as well as cells with karyotypic abnormalities (Fig. 4B, trisomy 8, 4 of 20 analyzed). Since the majority of the cells were with normal karyotypes, the observed karyotypic abnormalities were likely due to continuous in vitro passage. Our data (a 20% chromosomal abnormality) were in line with the recent study by Taapken et al. [41], which demonstrated frequent trisomy 8 and trisomy 12 in human iPSCs, made with or...
without integrating vectors, and ES cells (12.50% of 552 cultures from 219 human iPSC lines and 12.90% of 1,163 cultures from 40 human ESC lines).

Suppression of the Oxidative Stress Response and INK4/ARF Pathways in Sendai Virus-Mediated Cellular Reprogramming

Using a microarray representing the genome-wide transcriptome, we compared the global gene-expression patterns between primary HK cells and HK-derived SV-iPSC clones. Induction of key pluripotency genes, such as OCT4, SOX2, NANOG, LIN28, telomerase (TERT), DPPA4, and PODXL, was evident in SV-iPSC clones (Fig. 5A). Intriguingly, HK cells expressed very high levels of endogenous KLF4 and c-MYC, resulting in relative downregulation of these two key reprogramming factors in derived iPSCs (Fig. 5A). Sendai vector-mediated cellular reprogramming was also associated with suppression of genes involved in the senescence-associated, mitochondria/oxidative stress-response pathway (Fig. 5B). Potent inhibition of senescence/apoptosis-regulating INK4/ARF pathways, including more than 30-fold reduction in transcriptome levels in p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, and p21<sup>CIP1</sup>, was observed in SV-iPSC clones.
Overall Similarities in Global Gene Expression Profiles Between SV-iPSCs and LV-iPSCs

We further extended our study to examine the similarity between SV-iPSCs and other human pluripotent stem cells. The global gene expression patterns of SV-iPSC clones were overall similar to those of human ES H9 cells or HK-derived LV-iPSCs, but highly divergent from those of primary HK cells (Fig. 6A). Consistent with this observation, the dendrogram of unsupervised one-way hierarchical clustering analysis demonstrated that SV-iPSC clones formed a branch with LV-iPSC clones, distinct from the branch of nonreprogrammed HK cells (Fig. 6B). Notably, global gene expression profiles failed to distinguish between iPSCs...
made with Sendai vector and iPSCs made with lentiviral vector, or between those made from subjects with and without diabetes. To assess the similarity between SV- and LV-iPSC clones in more detail, we selected the top 100 differentially expressed genes between nonreprogrammed HK cells and SV-iPSC clones and generated a heat map, which also included the data from LV-iPSC clones. When listed next to the 200 differentially expressed genes between nonreprogrammed HK cells and SV-iPSCs (100 highest and 100 lowest, \( p < .05 \)), the transcriptome patterns of LV-iPSCs demonstrated patterns that were basically identical to those of SV-iPSCs (Fig. 6C), further confirming the overall similarity between SV-iPSCs and LV-iPSCs. When the genome-wide transcriptomes of SV-iPSC clones were compared with those of LV-iPSC clones, only 21 genes were identified as differentially regulated between the two groups \( (p < .05) \), more than fourfold differences in the levels of transcripts) (Fig. 6D).

**DISCUSSION**

In this study, we examined the feasibility of using nonintegrating Sendai viral vectors to reprogram primary fibroblasts and diabetic patient-derived keratinocytes, and we characterized the biological properties of the resulting cell lines. Sendai vector infection rapidly transduced both primary fibroblasts and keratinocytes, leading to reproducible generation of genomic modification-free iPSCs (SV-iPSCs), including generation of diabetes-specific iPSCs from an 85-year-old individual with T2D. SV-iPSCs lost Sendai viral genome and antigens within 8–12 passages, thus enabling derivation of transgene-free iPSCs. Genome-wide transcriptome analysis of SV-iPSCs indicated that Sendai vectors induce pluripotency through activation of endogenous pluripotency genes and suppression of genes involved in senescence-associated oxidative stress response and INF4/ARF pathways. Further studies with increased numbers of diabetic patients would be necessary to establish Sendai vector-mediated reprogramming as a routine method and to establish the existence or absence of any systematic differences of gene expression from ES cells. However, our present results clearly demonstrate proof of principle for the utility of the Sendai vector system for generating diabetic patient-specific, transgene-free iPSCs, which will provide a safe and regenerative platform for individualized approaches for diabetes research.

Derivation of T1D- or T2D-specific iPSCs and their differentiation into pancreatic \( \beta \) cells allows patient-specific disease modeling, drug screening, and, ultimately, customized tissue engineering and autologous cell replacement therapy for diabetes. However, multiple issues need to be addressed before autologous iPSCs can serve as a reliable foundation for those novel approaches. One of the major concerns associated with translational applications of diabetes-specific iPSCs is the use of integrating vectors for reprogramming [13, 18, 27]. Indeed, reactivation or sustained expression of vector-encoded pluripotency-associated genes, including proto-oncogene c-MYC, can compromise subsequent iPSC differentiation or increase the tumorigenicity of derived iPSCs [30, 31] (Thatta et al., manuscript in preparation). For therapeutic applications, we also need to pay attention to vector integration itself, because vector integration into host genome is inherently associated with the risks of insertional mutagenesis through disruption of essential gene repair pathways or activation of proto-oncogenes [28, 29]. Cellular reprogramming without integrating vectors can solve both problems. Various nonintegrating reprogramming strategies have been developed, including adenoviral vectors, episomal vectors, and introduction of reprogramming proteins, RNAs, or miRNAs [42–50]. Those reprogramming strategies will have a significant impact on future diagnostic and translation applications of iPSCs, because of their predictable biosafety and improved differentiation of derived iPSCs. Of note, some of those studies have demonstrated very high reprogramming efficiencies. However, those studies typically used murine embryonic fibroblasts or human neonatal or immortalized fibroblasts to demonstrate the capability. Indeed, somatic cells from aged organisms are more resistant to cellular reprogramming than those from their young counterparts [51], whereas immortalization or transformation of somatic cells significantly improves reprogramming efficiency [52]. Thus, the applicability of nonintegrating reprogramming strategies for reproducible generation of disease-specific iPSCs, particularly from elderly patients, remains largely unknown. Our results here demonstrate the feasibility of Sendai vector-mediated reprogramming, which leads to generation of iPSCs that are basically indistinguishable from those generated with conventional lentiviral vectors. The ability to reprogram cells from an 85-year-old patient underscores the power of this vector system for generating diabetes-specific iPSCs. Previously, using lentiviral vectors, we generated multiple T1D-specific iPSC lines and demonstrated considerable intrapatient variation in the propensity to differentiate into pancreatic progenitors and insulin-producing islet-like cells (Thatta et al., manuscript in preparation). The lines that failed to differentiate into insulin-producing cells showed aberrant regulation both of the pluripotency program and of stage-specific pancreatic factors (Thatta et al., manuscript in preparation). It is conceivable that the differences in vector integration sites or vector copy numbers play a critical role in this variation among iPSC clones and that the use of transgene-free iPSCs, made with the Sendai vector system, could minimize clonal variations. We are currently in the process of examining the differentiation propensities among multiple iPSCs from diabetic patients.

In this study, we used skin-derived keratinocytes as the somatic cell source. Intriguingly, our genome-wide transcriptome assay demonstrated high levels of c-MYC and KLF4 in human HK cells (Fig. 5). Indeed, when compared with parental HK cells, c-MYC and KLF4 expression was significantly downregulated in HK-derived iPSCs, suggesting that it may be possible to carry out c-MYC- and KLF4-free reprogramming of human HK cells. By using lentiviral vectors expressing OCT4, SOX2, and KLF4, we were able to generate c-MYC-free iPSCs from neonatal foreskin-derived HK cells (data not shown). However, it remains to be determined whether patient-derived adult HK cells can be reprogrammed without c-MYC or KLF4. Nevertheless, their properties, including easy procurement and animal component-free expansion, make HK cells an attractive source for clinical-grade iPSC derivation. One disadvantage of the use of epidermal keratinocytes, particularly for diabetes-specific iPSC derivation, is the requirement for a skin biopsy. Since poorly controlled diabetic patients often suffer from slowly healing wounds, a skin biopsy may not be an ideal procedure. In this regard, it is notable that Sendai vectors can also efficiently reprogram blood cells [50]. The use of Sendai vectors and blood cells for cellular reprogramming would allow minimally invasive, transgene-free iPSC derivation for future diabetes-specific iPSC generation.
Cellular senescence impairs successful nuclear reprogramming of primary cells from mice [51–53] or human cell lines [51, 54]. In contrast, suppression of cellular senescence, controlled by INK4 (p16INK4a and p15INK4b) and ARF/p19ARF/p21CIP1 pathways, significantly improves the reprogramming efficiency of murine and human somatic cells [51–53, 55]. Previously, we used lentiviral vectors to generate iPSCs from elderly patients with T2D [27]. Our data revealed that successful cellular reprogramming of HK cells from elderly patients is accompanied by telomere elongation and marked suppression of senescence-related genes, including p16INK4a, p15INK4b, and p21CIP1, indicating that HK-derived iPSCs are in a rejuvenated state [27]. Similar observations in SV-iPSCs from patients with diabetes indicate the conserved reprogramming mechanisms, through suppression of cellular senescence pathways, in both integrating and nonintegrating reprogramming strategies. Our results also highlight the critical roles of INK4/ARF/p53 components as a barrier to successful cellular reprogramming. Since RNA viral vectors can be modified to express artificial microRNAs [56], Sendai vectors, modified to coexpress miRNAs for those key senescence factors, may increase the efficiency of iPSC generation from elderly patients.

**CONCLUSION**

Our data demonstrate proof of principle of the utility of Sendai vectors to generate transgene-free iPSCs from patients with T1D or T2D. Diabetes-specific, genomic modification-free iPSCs should allow reliable and reproducible differentiation into disease-relevant cell types with predictable safety bios, providing a patient-specific platform for diagnostic and therapeutic iPSC applications.

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**AUTHOR CONTRIBUTIONS**

Y.C.K. and S.O.: study design and direction, data acquisition and analysis, manuscript discussion and writing; L.V.G. and J.R.D.: study design, data acquisition and analysis, contributions to discussion and manuscript editing; A.A., J.G.D.L., Y.K.K., and T.T.: sample acquisition; M.H. and N.F.: provision of materials, manuscript editing; J.M.W.S. and Y.I.: study design and direction, data acquisition and analysis, manuscript discussion and writing.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

M.H. and N.F. are employees of DNAVEC Corporation. The other authors indicate no potential conflicts of interest.

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