

Generation of human-induced pluripotent stem cells from burn patient-derived skin fibroblasts using a non-integrative method

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Abstract. Patient specific induced pluripotent stem cells (iPSCs) have been recognized as a possible source of cells for skin tissue engineering. They have the potential to greatly benefit patients with large areas of burned skin or skin defects. However, the integration virus-based reprogramming method is associated with a high risk of genetic mutation and mouse embryonic fibroblast feeder-cells may be a pollutant. In the present study, human skin fibroblasts (HSFs) were successfully harvested from patients with burns and patient-specific iPSCs were generated using a non-integration method with a feeder-free approach. The octamer-binding transcription factor 4 (OCT4), sex-determining region Y box 2 (SOX2) and NANOG transcription factors were delivered using Sendai virus vectors. iPSCs exhibited representative human embryonic stem cell-like morphology and proliferation characteristics. They also expressed pluripotent markers, including OCT4, NANOG, SOX2, TRA181, stage-specific embryonic antigen 4 and TRA-160, and exhibited a normal karyotype. Teratoma and embryoid body formation revealed that iPSCs were able to differentiate into cells of all three germ layers *in vitro* and *in vivo*. The results of the present study demonstrate that HSFs derived from patients with burns, may be reprogrammed into stem cells with pluripotency, which provides a basis for cell-based skin tissue engineering in the future.

Introduction

Previous studies have suggested that somatic cells from mice and humans may be reprogrammed to become induced pluripotent stem cells (iPSCs) (1,2). The reprogrammed cells were derived from a variety of different tissues and organs, including fibroblasts, keratinocytes and urine renal epithelial cells (3-7). The human skin fibroblast (HSF)-iPSCs demonstrated clear similarities with embryonic stem cells (ESCs) in their proliferation, pluripotency, clonal morphology, growth characteristics, surface markers, gene expression and epigenetics. HSF-iPSCs provide a reliable source of skin seed cells for specific patients, including individuals with large area burns or skin defects (8,9). HSF-iPSCs do not have the same ethical or immune rejection issues that are associated with ESCs (8,9). iPSCs may also be used in drug screening and experiments to aid the understanding of specific disease mechanisms (10-12).

In previous studies, somatic cells were transduced with vectors containing the human transcription factors NANOG, octamer-binding transcription factor 4 (OCT4), sex-determining region Y box 2 (SOX2), Kruppel-like factor 4 (Klf4) and c-Myc, which subsequently reprogrammed them to become iPSCs (1,2). Initially, lentivirus and retrovirus were used as vectors to introduce key transcription factors into target cells and thus induce reprogramming into iPSCs (13,14). However, lentivirus and retrovirus are integrating viruses (15), meaning that the virus gene may insert into the genome of the target cells, potentially causing the reactivation of transgenes, uncontrolled gene silencing and residual expression. These alterations may lead to undesirable consequences, which makes them unsuitable within a clinical setting (16). Using a non-integrating method is considered more appropriate and may eliminate the problem of insertional mutagenesis (17).

Previous research into epigenetics has confirmed that iPSCs retain the memory of epigenetic signatures from their original tissue and are more likely to differentiate toward donor-associated cells (18). Certain imprinted genes associated with growth, metabolism and neurological development of iPSCs and initial somatic cells share the same epigenetic and transcriptional statuses (19). The memory of the epigenetics

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may limit the full differentiation potential of iPSCs (18). In the present study, a non-integrating method was used to reprogram cells from the skin of patients with burns to generate iPSCs. The results of the present study may provide an experimental basis for the clinical use of iPSCs as seed cells.

Materials and methods

Isolation of fibroblasts and cell culture. Human dermal tissues were harvested from residual skin used for skin grafts in patients with burns. All protocols were approved by the Biomedical Ethics Committee of the Affiliated Hospital of Nanchang University (Nanchang, China) and written informed consent was obtained from all patients. Tissues were washed 2-3 times with penicillin/streptomycin and phosphate-buffered saline (PBS) (all from Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) within a vertical clean bench. The tissues were subsequently cut into 2x10 mm sections. The tissues were digested with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 4°C for ~5 h. High glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both from HyClone; GE Healthcare Life Sciences, Logan, UT, USA) was added to terminate the digestion process. The epidermis and dermis were separated using tweezers and dermal tissues were sliced into sections (0.5-1 mm³). The distance between each section was 0.3-0.5 cm, from which the fibroblasts came out. Culture dishes containing the dermal tissues were inverted for drying for 4 h, following which high glucose DMEM with 10% FBS was added and cultured at 37°C for 24 h in an atmosphere containing 5% CO₂. The medium was replaced every 3 days. After 24 days cultured, the fibroblasts were isolated from the dermal sections by 0.25% trypsin-EDTA digestion.

Passage of fibroblasts. To prevent cell density from inhibiting growth and to obtain a larger number of proliferating cells, cells were subcultured when they reached 80-90% confluence. The medium was replaced and cells were detached with 0.5 ml 0.25% trypsin-EDTA solution following washing with PBS. When the intercellular space increased and cells appeared round under an inverted phase contrast microscope (CTR6000; Leica Microsystems GmbH, Wetzlar, Germany), high glucose DMEM with 10% FBS was added to terminate digestion. The cells were centrifuged at 120 x g for 5 min at room temperature. The supernatant was removed and the remaining cell deposit was resuspended in 9 ml high glucose DMEM with 10% FBS and cultured at 37°C with 5% CO₂ following being subcultured at a rate of 1:3. The medium was replaced every 3 days.

Transduction of HSFs and generation of HSFs-iPSCs. At 2 days prior to transduction, HSFs at passage 3 were harvested and inoculated into 2 wells (1 well for counting and the other for transduction; 2.5x10⁵ cells/well) of a 6-well plate with high glucose DMEM and 10% FBS. The cells were completely adhered and stretched on the day of transduction. When cells reached 50-80% confluence they were transduced using the CytoTune™-iPS 2.0 Sendai reprogramming vectors (ID Pharma Co., Ltd., Tsukuba, Japan) containing the human transcription factors NANOG, OCT4 and SOX2 (10 µl of

each transcription tube). Cells were incubated at 37°C in an atmosphere containing 5% CO₂ overnight and the medium was replaced on the first day with fresh high glucose DMEM and 10% FBS to remove the Sendai reprogramming vectors. Following this, the medium was replaced every 2 days. At 6 days following transduction, the 6-well plates were coated with 1% Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). At day 7 post-transduction, the cells were detached with 0.5 ml 0.25% trypsin-EDTA solution following washing with PBS. When the cells were observed as round under the inverted phase contrast microscope, high glucose DMEM with 10% FBS was added to terminate the digestion. Cells were centrifuged at 200 x g for 4 min at room temperature, following which the cell pellet was resuspended in 1 ml high glucose DMEM with 10% FBS. The transduced cells were subsequently cultured in high glucose DMEM with 10% FBS under feeder-free conditions with 1% Matrigel-coated culture dish at a density of 1-5x10⁵ cells/100 mm at 37°C overnight in an atmosphere containing 5% CO₂. The medium was discarded the next day and replaced with reprogramming culture medium (ReproEasy; Beijing Cellapy Biotechnology Co., Ltd., Beijing, China). The medium was replaced every day and the culture was monitored until ESC-like colonies were observed under the inverted phase contrast microscope. At 4 weeks following transduction, the cell colonies were large and compacted enough to be picked out and expanded. They covered the majority of the surface area of the culture dish. The colonies were picked and transferred onto fresh 1% Matrigel-coated dishes with human PSCeasy medium (Beijing Cellapy Biotechnology Co., Ltd.) for expansion.

Picking out and transferring iPSCs colonies. Using a 1 ml syringe, cell colonies were broken into pieces and transferred onto a 1% Matrigel-coated 6-well plate containing 1 ml human PSCeasy medium. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. At 48 h post-transfer, colonies were attached to the culture plate and the medium was replaced. Following this, the medium was replaced every day. When the colonies covered 80-90% of the surface area of the culture plate they were considered ready for passaging.

Alkaline phosphatase (AP) staining. AP staining was performed using the BCIP/NBT Alkaline Phosphatase Color Development kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Briefly, iPSCs were washed with PBS 3 times for 3 min each time. The iPSCs were fixed at room temperature in 4% paraformaldehyde (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) for 20 min and washed again in PBS. BCIP/NBT solution was added to stain the iPSCs at room temperature for 20 min and the reaction was terminated by washing the cells twice with distilled water. Images were captured under an inverted phase contrast microscope (magnification, x100).

Immunofluorescence staining. A round coverslip was coated with 1% Matrigel overnight at 4°C. The following day it was picked out and iPSC colonies were transferred onto it. The colonies were subsequently cultured with human PSCeasy medium at 37°C for 48 h. When the colonies covered the

Table I. Sequences of the primers used in the polymerase chain reactions.

Gene	Primer sequence (5'-3')		Size (bp)
	Forward	Reverse	
OCT4	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT	164
GATA4	GACAATCTGGTTAGGGGAAGC	GAGAGATGCAGTGTGCTCGT	105
MSX1	TGCCTCGCTCTACGGTGCCT	GGCTGGAGGAATCGGCTGGC	154
SOX1	TTTCCCCTCGCTTTCTCA	TGCAGGCTGAATTTCGGTT	104
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	197

OCT4, octamer-binding transcription factor 4; MSX1, Msh homeobox 1; SOX1, sex-determining region Y box 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

majority of the surface area, they were considered ready for immunofluorescence staining. The iPSC colonies were fixed with 4% paraformaldehyde at room temperature for 15 min and subsequently washed 3 times with PBS. Colonies were treated with 0.5% Triton X-100 for 15 min at room temperature and blocked at room temperature using 3% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min. Following removal of the blocking buffer, colonies were incubated with primary antibodies directed against, OCT4 (sc-9081; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), TRA181 (MAB4381; EMD Millipore, Billerica, MA, USA), NANOG (ab109250; Abcam, Cambridge, UK), SSEA-4 (sc-21704; Santa Cruz Biotechnology, Inc.), SOX2 (630802; BioLegend, Inc., San Diego, CA, USA) and TRA-160 (sc-21705; Santa Cruz Biotechnology, Inc.) (all 1:100) at 4°C overnight with a blocking buffer (3% BSA). Following washing with PBS three times, the colonies were incubated with secondary antibodies conjugated with Alexa Fluor 488 (A-11034) or Alexa Fluor 594 (A-11032) (1:100; both from Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 50 min with a 3% BSA blocking buffer. The colonies were washed with PBS three times and a mounting medium with DAPI (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China) was used to stain the cell nuclei. Images were captured within 30 min using the inverted phase contrast microscope.

Karyotyping. When iPSCs cultured in 60 mm dishes reached 80-90% confluence, they were treated with 50 ng/ml colcemid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 7 h at 37°C before being harvested using trypsin-EDTA solution. The iPSCs were resuspended in 0.075 M KCl and incubated at 37°C for 20-40 min, then fixed in 3:1 methanol:glacial acidic acid (both from Sinopharm Chemical Reagent Co., Ltd.) at room temperature for 10 min. iPSCs were then centrifuged and fixed three times for (15 min at room temperature, 15 min at room temperature and overnight at 4°C). The harvested cells were stained with Giemsa (Sinopharm Chemical Reagent Co., Ltd.) at room temperature for 5 min. VideoTest-Karyo 3.1 software (Leica Microsystems GmbH) was used to analyze the karyotype of iPSCs at passage 16.

Differentiation of iPSCs in vitro. When the iPSCs cultured in 6-well plates reached 80-90% confluence, they were harvested using EDTA solution (Beijing Cellapy Biotechnology Co., Ltd.)

and resuspended with a differentiation medium [high glucose DMEM; 2 mM L-glutamine; 0.1 mM nonessential amino acid (Invitrogen; Thermo Fisher Scientific, Inc.); 0.1 mM b-mercaptoethanol (Sigma-Aldrich; Merck KGaA); 20% FBS] at 37°C with 5% CO₂. The medium was refreshed every 3 days and the iPSCs were cultured in suspension. Following 7 days in suspension culture, embryoid bodies (EBs) had formed. The following day the EBs were transferred to 1% Matrigel-coated 6-well plates and cultured in the high glucose DMEM for 7 days at 37°C with 5% CO₂. The medium was refreshed every 2 days. The cells were harvested and specific gene expression was measured by polymerase chain reaction (PCR). This was done to demonstrate that the cells underwent spontaneous differentiation.

PCR. Following 14 days of incubation as described above, genomic DNA from iPSCs and EBs was extracted using a TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. PCR was used to examine the expression of genes representative of the endoderm, mesoderm and ectoderm. The extracted genomic DNA of iPSCs and EBs was mixed with primers and a TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd.) and the thermocycling conditions were as follows: Pre-denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec for 35 cycles, followed by a final elongation at 72°C for 2 min and storage at 4°C. The amplified PCR products were resolved on 1.5% agarose gels (Thermo Fisher Scientific, Inc.) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The gels were run for 25 min at 100 V. Images were captured using a Bio-Rad Gel document system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences of all primers are listed in Table I.

Teratoma formation. To evaluate the pluripotency of iPSCs *in vivo*, the HSF-iPSCs were harvested and suspended with PBS in a 1.5 ml Eppendorf tube. A total of 1x10⁷ cells were injected subcutaneously into the hind legs of 4-week-old male Non-obese diabetic-severe combine immune deficiency (SCID) mice (n=7; 28-35 days old; 15-17 g; Charles River Systems, Inc., Burlington, MA, USA). Mice were housed at 22±2°C with 40-70% humidity and a 12 h light/dark cycle with free

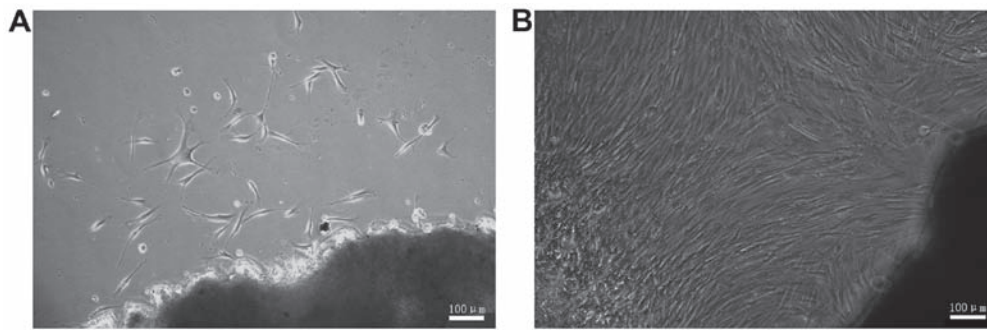


Figure 1. Morphology of human skin fibroblasts. Fibroblast cells following culture in high glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum for (A) 11 days and (B) 24 days.

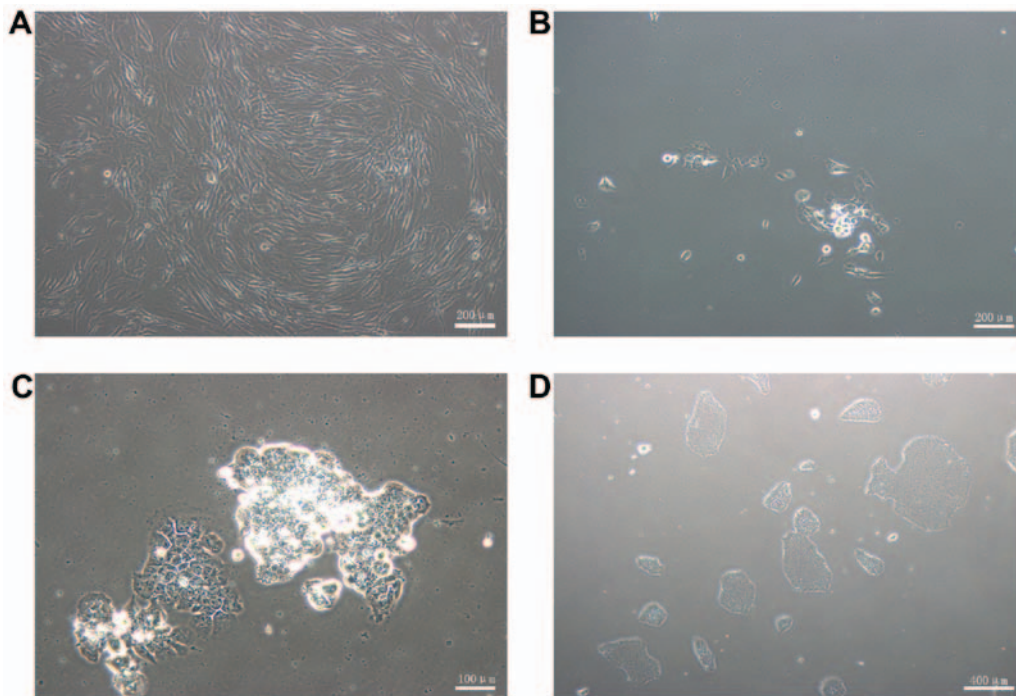


Figure 2. Generation of HSF-iPSCs. (A) Fibroblasts at passage 3 prior to transduction with Sendai virus and human transcription factors. (B) Embryonic stem cell-like colonies are visible 8 days following transduction. (C) HSF-iPSC colonies may be picked at week 4. (D) HSF-iPSCs at passage 2. HSF, human skin fibroblast; iPSCs, induced pluripotent stem cells.

access to food and water. At 8 weeks following injection, the formed tumors were dissected and harvested. The tumors were fixed in 4% paraformaldehyde at 4°C for 2 days, embedded in paraffin blocks, sliced into 3-4 μm sections and stained with hematoxylin and eosin (H&E) staining for 5 min each at room temperature. Tissue samples were observed using a light microscope at x100 magnification. All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University.

Results

The morphological characteristics of HSFs. HSFs were isolated from the skin tissues of patients with burns and cultured in high glucose DMEM with 10% FBS. Following culturing for 11 days, it was possible to see fibroblast cells that had broken off from the tissue segment (Fig. 1A). The cells exhibited branch- and spindle-shaped morphology. Following

culture for 24 days, the cells covered the majority of the surface area of the culture dish (Fig. 1B). Following passaging culture the HSFs demonstrated a marked proliferation ability.

Generation of HSF-iPSCs. To generate iPSCs using a non-integrating method, the HSFs were transduced with CytoTune-iPS 2.0 Sendai reprogramming vectors containing the human transcription factors NANOG, OCT4 and SOX2. The HSFs were cultured until they reached 50-80% confluence prior to transduction (Fig. 2A). At 7 days post-transduction, the cells were detached and transferred onto 1% Matrigel-coated 60 mm dishes. The following day, several small ESC-like colonies were observed (Fig. 2B). At 4 weeks post-transduction, the colonies exhibited representative human ESC-like morphology (Fig. 2C), which includes a large nucleoli and nucleus to cytoplasm ratio. The cells were packed tightly and the border was distinct. The ESC-like morphology and proliferation was maintained following

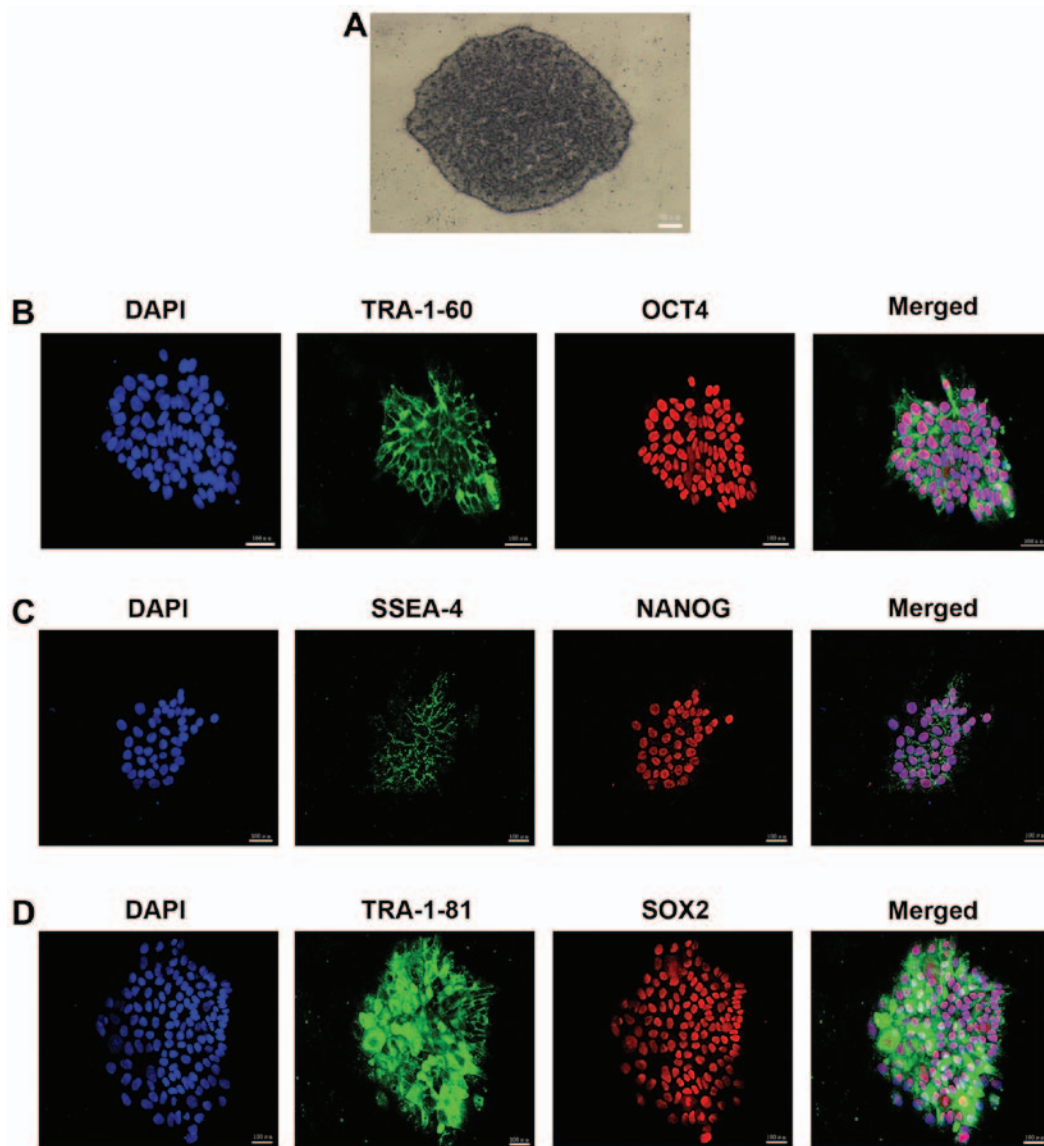


Figure 3. AP and immunofluorescence staining of HSF-iPSCs. (A) The HSF-iPSC colonies were positively stained with AP. Immunofluorescence staining was performed to detect pluripotent markers, including (B) TRA-1-60 and OCT4, (C) SSEA-4 and NANOG and (D) TRA-1-81 and SOX2. The nuclei of all cells were stained blue with DAPI. AP, alkaline phosphatase; HSF, human skin fibroblast; iPSCs, induced pluripotent stem cells; OCT4, octamer-binding transcription factor 4; SSEA, stage-specific embryonic antigen 4; SOX2, sex-determining region Y box 2.

passaging on 1% Matrigel-coated dishes with complete PSCeasy medium (Fig. 2D). A total of 100 ESC-like colonies per 1×10^5 HGFs was obtained, therefore the reprogramming efficiency was $\sim 0.1\%$.

Characteristics of HSF-iPSCs. The colonies exhibited positive AP staining (Fig. 3A), which indicated that pluripotent stem cells had been successfully developed. Immunofluorescence staining was used to examine the presence of pluripotency-associated proteins and revealed that the HSF-iPSCs strongly expressed surface pluripotency markers, including TRA181, SSEA-4 and TRA-160, as well as the intracellular pluripotency markers OCT4, NANOG and SOX2 (Fig. 3B-D). These results suggest that HSFs may be reprogrammed to form pluripotent stem cells. The HSF-iPSCs were confirmed by karyotype analysis (Fig. 4). They exhibited a normal karyotype of 46 XY as confirmed by chromosomal G-band analysis at passage 16.

Differentiation of HSFs-iPSCs *in vitro* and *in vivo*. To examine the differentiation potential of HSF-iPSCs *in vitro*, an experiment was designed to culture the HSF-iPSCs in suspension. The HSF-iPSCs differentiated to EBs spontaneously following 7 days in a suspension culture (Fig. 5A). The EBs were harvested and transferred to 1% Matrigel-coated 6-well plates and cultured for an additional 7 days in differentiation medium (Fig. 5B). DNA was isolated from the EBs and HSF-iPSCs and subsequently used for PCR to examine the expression of genes specific to the three germ layers. Msh homeobox 1 (MSX1; endoderm), GATA4 (mesoderm) and SOX1 (ectoderm) were revealed to be upregulated, whereas the ESC-specific gene OCT4 was downregulated in the EBs (Fig. 5C). Conversely, OCT4 was upregulated in HSF-iPSCs and MSX1, SOX1 and GATA4 were downregulated. The reference gene GAPDH was upregulated in the EBs and HSF-iPSCs. These results suggest that the HSF-iPSCs are capable of differentiating into various different cell types *in vitro*.

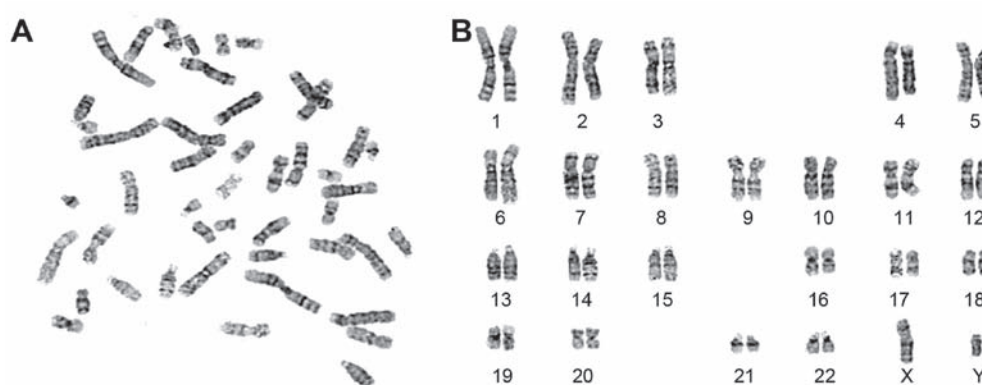


Figure 4. Karyotype analysis of human skin fibroblast-induced pluripotent stem cells. (A) Karyotype analysis revealed a normal karyotype of 46 chromosomes. (B) Analysis was organized according to the traditional format of chromosomes.

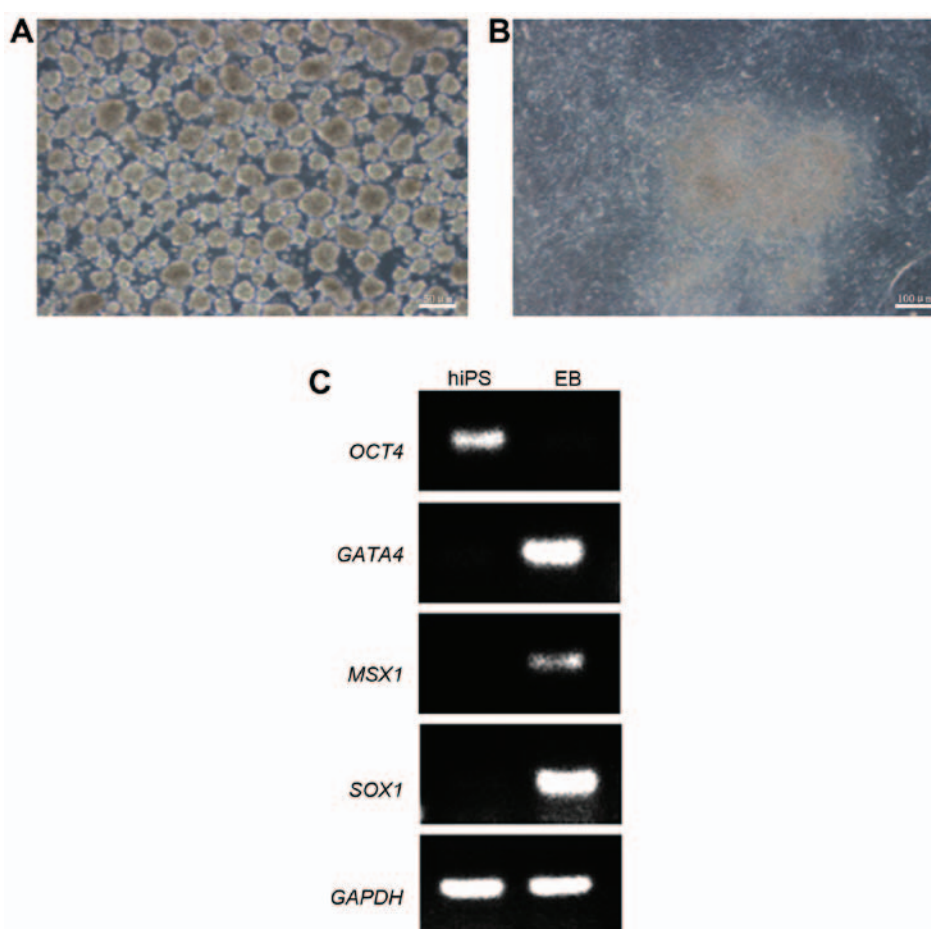


Figure 5. *In vitro* differentiation of HSF-iPSCs. (A) HSF-iPSCs were grown in suspension and EB formation was detected at 8 days. (B) EBs were grown in 1% Matrigel-coated 6-well plates for an additional 7 days and adherent cells were observed. (C) Polymerase chain reaction analysis was performed for differentiation markers of the three germ layers in HSF-iPSC clones and EBs. GAPDH was used as an internal control. HSF, human skin fibroblast; iPSCs, induced pluripotent stem cells; EB, embryoid bodies.

To examine the pluripotency of HSF-iPSCs *in vivo*, they were injected into the hind legs of SCID mice. At 8 weeks later, visible teratomas had formed (Fig. 6A and B). HE staining confirmed that the tumors contained derivatives of all three germ layers, including glands (endoderm), muscles (mesoderm) and nerves (ectoderm), as observed in Fig. 6C-E, respectively. These results indicate that HSF-iPSCs are able to differentiate into different cell types *in vivo*.

Discussion

Previous studies have successfully generated patient-specific iPSCs to treat a variety of diseases, including dystrophic epidermolysis bullosa, spinal muscular atrophy and Huntington's disease (20-22). In the present study, fibroblasts were isolated from the skin of patients with burns and patient specific iPSCs were developed following the reprogramming of fibroblasts.

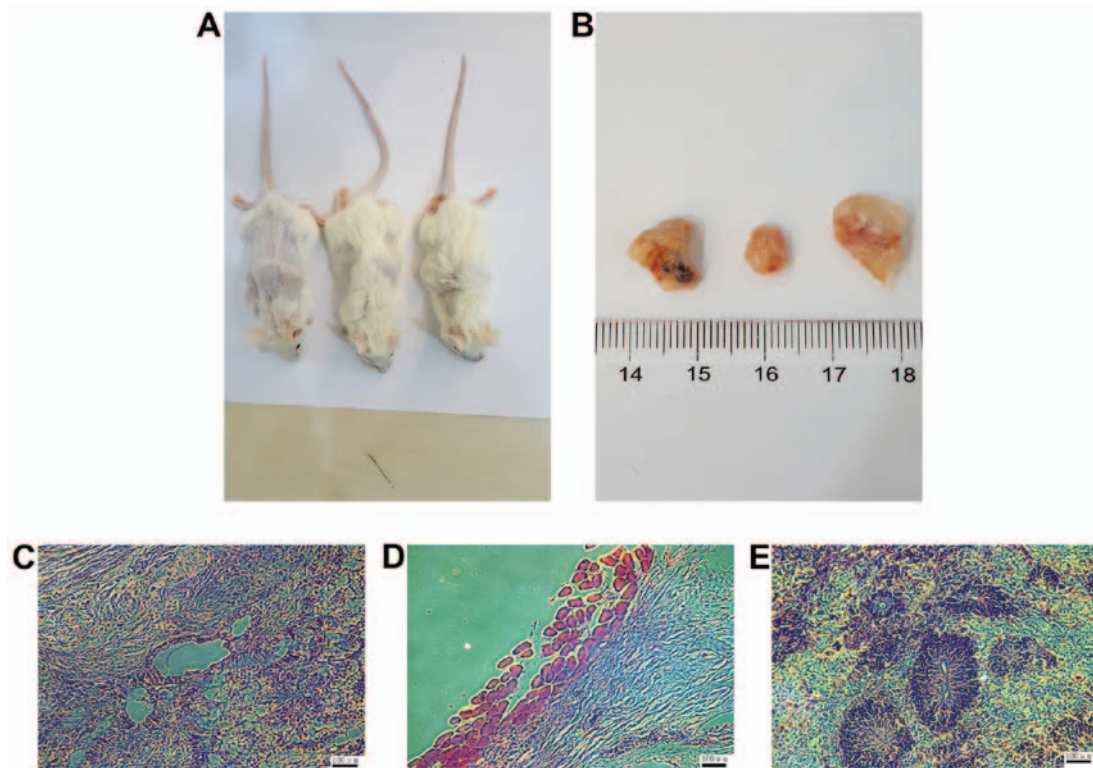


Figure 6. *In vivo* differentiation of HSF-iPSCs. (A) Injection of HSF-iPSCs into non-obese diabetic-severe combine immune deficiency mice gave rise to teratoma formation at 8 weeks. (B) The formed tumors were dissected from the mice. Hematoxylin and eosin staining of teratomas revealed the presence of differentiated tissues from all three germ layers, including (C) glands (endoderm), (D) muscles (mesoderm) and (E) nerves (ectoderm). HSF, human skin fibroblast; iPSCs, induced pluripotent stem cells.

Human dermal tissues were obtained from residual skin pieces following a skin graft on the patient with burns. Fibroblasts were harvested using the tissue block culture method and reprogrammed into iPSCs using the non-integration method. This process may provide a source of seed cells for patients with burns covering a large area, or individuals with skin defects.

Harvested cells demonstrated typical fibroblast morphology. In the present study, fibroblasts were transduced with Sendai virus reprogramming vectors containing the human transcription factors OCT4, SOX2 and NANOG, as opposed to OCT4, SOX2, Klf4 and c-Myc, as previous research has revealed that Klf4 and c-Myc are proto oncogenes, which may increase the tumor formation rate of iPSCs (23). The principal reason why the Sendai virus was selected to transduce the transcription factors was because it is a non-integrative virus and has a minimal effect on the cell genome following transduction (24-26).

Following transduction, the HSF-iPSCs morphology was observed as similar to ESCs. The immunofluorescence staining of the cells revealed the expression of pluripotency markers TRA181, SSEA-4, TRA-160, OCT4, NANOG and SOX2. Subsequently, it was demonstrated that cells were capable of differentiating into different cell types from the three germ layers *in vitro* and *in vivo*. These results suggest that HSF-iPSCs were successfully obtained. The HSF-iPSCs exhibited a normal karyotype of 46 XY as demonstrated using chromosomal G-band analysis. To avoid the pollution of heterogeneous cells and improve the safety of iPSCs, they were cultured on 1% Matrigel-coated dishes instead of mouse embryonic fibroblast feeder-cells (27). In the present study, it

was also revealed that skin tissue in skin grafts was thinner than regular skin tissue, as it did not contain subcutaneous tissue. The dermis and epidermis were isolated following 4-6 h digestion, which is a shorter time period than would be necessary for regular skin tissue and reduced the damage of the digestive enzymes to the cells (28).

The results of the present study demonstrate that fibroblasts harvested from patients with burns may be reprogrammed using Sendai virus vectors with OCT4, SOX2 and NANOG to form iPSCs with non-exogenous genomic integration (17,29). HSF-iPSCs were demonstrated to be pluripotent and remain in an undifferentiated state. Further study is required to develop the differentiation of HSF-iPSCs into specific cells or tissues of the skin, including fibroblasts, keratinocytes, melanocytes or vascular tissue, lymphocytic tissue and nerves (30-34). The results of the present study provide an experimental basis for the development of functional skin within a laboratory for use in a clinical setting. The development of this novel treatment for disease or injury may be of great significance to regenerative medicine and tissue engineering.

Acknowledgements

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