

ORIGINAL ARTICLE

Reprogramming human dermal fibroblast into induced pluripotent stem cells using non-integrative Sendai virus for transduction

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Abstract

Introduction: Induced pluripotent stem cells (iPSC) that exhibit embryonic stem cell-like properties with unlimited self-renewal and multilineage differentiation properties, are a potential cell source in regenerative medicine and cell-based therapy. Although retroviral and lentiviral transduction methods to generate iPSC are well established, the risk of mutagenesis limits the use of these products for therapeutic applications. **Materials and Methods:** In this study, reprogramming of human dermal fibroblasts (NHDF) into iPSC was carried out using non-integrative Sendai virus for transduction. The iPSC clones were characterised based on the morphological changes, gene expression of pluripotency markers, and spontaneous and directed differentiation abilities into cells of different germ layers. **Results:** On day 18-25 post-transduction, colonies with embryonic stem cell-like morphology were obtained. The iPSC generated were free of Sendai genome and transgene after passage 10, as confirmed by RT-PCR. NHDF-derived iPSC expressed multiple pluripotency markers in qRT-PCR and immunofluorescence staining. When cultured in suspension for 8 days, iPSC successfully formed embryoid body-like spheres. NHDF-derived iPSC also demonstrated the ability to undergo directed differentiation into ectoderm and endoderm. **Conclusion:** NHDF were successfully reprogrammed into iPSC using non-integrating Sendai virus for transduction.

Keywords: iPSC, Sendai virus, transduction, human dermal fibroblasts

INTRODUCTION

Direct reprogramming of human somatic cells to pluripotent state was achieved in 2007 through ectopic expression of defined transcription factors, Oct4, Sox2, Klf4 and c-Myc. The resulting cells, induced pluripotent stem cells (iPSC), exhibited embryonic stem cell-like properties with unlimited self-renewal capacity and the ability to differentiate into cell types of all three germ layers.^{1,2} Sharing similar features with embryonic stem cells (ESC), the therapeutic potential of iPSC in cell-based therapies, disease modelling, drug discovery and regenerative medicine is highly promising. Besides, iPSC are not controversial in their source and avoid the ethical issues and moral concerns surrounding ESC.^{3,4}

For the generation of iPSC, retroviral and lentiviral transduction methods are well established with high reprogramming efficiency.

Retroviral and lentiviral vectors deliver the transcription factors by integrating into the host genome randomly. The viruses maintain their transcriptional activity and could affect the host cell coding sequences, thus causing mutation. The risk of insertional mutagenesis and the oncogenic potential of the transcription factors, particularly Klf4 and c-Myc, render these methods unsuitable for therapeutic applications.⁵⁻⁷ Sendai viruses (SeV) which are made of RNA, do not integrate into the host genome or alter the genetic information of the host cell. They are able to generate integration-free iPSC that will be more suitable for future therapeutic applications.^{8,9} In this study, we generated iPSC with zero-footprint from normal dermal fibroblast cells using the non-integrating SeV method as proof-of-concept for future use in stem cell-based therapy.

MATERIALS AND METHODS

Cell culture

Normal human dermal fibroblasts (NHDF) were purchased commercially and maintained in FGM-2 - Fibroblast Growth Media 2 (Lonza, Switzerland) according to the manufacturer's protocol. Reprogrammed cells were maintained in Essential 8 Flex Medium Kit (Gibco, USA). iPSC colonies were manually picked and plated on vitronectin-coated (Gibco, USA) plates. The medium was changed every 48 h. All cells were cultured at 37°C in a 5% CO₂ incubator.

iPSC cell generation

NHDF were reprogrammed using the integration-free CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Invitrogen, USA) following the manufacturer's instruction. Briefly, NHDF were seeded at a density of 40-60% confluency two days before transduction. The calculated volumes of each of the three CytoTune 2.0 Sendai virus (SeV) vectors were added to the cells with Multiplicity of Infection (MOI) of 5-5-3 (KOS MOI = 5, hc-Myc MOI = 5, hKlf4 MOI = 3). The cells were incubated at 37°C and 5% CO₂ atmosphere for 24 h before being replaced with fresh fibroblast medium to remove the SeV vectors. The transduced cells were continuously cultured for another 6 days in fresh fibroblast medium. On day 7 post-transduction, the cells were harvested and plated on vitronectin-coated six-well plate at a cell density of 2.5x10⁵ cells/well. The medium was replaced with E8 Flex medium (Gibco, USA) the next day and every other day until the emergence of iPSC colonies. Colonies with embryonic stem cell-like appearance were manually picked and maintained on vitronectin-coated plates in E8 Flex medium.

Real time RT-PCR

Total RNA was isolated from NHDF and iPSCs using TRIzol (Invitrogen, USA). One microgram of RNA was used for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Ten times diluted cDNA was used for quantitative RT-PCR using a SYBR select master mix kit (Applied Biosystems, USA) in StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). The primers used for qRT-PCR analysis are shown in Table 1. PCR was carried out under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15

s and 60°C for 1 min. The quality of qRT-PCR was validated by single-peaked melting curves of the PCR products. Data was analysed by using the comparative $\Delta\Delta C_t$ method, and the mRNA expression was normalised to the expression level of GAPDH. Reactions were performed in triplicates, and the data presented were derived from three independent experiments. The data were evaluated by Student's *t* test. *p*<0.05 was considered statistically significant.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) at room temperature for 15 min and washed twice in PBS containing 1% BSA. For intracellular staining, cells were permeabilised with 0.2% Triton-X 100 (Pharmacia Biotech, Sweden) for 15 min. Non-specific binding was blocked by using 1% BSA in PBS. The cells were then incubated overnight at 4°C with specific antibodies for either OCT4 (dilution 1:100), SSEA4 (dilution 1:100), NANOG (dilution 1:100) or TRA-1-81 (dilution 1:100) (Merck Millipore, USA). After washing, cells were incubated with a secondary antibody containing fluorescein-conjugated rabbit anti-mouse IgG (Chemicon, Millipore, USA) for 1 h at room temperature. After several washes in PBS, cells were viewed for fluorescence using an inverted fluorescence microscope (Carl Zeiss, Germany).

In vitro spontaneous and lineage-directed differentiation

For *in vitro* spontaneous differentiation, iPSCs were cultured in suspension culture in a standard hESC medium [DMEM/F12 (Gibco, USA), 20% knock-out serum replacement (Gibco, USA), 0.1 mM nonessential amino acids (Gibco, USA), 4 mM L-glutamine (Sigma-Aldrich, USA), 10 ng/mL fibroblast growth factor (bFGF) (Invitrogen) and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, USA)] for 8 days. The embryoid bodies formed were transferred to vitronectin-coated plates for attachment and further differentiation in DMEM/F12, 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) for another 8 days before being harvested. For ectoderm- and endoderm directed differentiation, the iPSCs were cultured in DMEM/F12 medium, 10% FBS supplemented with 100 ng/mL Noggin (Gibco, USA) and 100 ng/mL Activin A (Sigma-Aldrich, USA), respectively. Eight days later, the differentiated cells were harvested. All cells were cultured at 37°C in a 5% CO₂ incubator.

TABLE 1: Sequences of primers used in the study

Gene	Primer sequences (5'-3')
<u>Pluripotency markers</u>	
OCT4	F: GACAGGGGGAGGGGAGGAGCTAGG R: CTTCCCTCCAACCAGTTGCCCAAAC
SOX2	F: GGGAAATGGGAGGGGTGCAAAAGAGG R: TTGCGTGAGTGTGGATGGGATTGGTG
cMyc	F: GCGTCCTGGGAAGGGAGATCCGGAGC R: TTGAGGGGCATCGTCGCGGGAGGCTG
Nanog	F: TTTGGAAGCTGCTGGGGAAG R: GATGGGAGGAGGGGAGAGGA
Rex1	F: GTGGATGCGCACGTGCGTACGC R: CTGGAGGAATACCTGGCATTG
<u>Mesoderm, Endoderm and Endoderm specific markers</u>	
MSX1	F: GAGTTCTCCAGCTCGCTCAG R: TCTCCA GCTCTGCCTCTTGT
GATA2	F: GCTGCACAATGTTAACAGGC R: TCTCCTGCATGCACTTTGAC
AFP	F: AAATGCGTTTCTCGTTGC R: TTTCATCCACCACCAAGC
GATA4	F: CCAAGCAGGACTCTTGGAAC R: GGGAAAGAGGGAAGATTACGC
GATA6	F: TCTACAGCAAGATGAACGGCCTCA R: TCTGCGCCATAAGGTGGTAGTTGT
PAX6	F: TGCTGGAGGATGATGACAGAGGAAT R: TGCTGCTGTTGTTGCTTGAAGACC
CDX2	F: AAAGGCTTGGCTGGTGTATG R: GTCAGGCCTGGAGTCCAATA
TUJ1	F: AGTGATGAGCATGGCATCGACCC R: GGCACGTA CTTGTGAGAAGAGGC
MAP2	F: CAGGCAAAGGACAAAGTCTCTGACG R: CGCCGAGGAGGGAGAATGGAGG
GAPDH	F: GAAATCCCATCACCATCTTCCAGG R: GAGCCCCAGCCTTCTCCATG

RESULTS

Normal human dermal fibroblasts (NHDF) was reprogrammed with non-integrating Sendai virus (SeV) vectors carrying Yamanaka factors, OCT4, SOX2, KLF4 and c-MYC (OSKM). At day 15 post-transduction, embryonic stem cell (ESC)-like clusters started to appear (Fig. 1A).

At day 18-25, the colonies that showed ESC-like morphology with flatter cobblestone-like appearance and clearly defined border were picked and transferred to a new vitronectin-coated plate (Fig. 1B). Gene expression study showed that the endogenous expression level of pluripotency markers, Oct4, Sox2, cMyc, Nanog and Rex1, were up-regulated in NHDF-iPSC

(Fig. 1C). The colonies also stained positively for the stem-cell specific proteins, Oct4, Nanog, SSEA-4 and TRA-1-81 (Fig. 1D). To confirm SeV-based reprogramming method is integration free, RT-PCR was carried out to detect the SeV genome and transgenes (Fig. 1E). At passage 7, vectors carrying SeV and cMyc were still present in NHDF-iPSC; at passage 10, all the SeV genome and transgenes were not detectable, validating the virus-free status in NHDF-iPSC.

On further characterisation on the differentiation capacity of the reprogrammed NHDF, the iPSC were spontaneously differentiated into cells of three germ layers via embryoid body (EB) formation. When cultured in suspension for

up to 8 days in low attachment plate, the colonies formed floating spherical EB (Fig. 1F). When the EB were transferred to bFGF-free hESC medium for another 8 days, the cells experienced spontaneous differentiation and demonstrated enhanced expression levels for markers from mesoderm, endoderm and ectoderm, as shown by qPCR analysis (Fig. 1G). In endoderm and ectoderm directed differentiation assay, qPCR results also showed enhanced expression of ectoderm and endoderm markers in NHDF-iPSC as compared to the parental NHDF cells (Fig. 1H), further confirming the differentiation capacity of the reprogrammed NHDF.

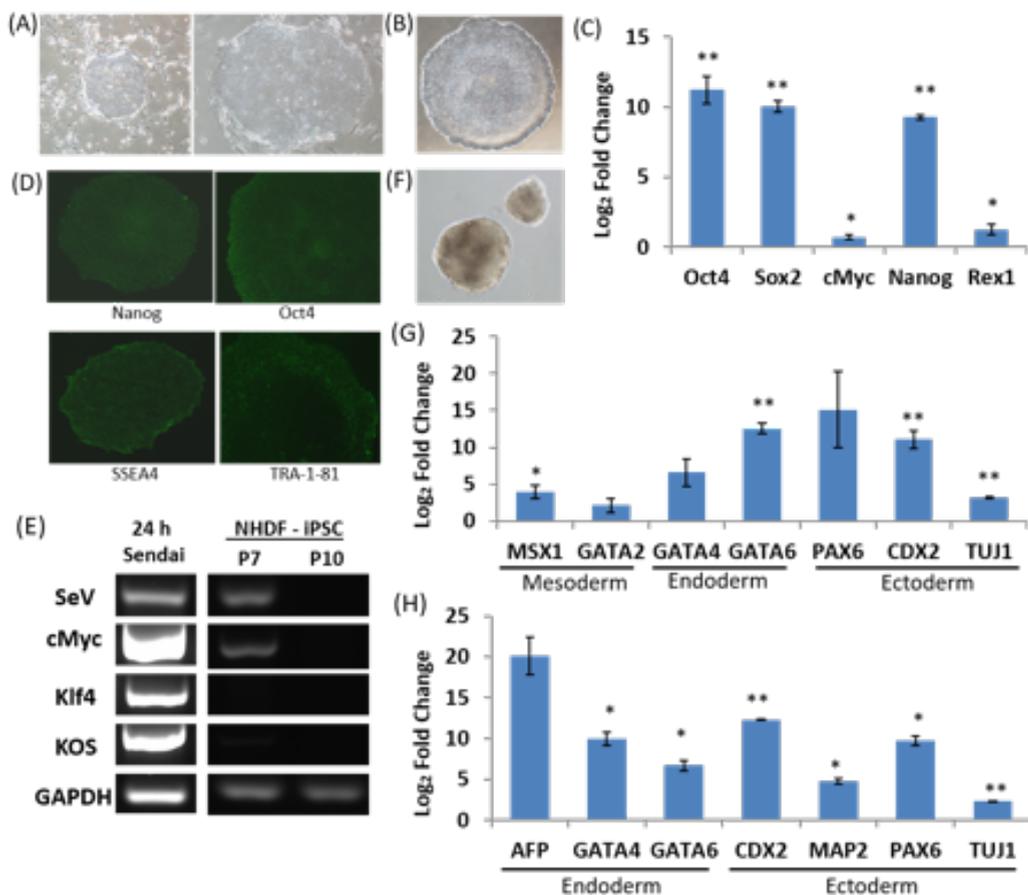


FIG. 1: Reprogrammed NHDF-iPSC showed ESC-like features and pluripotency. (A-B) Cell morphology of iPSC colonies before selection (A) and on culturing (B). (C-D) Expression of pluripotency markers in NHDF-iPSC, quantified by qPCR (C) and immunofluorescence analysis (D). (E) Validation of virus-free NHDF-iPSC by RT-PCR. Cells collected at 24 h post-transduction were used as positive control. (F) Formation of embryoid bodies (EB) when the colonies were cultured in suspension for 8 days. (G) Expression of germ-layer markers in EB that undergo spontaneous differentiation, quantified by qPCR. (H) Expression of ectoderm and endoderm markers in directed differentiated NHDF-iPSCs, quantified by qPCR. In qPCR analysis, the data presented were mean ± SEM from three independent experiments. The gene expression levels were normalised to parental NHDF cells. *p<0.05 and **p<0.01

DISCUSSION

In this work, we show that NHDF was successfully reprogrammed into iPSC by using non-integrating SeV-based reprogramming vector that carried the four Yamanaka factors. Unlike other vectors, SeV effectively deliver the transcription factors into the cells cytoplasm without integrating into the host genome.^{8,9} We confirmed the clearance of the vectors and transgenes in our NHDF-iPSC after ten culture passages. A previous study proved that non-integrating reprogramming method preserved the genomic integrity and displayed lower single nucleotide polymorphism as compared to DNA-integrating reprogramming methods.¹⁰ This makes the virus-free iPSC a potential cell source for applications in cell-based therapy and regenerative medicine. A previous study by Ou *et al.* (2016) demonstrated gene therapy strategy of combining iPSC and CRISPR/Cas9 tool to treat β -thalassaemia in mice model with severely impaired immune system. The study suggested a potential personalised treatment that could be applied in thalassaemic patients using iPSC, eliminating the risk of immune rejection. However, further investigations on the response of immune system on the engraftment are required.¹¹

A pilot clinical study using autologous iPSC-derived retinal pigment epithelial cell sheet in two Japanese patients with neovascular age-related macular degeneration was reported in 2017. The iPSC were derived using non-integrative episomal vectors. The outcome was disappointing. For the first patient at one year after transplantation, the visual acuity had not improved, though the transplanted cell sheet remained intact, and for the second patient, transplantation did not proceed due to concerns on the genetic changes detected in the cell products.^{12,13} Thus, extensive research and guidelines on the safety issue concerning iPSC-based cell therapy, including the genetic abnormalities and disease risks, are critical for future clinical application. Moreover, the defined protocols for iPSC cell production and characterisation, mode of delivery, zero-footprint gene editing protocol, and basic regulation for stem cell therapeutics are to be addressed appropriately. Other requirements such as targeted differentiation into specific cell types and cell maturation remained to be overcome.¹⁴

In conclusion, NHDF were successfully reprogrammed into iPSC using non-integrating Sendai virus for transduction. Generation of iPSC is a promising tool for stem cell-based therapy, but there much work needs to be done before

iPSC can be used for translational purposes.

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