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Original Paper

Efficient Generation of Non-Integration and Feeder-Free Induced Pluripotent Stem Cells from Human Peripheral Blood Cells by Sendai Virus

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Abstract

Background/Aims: Induced pluripotent stem cells (iPSCs) hold great promise for regenerative medicine, disease modeling, and drug development. Thus, generation of non-integration and feeder-free iPSCs is highly desirable for clinical applications. Peripheral blood mononuclear cells (PBMCs) are an attractive resource for cell reprogramming because of their properties of easy accessibility and the limited invasiveness of blood collection. However, derivation of iPSCs is technically demanding due to the low reprogramming efficiency and nonadherent features of PBMCs. ***Methods:*** iPSCs were generated from PBMCs using non-integrative Sendai viruses carrying the reprogramming factors Oct4, Sox2, Klf4, and cMyc. The derived iPSCs were fully characterized at the levels of gene and protein, and then they were transplanted into immunocompromised mice for evaluation of *in vivo* differentiation potential. Three types of extracellular substrates (Geltrex, vitronectin, and rhLaminin-521) were tested for their influences on cell reprogramming under feeder-free conditions. We also sought to establish approaches to efficient cell recovery post-thaw and single cell passaging of iPSCs employing Rock inhibitors. ***Results:*** iPSCs were efficiently generated from PBMCs under feeder-free conditions. The derived iPSCs proved to be pluripotent and transgene-free. Furthermore, they demonstrated multi-lineage differentiation potentials when transplanted into immunocompromised mice. Among the three substrates, Geltrex and rhLaminin-521 could effectively support the initial cell reprogramming process, but vitronectin failed. However, the vitronectin, similar to Geltrex and rhLaminin-521, could effectively maintain cell growth and expansion of passaged iPSCs. In addition, RevitaCell supplement (RVC) was more potent on cell recovery post-thaw than Y-27632. And RVC and Y-27632 could significantly increase the cell survival when the cells were passaged in single cells, and they showed comparable effectiveness on cell recovery. ***Conclusion:*** We have successfully derived non-integration and feeder-free human iPSCs from

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peripheral blood cells, and established effective strategies for efficient cell recovery and single cell passaging. This study will pave the way to the derivation of clinical-grade human iPSCs for future clinical applications.

Introduction

Induced pluripotent stem cells (iPSCs) were first established from mouse fibroblasts by Yamanaka in 2006 [1]. Subsequently, human iPSCs were successfully derived by the Yamanaka and Thomson groups [2, 3]. By ectopic-expression of a defined set of reprogramming factors (Oct4, Sox2, Klf4, and cMyc/Oct4, Sox2, Nanog, and Lin28), iPSCs have been generated from many cell types of different species [1-8]. Retrovirus or lentivirus was initially employed to deliver reprogramming factors for iPSC generation [1, 3]. These genome-integrating methods could potentially produce tumorigenic insertional mutations, and residual or reactivation of transgene expression during iPSC differentiation could influence their *in vitro* differential capacity and the functionality of iPSC derivatives [3, 9]. To solve these problems, various methods were explored to derive transgene-free iPSCs, such as plasmid vectors, minicircle DNA vectors, piggyBac, mRNA, adenovirus, Sendai virus, proteins, and small molecules [10-17].

Transient expression of reprogramming factors by episomal plasmids would allow for the derivation of transgene-free iPSCs. However, transient transfection with a standard plasmid vector cannot provide a long enough expression period of time for cell reprogramming [14]. OriP/EBNA-1 (Epstein-Barr nuclear antigen-1)-based plasmids are stably expressed for a longer period of time than standard plasmids, and higher transfection efficiency can be achieved with these vectors due to the oriP/EBNA-1-mediated nuclear import and retention of vector DNA [18]. A single transfection of episomal plasmid vectors is sufficient for the derivation of iPSCs.

Expression of reprogramming factors using mRNA provides another method to make transgene-free iPSCs. It was shown that *in vitro* transcribed mRNAs were able to efficiently express reprogramming factors when transfected into human fibroblasts [19]. Although reprogramming factor mRNAs are commercially available, this method suffers from the limitations that it is labor-intensive, requires daily transfection of mRNA for 7 successive days, and there are no successful reports regarding the reprogramming of blood cells.

Sendai virus is a single chain RNA virus that does not integrate into the host genome or alter the genetic information of the host cells. The virus remains in the cytoplasm and is therefore diluted out of the host cells ~10 passages after virus infection. Sendai virus can infect a wide range of cell types in proliferative and quiescent states with high transduction efficiency. Expression of transgenes delivered by Sendai virus is detectable as early as 6-10 hrs after transduction, with maximum expression detected more than 24 hrs after transduction. Sendai-based reprogramming vectors have been used to successfully reprogram neonatal and adult fibroblasts as well as blood cells with high efficiency [10, 20, 21].

Overall, episomal vectors, mRNA, and Sendai virus are the most often used non-integrating methods for iPSCs generation among the aforementioned methods. However, mRNA has a limited applicability to somatic cell types, and there is not yet any report demonstrating successful reprogramming of blood cells. Episomal vectors have relatively low reprogramming efficiencies compared to the Sendai virus method. In this report, we successfully generated non-integration and feeder-free iPSCs from human peripheral blood cells by Sendai virus. We tested three different types of substrates (Geltrex, vitronectin, and rhLaminin-521) for their influences on cell reprogramming. Among these substrates, Geltrex and rhLaminin-521 could effectively support the initial cell reprogramming process, and the passaged iPSCs could grow and expand well on all three substrates. We also compared the effects of two Rock inhibitors, RevitaCell supplement (RVC) and Y-27632 on cell recovery after cell thawing and demonstrated that RVC was more effective on cell recovery than Y-27632. Furthermore, RVC and Y-27632 showed comparable effectiveness on single cell

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passaging, and they all provided great improvements in cell survival and recovery of iPSCs. Together, the current study demonstrates that non-integration and feeder-free iPSCs can be effectively derived from human peripheral blood cells by Sendai virus. Considering safety issues, derivation of footprint-free and xeno-free iPSCs is highly important for their future clinical applications.

Materials and Methods

Ethical approval

Blood samples were collected from five healthy voluntary donors, ranging from 32 to 53 years old, with written consent permitted by the ethical committee of the Beijing Institute of Biotechnology.

Isolation and culture of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by density gradient centrifugation with Ficoll-paque Plus (p = 1.077±0.001 g/ml) (GE Healthcare,17-1440-02) from 5 ml whole blood according to the manufacturer's instructions and cryopreserved in the liquid nitrogen tank for future application. Four days before transduction, frozen PBMCs were quickly thawed and cultured in complete PBMC medium containing complete StemPro-34 SFM medium supplemented with 100 ng/ml stem cell factor (SCF, Life Technologies, PHC2111), 100 ng/ml FLT3 (Life Technologies, PHC9414), 20 ng/ml interleukin-3 (IL3, Life Technologies, PHC0034), and 20 ng/ml interleukin-6 (IL6, Life Technologies, PHC0065). Medium was half replaced with fresh complete PBMC medium everyday for 3 days (Day -3 to Day -1). Complete StemPro-34 SFM medium consisted of StemPro-34 SFM medium (1X) (Life Technologies, 10639-011) supplemented with StemPro-34 Nutrient Supplement and 2 mM L-Glutamine (Life Technologies, 25030-081).

Generation of iPSCs from PBMCs with Sendai virus

The scheme of generating iPSCs is shown in Fig. 1A. The process was as follows. After 4 days cell culture (Day 0), the cells were collected and the number of cells were counted. 3 X 10^5 live cells were transduced by the Klf4, Oct4, and Sox2 (KOS) virus, and the c-Myc virus at a multiplicity of infection (MOI) of 5 (KOS MOI=5, hc-Myc MOI=5), and the Klf4 virus at an MOI of 3 (hKlf4 MOI=3) (CytoTune®-iPSC2.0 Sendai Reprogramming Kit, Life Technologies, A16517) in a total volume of 1 ml. The transduction was performed in complete PBMC medium with 4 μg/ml of Polybrene by centrifugation at 2250 RPM for 30 min at room temperature. And then the pellet was resuspended in the same medium containing the virus and plated in a 24-well plate and incubated overnight at 37°C. The day after transduction (Day 1), Sendai viruses were removed by centrifuging the cell suspension. The cells were resuspended in a 24-well plate with fresh complete PBMC medium for 2 days. At day 3, the cells were collected by centrifugation, resuspended with complete StemPro-34 SFM medium without cytokines, and plated onto Geltrex-, vitronectin-, and rhLaminin-521-coated 6-well plates (Life Technologies, A1413301, A14700, and A29248). The medium was refreshed every other day. At day 7, transitioning into iPSC medium began by replacing half of the StemPro-34 SFM medium without cytokines with Essential 8 (E8) medium (Life Technologies, A151700). From day 8 on, the full volume of medium was changed to E8 medium. The medium was replaced everyday thereafter and the emergence of iPSC colonies was monitored. Around day 15 to 21 after transduction, colonies were ready for transfer. The colonies were manually picked and transferred onto different substrate-coated 12-well plates for expansion.

RNA isolation and reverse transcription

Total RNA was purified with TRIzol reagent (Life Technologies, 15596026) and digested with DNase to remove genomic DNA contamination. 1 μg of total RNA was used for reverse transcription reaction with Superscript IV first-strand synthesis system (Life Technologies, 18091050), according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with *Taq* DNA polymerase (Life Technologies, 10342053). The PCR conditions were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 56°C for 30s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Primer information is shown in Table 1.

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Table 1. Primers for RT-PCR

Genes	Accession	Forward	Reverse	Size	Tm	Cycles
Oct4	NM_002701	tctcccatgcattcaaactg	gttttctttccctagctcctc	80	56	30
Sox2	NM_003106	cccggcacggccattaac	acccctcccatttccctc	109	56	30
Nanog	NM_024865	cctgtgatttgtgggcctg	gacagtctccgtgtgaggcat	78	56	30
Klf4	NM_001314052	gggttcaggtgccccagc	tgtcagactcgccaggtg	85	56	30
cMyc	NM_002467	ctgccaggacccgcttct	gttttccactacccgaaa	76	56	30
GAPDH	NM_002046	cgagatccctccaaaatcaagt	tgaggctgttgtcatacttctcat	196	56	30
Sev	AB855655	ggatcactaggtgatatcgagc	accagacaagagtttaagagatatgtatc	181	56	30

Flow cytometry analysis

Cells were analyzed for Oct4, Sox2, and SSEA-4, using Human/Mouse Pluripotent Stem Cell Multi-Color Flow Cytometry Kit (R&D Systems, FMC001) according to the manufacturer's instructions. The corresponding isotype-matched antibodies were used as negative controls. Cells (20, 000 events per sample) were acquired on a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using FCS express 4 plus software.

Table 2. Primers for DNA methylation

Genes	Sequence	Position (from TSS)
Oct4-OF	gaggagttgagagggtgattggtttt	-662 ~ -637
Oct4-OR	cgaaaaaactactcaacccct	+5 ~ +25
Oct4-IR	ggagaggggggttaagtatttgggtttt	-611 ~ -585
Oct4-IF	tcactttattacccaaactaa	-207 ~ -227

Alkaline phosphatase (AP) staining, karyotyping, and immunofluorescence (IF) analysis

After 3 passages, cells were subjected to AP staining according to the manufacturer's protocol using the AP Detection Kit (Stemgent, 00-0055) for pluripotency detection. Briefly, cells were fixed in Fix Solution for 2-5 min at room temperature. Then cells were incubated in freshly prepared AP Substrate Solution for 10-20 min in the dark. Authentic iPSC colonies were stained positive for alkaline phosphatase, displaying a red or purple stain. For karyotype analysis, cell division was blocked in mitotic metaphase using colcemid-spindle formation inhibitor (Invitrogen, 15212). G-band standard staining (Giemsa, Invitrogen, 10092013) was used for the chromosome visualization. For the detection of pluripotency markers by IF, cells were grown in 6-well plates and fixed with 4% paraformaldehyde for 30 min at 4°C. After that, cells were permeabilized and blocked with 10% calf serum and 0.1% Triton X-100 in PBS. Subsequently, cells were stained with primary antibodies overnight at 4°C and finally incubated with fluorochrome-labeled secondary antibodies. The primary antibodies used for detecting pluripotency markers were anti-Oct4 (Santa Cruz, sc-5279), anti-Sox2 (Calbiochem, sc1002), anti-Nanog (Santa Cruz, sc-33759), and anti-SSEA-4 (Santa Cruz, sc-59368). The secondary antibodies used were donkey anti-mouse AF488, donkey anti-mouse AF594, and donkey anti-rabbit AF594 (Life Technologies, A21202, A21203, and A21207). Nuclei were stained by Hoechst 33258 (Sigma Aldrich, 94403). Negative staining controls were carried out by replacing the primary antibody with rabbit or mouse sera. Images were captured using Nikon Eclipse Ti-U microscope, and images were processed using Adobe Photoshop CS5.

Bisulfite sequencing

Genomic DNA was isolated from the cells and modified with sodium bisulfite using the Epitect Bisulfite Kit (Qiagen, 59104) according to the manufacturer's instructions. Methylation-specific PCR primers are shown in Table 2. Amplified products were cloned into pGEM®-T Vector System (Promega, A3600). Randomly selected clones were sequenced with the T7 forward primer for the gene and analyzed for methylation changes at CpG sites of the *Oct4* promoter (-662 to +25, from transcriptional start site (TSS)).

Teratoma formation

Cells were harvested by 0.5 mM EDTA treatment, collected into conical tubes, and centrifuged, and the pellets were resuspended in E8 medium. 3 X 10^6 cells/mouse were injected subcutaneously into the dorsal flank of immunocompromised mice (n=6/group). Around 12 weeks, mice were euthanized when a visible tumor mass was detected. The teratomas were dissected and fixed in 10% formalin. Samples were

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embedded in paraffin and processed with hematoxylin and eosin staining. The teratomas were confirmed by histological analysis. The remaining mice, without visible tumors, were euthanized at week 16. All mice were examined for teratoma formation by gross and microscopic inspection. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Beijing Institute of Biotechnology.

Freezing and thawing of iPSCs

When cells reached a confluency of 70-80%, the spent medium was aspirated out and the cells were treated with 0.5 mM EDTA. Then the cells were incubated at 37°C for 3-5 min and gently transferred into conical tubes, leaving the cells in clumps. The cell suspension was centrifuged and the pellet was resuspended in freezing medium containing E8 medium supplemented with 10% DMSO. The cell suspension was aliquoted into cryovials and placed in a cryofreezing container at -80°C. After overnight storage at -80°C, the cells were transferred to a liquid nitrogen tank for long-term storage. For thawing and recovering cells, the cryovial was immersed in a 37°C water bath for 1-2 min. Then the cells were gently transferred to a 15 ml conical tube containing pre-warmed E8 medium. The cells were centrifuged and the pellet was resuspended in 2 ml of E8 medium supplemented with either 1X RVC (Life Technologies, A2644501) or 10 μM Y-27632 (Calbiochem, 688000). The cells were cultured on vitronectin-coated 6-well plates. The next day, the cells were refreshed with fresh E8 medium. The medium was replaced daily thereafter until the cells are approximately 80% confluent. To determine the efficiency of the cell recovery following cell thawing, images were captured by phase-contrast microscopy at regular intervals. For measuring the proliferation of cells after cell thawing, cell masks were calculated by NIS-Elements AR analysis (Nikon) to determine the percentage change in confluency of the cells over time.

Kinetic growth of single iPSCs

For single cell passaging, cells were rinsed with DPBS and treated with TrypLE™Express Enzyme (Life Technologies, 12604-013) for 5 min. Next, the cells were triturated several times to efficiently singularize the cells. Finally, the cells were centrifuged and resuspended, and cultured (10, 000 cells/cm^2) in E8 medium supplemented with either 1X RVC or 10 μM Y-27632 for 24 hrs post-split, followed by daily feeding with E8 medium alone for the remainder of the culture. To determine the kinetic growth of the cells following single cell passaging, images were captured by phase-contrast microscopy at regular intervals, and cell masks were calculated to determine the proliferation of cells over time.

Statistical analysis

All the experiments were carried out at least three times. Data were displayed as mean ± SD. To assess statistical significance, two-tailed, unpaired Student's t test was performed and $p < 0.05$ was considered significant.

Results

iPSC generation from peripheral blood

PBMCs were isolated from 5 ml whole blood of healthy donors by density gradient centrifugation, and the cells were cryopreserved for the following iPSC generation. In general, 1×10^6 PBMCs could be efficiently purified from 1 ml whole blood and that is sufficient for iPSC derivation. Four days before cell reprogramming, the frozen PBMCs were quickly thawed and cultured in complete PBMC medium with cytokines SCF, FLT3, IL3, and IL6 (Fig. 1A). After 4 days recovery, 3×10^5 live cells were transduced by the Klf4, Oct4, Sox2, and c-Myc viruses with the manufacturer recommended MOI. To increase the transduction and reprogramming efficiencies, the transduction was performed by centrifugation at 2250 RPM for 30 min at room temperature, and this greatly increased the number of colonies. For derivation of feeder-free iPSCs, we employed extracellular substrates to replace feeder cells for iPSC generation. Three types of substrates were evaluated for supporting cell reprogramming. At day 3 after transduction, the cells were replated onto Geltrex (1:100)-, vitronectin (0.5 μg/cm^2)-, and rhLaminin-521 (0.5 μg/cm^2)-coated 6-well plates, respectively.

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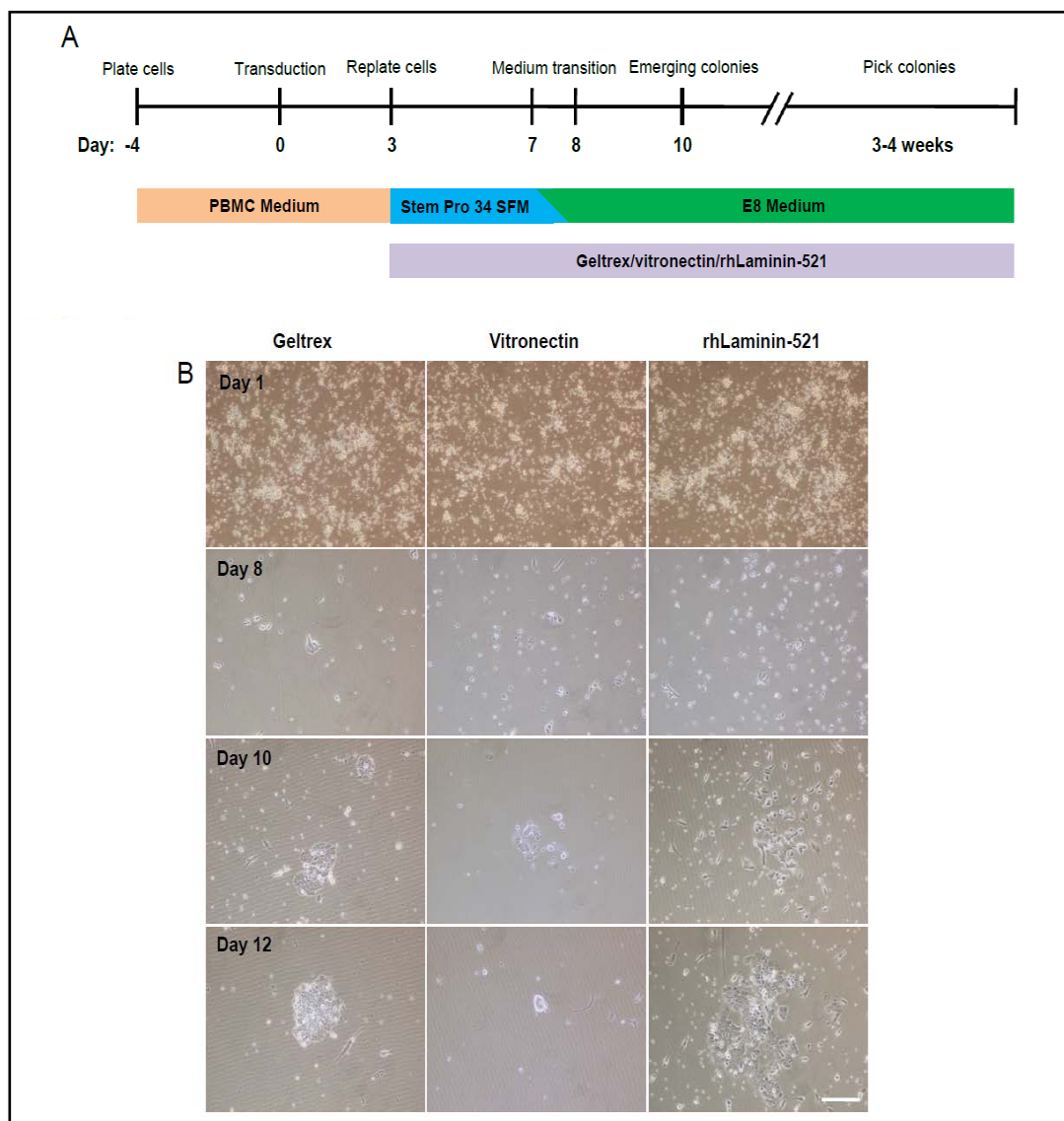Ye et al.: Peripheral Blood Cells-Derived Non-Integration and Feeder-Free iPSCs

Fig. 1. Generation of human iPSCs from PBMCs by Sendai virus. A. Schematic diagram of derivation of iPSCs. After 4 days cell culture, PBMCs were transduced by Sendai viruses at MOI=5-5-3 (KOS MOI=5, hc-Myc MOI=5, and hKlf4 MOI=3). One day after transduction, Sendai viruses were removed and the cells were cultured in complete PBMC medium for 2 days. At day 3, the cells were replated onto Geltrex-, vitronectin-, and rhLaminin-521-coated plates, respectively. At day 7, transitioning into E8 medium began. Around day 15 to 21 after transduction, colonies were manually picked and transferred onto different substrate-coated plates for expansion and characterization. B. Effects of extracellular substrates on the initial cell reprogramming stage. About 1 week after transduction, some small colonies began emerging on all three types of substrate-coated plates. The number of colonies on vitronectin substrate was less than those on Geltrex and rhLaminin-521 substrates, respectively. The numbers of colonies on Geltrex and rhLaminin-521 substrates were comparable. The colonies on vitronectin substrate were easy to detach from the plates when changing the medium. Bar is 200 μm.

Around 1 week after transduction, some small colonies began emerging on all three types of substrate-coated plates. However, the number of colonies on vitronectin substrate was less than those on Geltrex and rhLaminin-521 substrates, respectively. The numbers of colonies on Geltrex and rhLaminin-521 substrates were comparable. Furthermore, the colonies on vitronectin substrate were not firmly attached to the plates during the reprogramming,

and easy to detach from the plates when changing the medium (Fig. 1B). Even though we increased the concentration of vitronectin to 1 μg/cm^2, there was not much difference on reprogramming compared to using 0.5μg/cm^2. Therefore, it might be difficult to successfully reprogram PBMCs on vitronectin substrate due to loosing attachment between the colonies and vitronectin substrate during reprogramming. Around 2-3 weeks, the size of the colonies was suitable for picking. The flat and compact colonies with clear boundaries were selected for transfer. In our experiments, around 30 human embryonic stem cell (ESC)-like colonies per 3 X 10^5 PBMCs were obtained (33 colonies for Geltrex-iPSCs and 29 colonies for rhLaminin-521-iPSCs, respectively). The reprogramming efficiency was approximately 0.01% (The efficiency was calculated as the number of emerging human ESC-like colonies per starting cell number). Although the vitronectin substrate was inefficient to support the initial cell reprogramming process, the passaged colonies could successfully grow and expand on this substrate. There was not much difference observed on cell growth and expansion during the cell culturing on the three types of substrate (Geltrex, vitronectin and rhLaminin-521)-coated plates. In addition, we also successfully derived iPSCs from the other four donors' PBMCs with similar reprogramming efficiencies and timing.

Characterization of iPSCs

When picked and expanded in E8 medium, iPSCs showed typical human ESC morphology, i.e. flat and compact colonies with clear boundaries, high nucleus/cytoplasm ratios and prominent nucleoli, and they robustly proliferated for more than 20 passages and maintained normal karyotypes (Fig. 2A). These iPSCs were positive for AP staining (Fig. 2A). To test pluripotency gene expression, total RNA was extracted from iPSCs. The results showed that iPSCs expressed pluripotency markers Oct4, Sox2, Nanog, Klf4, and cMyc (Fig. 2B). RT-PCR analysis also indicated that Sendai virus was retained in these iPSCs up to passage 10, which is consistent with the previous report [22]. To obtain virus-free iPSCs, we performed a temperature shift to remove the virus. After incubating the cells at 38°C for 5 days, Sendai virus was completely absent in passage 12 cells (Fig. 2B). To analyze the methylation status of the pluripotency gene promoter, genomic DNA was isolated from the cells. The Oct4 promoter showed differential methylation patterns between iPSCs and PBMCs detected by bisulfite sequencing. The Oct4 promoter was demethylated in these iPSCs, similar to human ESCs [23] and in contrast to the parental PBMCs (Fig. 2C). Flow cytometry analysis for pluripotency-associated protein expression revealed that they expressed typical human ESC-specific antigens Oct4, Sox2, and SSEA-4 (Fig. 2D). IF analysis confirmed the expression of pluripotency markers Oct4, Sox2, Nanog, and SSEA-4 (Fig. 2E). In order to investigate *in vivo* differentiation potential, iPSCs were digested, collected, and injected into immunocompromised mice. Around 12 weeks, a visible tumor mass was detected. Histological analysis showed that they formed teratomas consisting of derivatives of all three embryonic germ layers (Fig. 3). Overall, these results suggest that we successfully derived non-integration and feeder-free iPSCs from PBMCs using Sendai viruses carrying Oct4, Sox2, Klf4, and cMyc. The cells express pluripotency markers as confirmed in gene and protein levels. Furthermore, the cells possess multi-lineage differentiation potentials *in vivo*.

Rock inhibitors promote cell recovery after cell thawing

Human iPSCs display different passaging properties compared with mouse iPSCs. Unlike mouse iPSCs, they have to be passaged in cell clumps (e.g., using reagents such as dispase, collagenase, or EDTA), instead of single cells. Furthermore, they have very poor cell survival efficiency after cell cryopreservation. The major reason for the low survivability of iPSCs after cell thawing was apoptosis rather than cellular necrosis induced directly by cryoinjury [24]. To increase the cell survival, we tested two Rock inhibitors, RVC and Y-27632, and observed their influence on cell survival post-thaw. In the control group (without Rock inhibitors), the cells displayed very poor survival efficiency, and most cells were dead (Fig. 4A). The cells grew and expanded slowly and only reached 8.69% confluency after 5 days culturing (Fig. 4B &C). As expected, both Rock inhibitors dramatically enhanced cell survival efficiency, and RVC
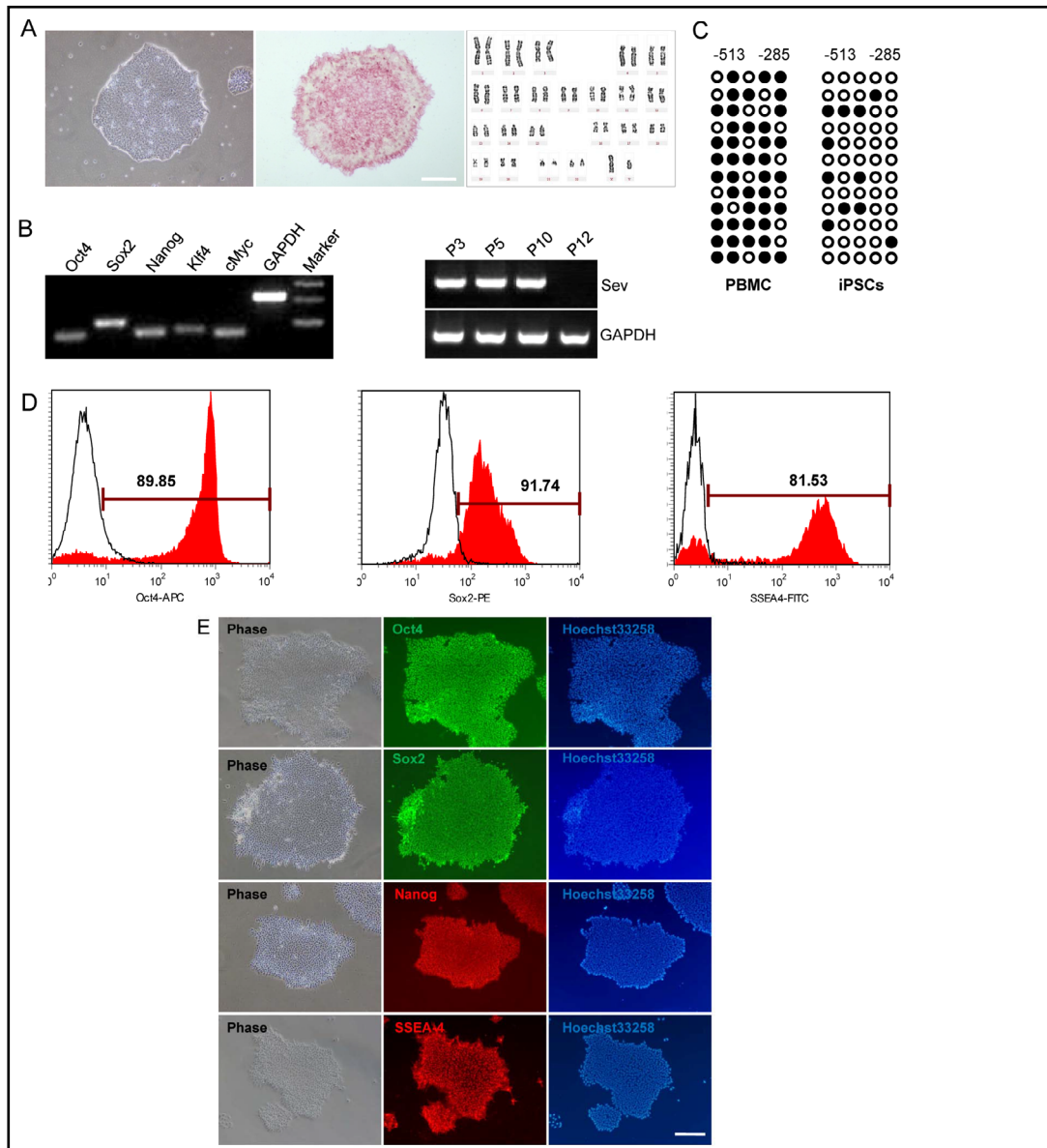
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Fig. 2. Characterization of iPSCs. A. Morphology assessment, AP staining, and karyotyping of iPSCs. iPSCs at passage (P) 3 displayed typical human ESC morphology, flat and compact colonies with clear boundaries, high nucleus/cytoplasm ratios, and prominent nucleoli (left panel). These colonies were positive for alkaline phosphatase, displaying a red or purple stain (middle panel). A representative karyogram of an iPSC clone is shown on the right panel. This iPSC clone maintained a normal karyotype (46, XY) after 20 passages of expansion. Bar is 200 μm. B. Expression of pluripotency and Sendai virus genes by RT-PCR. iPSCs expressed pluripotency genes Oct4, Sox2, Nanog, Klf4, and cMyc (left panel). Sendai virus (Sev) was retained in iPSCs up to P10. After incubating the cells at 38°C for 5 days, Sendai virus was completely absent in P12 cells (right panel). C. Analysis of Oct4 promoter DNA methylation status by bisulfite sequencing. The Oct4 promoter of iPSCs was less methylated than that of the parental PBMCs. Methylated CpG sites within the Oct4 promoter are designated by filled circles and unmethylated CpG sites by unfilled circles. D. Flow cytometric analysis. iPSCs were analyzed for expression of pluripotency markers Oct4, Sox2, and SSEA-4. Unfilled histograms represent matched antibody isotype controls and filled histograms represent expression of markers on the tested cells. E. IF analysis of pluripotency markers in iPSCs. iPSCs were stained in situ by anti-Oct4 (1:100), anti-Sox2 (1:100), anti-Nanog (1:100), and anti-SSEA-4 (1:50) antibodies. Nuclei were highlighted with Hoechst 33258 staining. Bar is 200 μm.
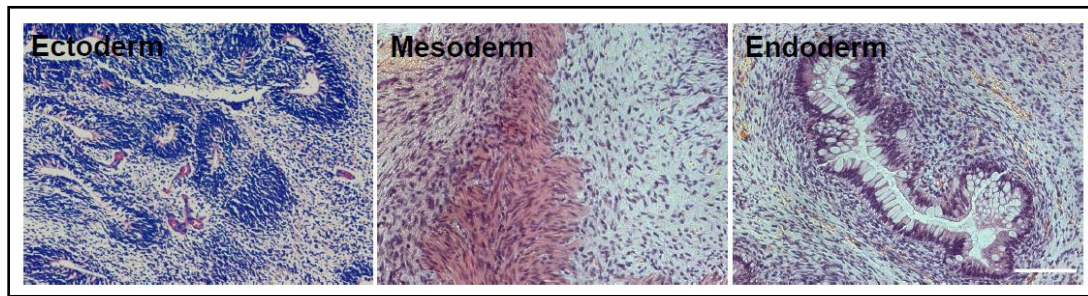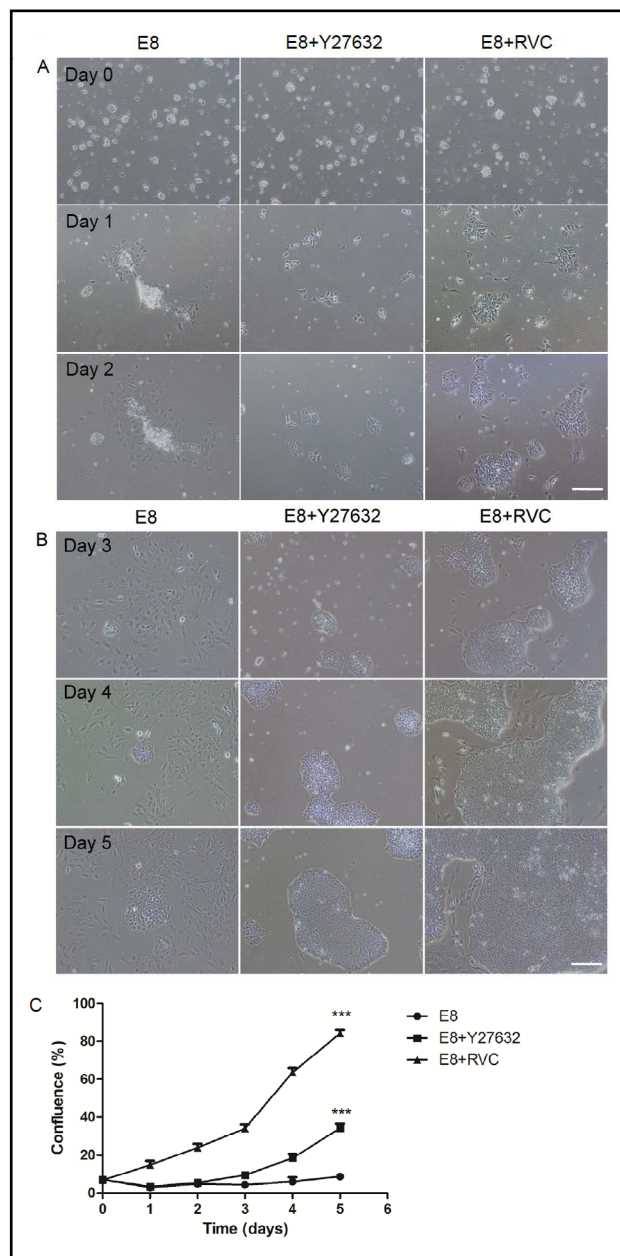
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Fig. 3. Teratoma formation. 3 X 10^6 cells/mouse were injected subcutaneously into the dorsal flank of immunocompromised mice (n=6/group). Around 12 weeks, a visible tumor mass was detected. The teratomas were dissected and processed with hematoxylin and eosin staining. The teratomas consisted of derivatives of ectoderm, mesoderm, and endoderm. Bar is 100 μm.

Fig. 4. Rock inhibitors promote cell recovery after cell thawing. A & B. Analysis of cell morphology and recovery post-thaw. iPSCs (cell clumps) were thawed and plated on vitronectin-coated plates in E8 medium supplemented with either 10 μM Y-27632 (middle panel) or 1X RVC (right panel). Following a 24-hour recovery, iPSCs were fed with E8 medium alone for the remainder of the culture. The control group (left panel) was maintained in E8 medium alone for the entire experiment. Bar is 200 μm. C. Analysis of cell proliferation after cell thawing. Undifferentiated cell masks were calculated to determine the percentage change in confluency of the cells over time. Data are mean ± SD of triplicate experiments. * p<0.05; ** p<0.01; *** P<0.001.

was more effective on cell recovery post-thaw than Y-27632. After 5 days culturing, the cells reached 84.19% and 34.21% confluency in the RVC and Y-27632 groups, respectively (Fig. 4B&C). In all, the results indicate that Rock inhibitors can significantly promote cell recovery and increase the cell survival.

Single cell passaging achieved by Rock inhibitors

iPSCs are fundamental tools for basic research and applied applications, including cell therapy, drug discovery, and toxicological evaluation. Generally human iPSCs need to be passaged in cell clumps because breaking down the clumps into single cells may

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substantially decrease the cell survival. However, employing clump passaging approach has often been incompatible with downstream experimental applications, such as high-throughput screening, gene editing, and directed lineage differentiation. Thus, we sought to establish an applicable single cell passaging approach for human iPSCs. The major cause for the vulnerability of iPSCs was apoptosis or anoikis (detachment-induced apoptosis) upon cellular detachment and dissociation. They underwent massive cell death particularly after complete dissociation [25-28]. Therefore, we investigated whether Rock inhibitors could effectively support single cell passaging. The results showed that addition of either RVC or Y-27632 for 24 hrs post-split could significantly increase the cell survival when the cells were passaged in single cells (Fig. 5A). They showed comparable effectiveness on cell recovery (Fig. 5B). After passaging, the cells grew and expanded rapidly, and maintained the normal iPSC colonies morphology. The colony formation efficiency was markedly increased. Altogether, single cell passaging of human iPSCs could be achieved by addition of Rock inhibitors.

Fig. 5. Single cell passaging achieved by Rock inhibitors. A. Morphology assessment of iPSCs. The cells were dissociated into single cells and plated at a density of 10,000 cells/cm^2 on vitronectin-coated plates in E8 medium supplemented with either 10 μM Y-27632 or 1X RVC. Following a 24-hour recovery, iPSCs were fed with E8 medium alone for the remainder of the culture. Bar is 200 μm. B. Kinetic growth of single iPSCs. Undifferentiated cell masks were calculated to determine the percentage change in confluency of the cells over time. Data are mean ± SD of triplicate experiments. * $p<0.05$; ** $p<0.01$; *** $P<0.001$.

Discussion

The advent of the iPSC technique is a recent breakthrough of stem cell research and application. iPSCs are self-renewable and have multi-lineage differentiation potentials, similar to ESCs. The development of cellular reprogramming techniques overcomes the two major issues hindering fulfillment of the great potential of human ESCs [1-3]. One is the ethical issue associated with the human ESCs derivation, and the other is the immunological incompatibility induced by histocompatibility–antigenic factors. iPSCs hold great promise for regenerative medicine, disease modeling, and drug development. Thus, there is a need to develop an efficient method to enable the routine derivation of footprint-free and xeno-free iPSCs from many human donor samples, and eventually the derivation of clinical-grade human iPSCs.
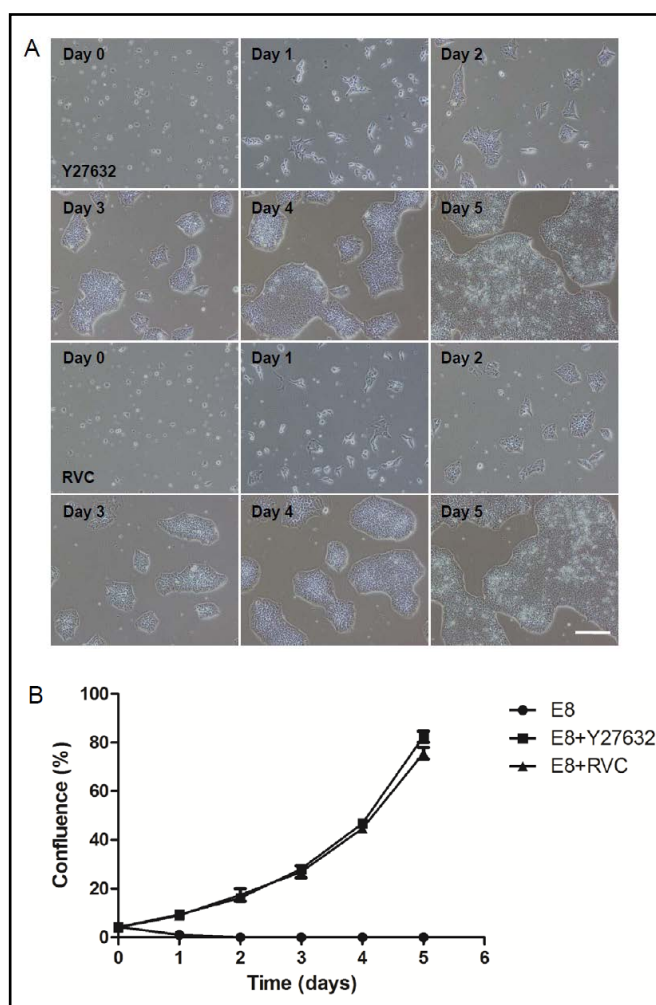
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Although fibroblasts from different sources were initially employed in many studies for the derivation of iPSCs, PBMCs have been widely accepted as a more convenient and almost unlimited resource for cell reprogramming [6, 29-31]. Here, we routinely employed 5 ml peripheral blood for PBMCs isolation and derivation of iPSCs. To make this method easily accessible and minimally invasive, we attempt to cut down the initial volume of peripheral blood. In general, 1×10^6 PBMCs could be efficiently purified from 1 ml peripheral blood. Freshly purified PBMCs from as little as 500 µl of peripheral blood were enough for the successful derivation of iPSCs without addition of any reagent for enhancing reprogramming (data not shown). We also found that freshly purified PBMCs cultured for 4-6 days enhanced the cell reprogramming efficiency, consistent with the previous report [32]. Soares, et al. reported generation of hiPSCs from PBMCs using Sendai virus. They first isolated PBMCs from blood and cultured them for 9 days to allow expansion of the erythroblast population before the initiation of cell reprogramming. Then the PBMCs were successfully reprogrammed into iPSCs in the presence of mouse embryonic fibroblasts [33]. Our work further optimized the previous protocol. Before cell reprogramming, there was no need to expand the erythroblasts from PBMCs, and only 4 days culturing were required for cell recovery after cell thawing. Generally, 3×10^5 live cells were enough for the successful reprogramming, which is less than the amount (5×10^5 live cells) used in the previous protocol [33]. Of note, we could efficiently derive iPSCs under feeder-free conditions, which is favorable for future clinical applications.

Sendai virus reprogramming is an efficient and robust approach with a complete clearance of viral sequences at higher cell passages. The new generation of CytoTune®-iPSC 2.0 showed less cytoxocity and higher reprogramming efficiency compared to CytoTune®-iPSC 1.0. However, the cost of the reprogramming kit is very expensive. It was reported that one kit might apply to 24-48 blood samples (20, 000 cells each). The viral activity was maintained close to 80% activity of its original stock solution after 1 freeze and thaw cycle, and >50% activity even after 3 cycles [34]. This allows for thawing Sendai virus and preserving it in small aliquoted frozen stocks, which can greatly decrease the cost of reprogramming. In the current study, we obtained a similar result that efficient reprogramming of PBMCs could be achieved by the virus after 1 freeze and thaw cycle preservation.

Generation of feeder-free iPSCs from PBMCs is technically demanding due to the low reprogramming efficiency under feeder-free conditions. To generate feeder-free iPSCs, we determined whether three types of extracellular substrates could efficiently support cell reprogramming. Geltrex is a reduced growth factor basement membrane extract from murine Engelbreth-Holm-Swarm tumor used routinely for attachment and maintenance of hESCs. The major components of Geltrex include laminin, collagen IV, entactin, and heparin sulfate proteoglycan which provide the foundation for three-dimensional culture studies. Vitronectin and rhLaminin-521 are two recombinant human proteins. Vitronectin is a truncated recombinant protein, corresponding to the amino acid fragment 62-478 of human vitronectin used as a substrate for the feeder-free culture of human pluripotent stem cells (PSCs). rhLaminin-521 is a recombinant protein that provides a defined surface for feeder-free culture of PSCs. Laminin-521 is a natural component of the stem cell niche *in vivo*. rhLaminin-521 recapitulates a natural environment for maintenance of self-renewal, normal morphology, pluripotency, and karyotype of PSCs. In the current study, we employed Geltrex, vitronectin, and rhLaminin-521 to replace feeder cells for cell reprogramming. The results revealed that only Geltrex and rhLaminin-521 could efficiently support the initial cell reprogramming process, but vitronectin failed due to loss of attachment between the colonies and vitronectin substrate during the initial reprogramming stage. PBMCs are nonadherent cells, and therefore, tight adherence of PBMCs to the culture well or substrate is particularly important for successful reprogramming. In addition, although the vitronectin failed to support the initial reprogramming, the passaged iPSCs could grow normally and expand on vitronectin substrate, which is consistent with the published report [35]. Previously a similar study reported generation of hiPSCs from PBMCs under feeder-free conditions using Sendai virus [36]. They successfully derived iPSCs using Matrigel matrix in chemically defined conditions, however, Matrigel contained xeno-components. Matrigel

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and Geltrex are both extracted from murine tumor, and use of animal-derived products in derivation, maintenance, and differentiation of iPSCs risks zoonotic disease transmission and immune rejection when transplanted into humans. To avoid potential incorporation of xenogenic products, human substrate proteins are highly desirable. Our study identified that rhLaminin-521 was an ideal substrate for the routine derivation of xeno-free iPSCs.

Efficient cryopreservation and recovery of iPSCs is the first step for the basic research and applications. iPSCs have extremely poor survival rates after cell cryopreservation due to cell apoptosis [24]. In the current study, we found that the iPSCs displayed very poor survival efficiency after thawing with E8 medium alone, and the surviving cells were easy to differentiate. Very few cells could maintain their pluripotency. The reasons for this might be cell apoptosis or some unknown mechanisms, and this is an interesting question worth further investigation. To improve cell recovery, two Rock inhibitors were tested. Both RVC and Y-27632 dramatically enhanced the survival rates post-thaw, and RVC displayed a higher survival rate than that of Y-27632, as demonstrated by the confluency after cell culturing. In addition, unlike Y-27632, there was no requirement for pretreating iPSCs with RVC before cryopreservation [37, 38]. RVC contains a Rock inhibitor that is more selective than traditional Rock inhibitor Y-27632 and is coupled with small molecules including antioxidant and free-radical scavenger properties. This cocktail minimizes apoptosis and necrosis over the first 24 hrs after cell thawing and the off-target pathways affected. Those may be the major reasons for the higher survival rate under the condition of RVC and E8 medium combination. Single cell culture is a standardized and accepted practice in stem cell culture research. By employing Rock inhibitors, we successfully established a single cell passaging approach. Both RVC and Y-27632 could significantly improve the cell survival after single cell passaging while retaining normal cell growth kinetics, morphology, and pluripotency.

Conclusion

In the present study, we have successfully derived non-integration and feeder-free human iPSCs from peripheral blood cells. Of note, we established effective strategies for efficient cell recovery and single cell passaging, which play pivotal roles on iPSC applications. This study will pave the way to the derivation of clinical-grade human iPSCs for future clinical applications.

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Disclosure Statement

The authors declare no conflicts of interest.

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