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Establishment of integration-free induced pluripotent stem cells from human recessive dystrophic epidermolysis bullosa keratinocytes

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ABSTRACT

Background: Induced pluripotent stem cell (iPSC) technology enables patient-specific pluripotent stem cells to be derived from adult somatic cells without the use of an embryonic cell source. To date, recessive dystrophic epidermolysis bullosa (RDEB)-specific iPSCs have been generated from patients using integrating retroviral vectors. However, vector integration into the host genome can endanger the biosafety and differentiation propensities of iPSCs. Although various integration-free reprogramming systems have been reported, their utility in reprogramming somatic cells from patients remains largely undetermined.
Objective: Our study aims to establish safe iPSCs from keratinocytes of RDEB patients using non-integration vector.
Method: We optimized and infected non-integrating Sendai viral vectors to reprogram keratinocytes from healthy volunteers and RDEB patients.
Results: Sendai vector infection led to the reproducible generation of genomic modification-free iPSCs from these keratinocytes, which was proved by immunohistochemistry, reverse transcription polymerase chain reaction, methylation assay, teratoma assay and embryoid body formation assay. Furthermore, we confirmed that these iPSCs have the potential to differentiate into dermal fibroblasts and epidermal keratinocytes.
Conclusion: This is the first report to prove that the Sendai vector system facilitates the reliable reprogramming of patient keratinocytes into transgene-free iPSCs, providing another pluripotent platform for personalized diagnostic and therapeutic approaches to RDEB.

1. Introduction

Induced pluripotent stem cells (iPSCs) have a pluripotency that is biologically homologous to that of embryoid stem cells, which are generated from various somatic cells by the induction of reprogramming factors such as *Nanog*, *Klf4*, *Oct3/4* (*Pou5f1*), *Sox2*, and *c-Myc* [1]. Since iPSCs have an unlimited proliferative capacity, they are regarded as a potential source of regenerative therapies for human diseases including Parkinson's disease, spinal cord injury, osteoarthritis, heart failure due to ischemic heart disease or genetic disorders, and diabetes mellitus [2–6]. Patient-specific iPSCs have also been established from patients with various disorders, and these are likely to be useful tools to elucidate pathomechanisms, to screen for effective drugs, and to develop custom-made iPSC-based therapies for each individual. Patient-specific iPSCs have been reported from patients with inherited skin

Abbreviations: bFGF, basic fibroblast growth factor; BJ-iPSC, BJ fibroblast-derived induced pluripotent stem cell; CRISPR/Cas9, clustered regularly interspaced palindromic repeats/CRISPR-associated; DAPI, 4′,6-diamidino-2-phenylindole; EB, epidermolysis bullosa; EBKC-iPSCs, epidermolysis bullosa-specific keratinocyte-derived induced pluripotent stem cells; iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblasts; MMC, mitomycin C; MOI, multiplicity of infection; NHEK, normal human epidermal keratinocyte; NHEK-iPSC, Sendai viral vector-driven induced pluripotent stem cell from normal human epidermal keratinocytes; OCT4, octamer-binding transcription factor 4; PBS, phosphate-buffered saline; RDEB, recessive dystrophic epidermolysis bullosa; RT-PCR, reverse transcription polymerase chain reaction; SSEA-3, stage-specific embryonic antigen-3; SVV, Sendai viral vector.

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disorders such as xeroderma pigmentosum, Hutchinson–Gilford progeria syndrome, Hermansky–Pudlak syndrome, and lipodystrophy [7–10]. Patient-specific iPSCs from epidermolysis bullosa (EB) also have been reported from several group as EB patients-iPSCs from fibroblasts, keratinocytes and also revertant mosaicism, most of such iPSCs have been generated by using integrating retroviral vectors, such as Yamanaka et al. first developed [1,11–14]. However, vector integration into the host genome can compromise the biosafety and differentiation propensities of derived iPSCs when these cells are considered for clinical usage, such as potential tumorigenesis [15]. In light of this, several integration-free reprogramming systems have been recently described for the establishment of iPSCs, such as Sendai virus vectors and episomal vectors [16,17].

The Sendai virus is an enveloped virus with a nonsegmented negative-strand RNA genome [18]. Since viral genome does not integrate into host DNA, the Sendai virus vector (SVV) is a reasonable source for introducing Yamanaka factors (*Oct4, Klf4, Sox2, c-Myc*) to develop iPSCs [18]. Although one group has generated iPSCs from keratinocytes using SVV [19], little investigation has addressed the optimal conditions for establishing iPSCs from keratinocytes, especially those derived from patients with skin disorders. In this study, we elucidated the optimization of SVV-driven iPSC introduction from epidermal keratinocytes. Furthermore, we achieved the first generation of transgene-free iPSCs from the keratinocytes of RDEB patients. These iPSCs are confirmed to differentiate into multiple lineages, including epidermal keratinocytes and dermal fibroblasts as conventional retrovirus-driven iPSCs, which could have potential for safe cell therapies for various skin disorders.

2. Material and methods

2.1. Patients

This study was approved by the Internal Review Board on Ethical Issues of Hokkaido University Hospital (approval number: 014-0041). Written informed consent was obtained from the participants before the processes conducted in this study. NHEKs were cultured from the face skin of a healthy volunteer. In this study, two patients with the RDEB generalized intermediate subtype were enrolled: a 24-year-old Japanese male who is compound heterozygous for c.7723G>A and c.8569G>T (case 1) [20], and a 58-year-old female who is compound heterozygous for c.5444G>A and c.5818delC (case 2) [21]. Both patients have widespread erosions and ulcers on the whole body (Fig. 1).

2.2. Cells and cell culture

To isolate keratinocytes from the skin specimens, we immersed the samples in dispase (1000–13,000 PU/ml, Wako Pure Chemical. Osaka, Japan) solution for 16 h and separated the epidermis mechanically with tweezers. Then we minced epidermis into very small pieces using scissors, and put them into 15 ml conical tube containing 2 ml of 0.25% trypsin and incubated at 37 °C for 7 min. Next, we neutralized the trypsin using 6 ml of phosphate-buffered saline (PBS) containing 10% fetal bovine serum, and centrifuged at 300 × *g*, for 5 min at 25 °C. The medium was aspirated and the cell pellet was resuspended with a desired volume of medium (CnT-PR; CELLnTEC, Bern, Switzerland), and the cells were counted. The cells were seeded at 5000–10,000 cells/cm^2 and placed in a humidified incubator at 37 °C and 5% CO_2. The medium was replaced 24 h after plating and again every 2–3 days until 70–80% confluence. We used the keratinocytes at passage 3–4 for further experiments. Mitomycin C (MMC)-treated mouse embryonic fibroblasts (MEFs) were purchased as feeder cells (ReproCELL Inc., Yokohama, Japan). BJ-iPSCs (RBRC-HPS0063) were purchased from RIKEN BioResource Center (Tsukuba, Japan) through the National Bio-Resource Project of MEXT as a positive control of iPSCs [22].

2.3. Generation of iPSCs from keratinocytes

Since the generation of iPSCs from human fibroblasts using SVVs(Cyto-tune®-iPS2.0, ID Pharma, Tsukuba, Japan) which contain three vectors, polycistronic *Klf4-Oct3/4-Sox2*, *cMyc* and *Klf4* had been already established, we modified the protocol for the reprogramming of keratinocytes. The keratinocytes were seeded at 50,000 cells/cm^2 with CnT-PR medium and placed in a humidified incubator at 37 °C and 5% CO_2. The next day, keratinocytes were transduced by SVVs with four transcription factors: *c-MYC*, *SOX2*, *OCT3/4* and *KLF4* for 48 h. After 6 days, the transduced cells were reseeded on MMC-treated MEFs. The next day, medium changed to human embryonic stem cell medium (Primate ES Cell Medium, ReproCELL) with 5 ng/ml basic fibroblast growth factor (bFGF, ReproCELL) until colonies appeared. On day 28 after transduction, colonies of iPSCs were mechanically picked up and placed on MMC-treated MEFs, and then these iPSCs clones were further cultured with embryonic stem cell medium including 5 ng/ml bFGF. We used iPSCs at passages 8–30 for further investigations.

2.4. In vitro *differentiation of iPSCs*

The protocol for spontaneous *in vitro* differentiation has been described previously [11]. Briefly, iPSC colonies were treated with

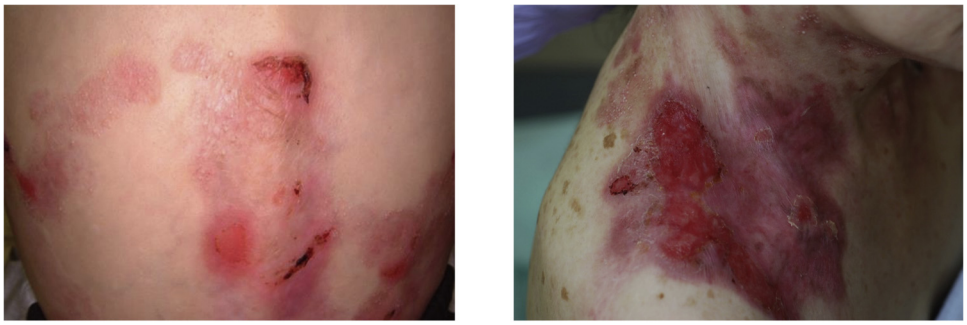

Fig. 1. The clinical manifestations of the generalized intermediate RDEB patients. Case 1: a 24-year-old Japanese male with compound heterozygous mutations for c.7723G>A and c.8569G>T, Case 2: a 58-year-old female with compound heterozygous mutations for c.5444G>A and c.5818delC.

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