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The Establishment of *In Vitro* Human Induced Pluripotent Stem Cell-Derived Neurons

(Penubuhan Neuron Berpunca Sel Stem Pluripoten *In Vitro* Manusia)

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ABSTRACT

Induced pluripotent stem cells (iPSCs) have been generated using different reprogramming strategies. Lentiviruses remain a strategic method for cell reprogramming as it is highly efficient in gene transfer. The latest fourth-generation lentiviral packaging systems claimed to be efficient and safe. However, modifications made to enhance safety of lentiviral vectors have been shown to affect vector performance. In this study, we established that the fourth-generation lentiviral packaging system can produce high-titre lentiviruses with high-transduction efficiencies. Subsequently, the robustness and reproducibility of generating iPSCs from adult human dermal fibroblasts were tested using these lentiviruses. The use of fourth-generation lentiviruses consistently generates iPSCs with similar efficiency and quality in different primary cell lines. This study demonstrated that the human-derived iPSCs can be maintained using mitomycin-C inactivated feeder cells. The iPSCs clones highly expressed key pluripotency markers and can spontaneously differentiate into cells from the three embryonic germ layers. The iPSCs generated were able to differentiate into neural stem cell lineages, producing cells expressing Nestin and Sox2 as well as able to further differentiate into neurons with more than 70% efficiency. The data demonstrated that the use of the fourth-generation lentiviral packaging to produce lentiviruses for iPSCs generation is robust and reproducible as it can generate iPSCs from different adult dermal fibroblasts with the potential to differentiate into neural stem cells and neurons. The use of safer lentiviral packaging systems combined with established vector plasmids will help to expedite the generation of iPSCs for clinical applications.

Keywords: Induced pluripotent stem cells; lentivirus; neural stem cells; neurons; reprogramming

ABSTRAK

Sel induk pluripoten teraruh (iPS) telah dihasilkan menggunakan strategi pengaturcaraan semula yang berbeza. Lentivirus kekal sebagai kaedah strategik untuk pengaturcaraan semula sel kerana ia sangat cekap dalam pemindahan gen. Sistem pembungkusan lentivirus generasi keempat terkini dikatakan lebih cekap dan selamat. Walau bagaimanapun, pengubahsuaian yang dibuat untuk meningkatkan keselamatan vektor lentivirus telah ditunjukkan boleh menjejaskan prestasi vektor. Dalam kajian ini, kami mendapati bahawa sistem pembungkusan lentivirus generasi keempat boleh menghasilkan lentivirus dengan titer tinggi serta kecekapan transduksi yang tinggi dan seterusnya menguji keteguhan dan kebolehulangan penjaanan sel iPS daripada fibroblas kulit manusia menggunakan lentivirus ini. Penggunaan lentivirus generasi keempat secara tekal menjana sel iPS dengan kecekapan dan kualiti yang sama dalam talian sel primer yang berbeza. Kami menunjukkan bahawa iPS yang dihasilkan di atas sel penyuap yang dinyahaktifkan

menggunakan mitomisin-C yang berasal daripada manusia boleh menyokong dan mengekalkan sel iPS. Klon sel iPS yang diperoleh mengekspresikan penanda pluripotensi utama dan boleh secara spontan membezakan menjadi sel daripada tiga lapisan sel embrio. Sel iPS yang diperoleh dapat dibezakan kepada keturunan sel induk saraf yang mengekspresikan Nestin dan Sox2 dan boleh matang menjadi neuron dengan kecekapan lebih daripada 70%. Data kami menunjukkan bahawa penggunaan pembungkusan lentivirus generasi keempat untuk menghasilkan lentivirus untuk penjanaan sel iPS adalah teguh dan boleh dihasilkan semula kerana ia boleh menjana sel iPS daripada fibroblas kulit dewasa yang berbeza dengan potensi untuk membeza menjadi sel stem saraf dan neuron. Penggunaan sistem pembungkusan lentivirus yang lebih selamat dalam gabungan dengan plasmid vektor yang mantap akan membantu mempercepatkan penjanaan sel iPS untuk terjemahan klinikal.

Kata kunci: Lentivirus; neuron; pengaturcaraan semula; sel induk pluripoten terjana; sel induk saraf

Introduction

Reprogramming of patient-derived adult somatic cells into a pluripotent state resembling embryonic stem cells have allowed the development of *in vitro* models for studying the mechanism of various diseases. Viral-based gene delivery methods have been used to generate iPSCs, including retroviruses (Takahashi et al. 2007) and lentiviruses (Yu et al. 2007), as well as non-integrative viral methods, such as adenoviruses (Zhou & Freed 2009) and Sendai viruses (Fusaki et al. 2009). Non-integrative gene delivery systems are often complicated with low reprogramming efficiencies, rendering integrative viral delivery systems, such as lentiviruses, to remain relevant in iPSC generation (González, Boué & Belmonte 2011; Malik & Rao 2013). iPSC-based *in-vitro* disease models have been created using lentiviruses for diseases, such as Duchenne Muscular Dystrophy (Teotia et al. 2015), schizophrenia (Liu et al. 2018) and diabetes (Kudva et al. 2012).

A major concern when using lentiviral technology is the potential generation of replication-competent viruses. The first-generation lentiviral vectors utilized human immunodeficiency virus (HIV) genes in a single plasmid, separating only the envelope protein in a separate plasmid. The second and third-generation systems worked towards enhancing safety by removing the more accessory viral proteins and the separation of vector plasmids into 3 or 4 separate plasmids, requiring an increase in recombination events to generate replication-competent viral particles. The latest fourth-generation system further split the helper plasmids into 5 separate plasmids, aimed at increasing safety during lentiviral production (Wu et al. 2000a). The separation of gene sequences over several plasmids has resulted in lower viral titres due to the need for an additional plasmid (Merten et al. 2016).

The combination of transcription factors also plays a role in the successful reprogramming of somatic cells into iPSCs. The most commonly used combination is known as the Yamanaka factors, consisting of Oct4, Sox2, Klf-4 and c-Myc (OSKM), which has been successful in reprogramming mouse and human somatic cells (Takahashi et al. 2007; Takahashi & Yamanaka, 2006). Around the same time, another combination of transcription factors Oct4, Sox2, Nanog, and LIN28 by Thomson Laboratory had shown similar reprogramming efficiency to the OSKM combination (Yu et al. 2007). The choice of reprogramming method and factors depend on the outcome for downstream applications, with the main aim being to obtain the highest reprogramming efficiency.

The culture conditions of iPSC reprogramming have also been shown to influence reprogramming efficiency. Defined culture conditions, such as using xeno-free iPSC culture media and synthetic matrix, have allowed standardization of culture conditions. However, this method is not only expensive but has been shown to have a lower reprogramming efficiency than feeder-based culture systems (Malik & Rao 2013). Feeder-based systems have been shown to support and maintain iPSC during reprogramming and expansion in an undifferentiated state by constant supplementation of bFGF (Teotia et al. 2016). Despite advances in xeno-free culture systems, the accessibility of inactivated feeder cell co-culture systems that utilize mouse or human cells made it relevant in achieving high reprogramming efficiencies.

For iPSC to be useful for downstream applications, reprogrammed iPSC must be able to differentiate into specific lineages. Zhou et al. (2018) demonstrated that the method of reprogramming somatic cells into iPSC, whether retroviral, episomal or mRNA-based

reprogramming, did not affect the gene expression of pluripotency markers or the differentiation potential of the derived iPSCs into smooth muscle cells (Zhou et al. 2018). In using iPSCs for *in vitro* disease modelling, the cells must be able to differentiate effectively into the terminal fate with less stringent attention placed on the remaining viral transgene. Therefore, a more robust reprogramming method is preferred.

To date, the efficiency of combining fourth-generation lentiviral packaging system with plasmids carrying a six-factor combination of transcription factors to produce iPSCs for neuron generation have not been tested. This study aims to investigate the efficiency of a fourth-generation lentivirus system in reprogramming human dermal fibroblasts into iPSCs, and subsequently evaluate the generated iPSCs ability to generate neurons. This study showed that the use of the fourth-generation lentiviral packaging system can produce lentiviruses carrying a combination of six reprogramming factors with high viral titres and are highly efficient in reprogramming human fibroblasts into high-quality iPSCs in a human feeder-based co-culture system. This study then demonstrated the efficiency of lentiviral reprogrammed iPSCs to differentiate into neural cells that can potentially be used for further disease modelling purposes.

Materials & Methods

Lentiviral production and titration

Bicistronic plasmids pSIN4-EF-O2S (Addgene plasmid # 21162; http://n2t.net/addgene:21162;RRID:Addgene_21162), pSIN4-CMV-K2M (Addgene plasmid # 21164;http://n2t.net/addgene:21164;RRID:Addgene_21164) and pSIN4-EF2-N2L (Addgene plasmid #21163;http://n2t.net/addgene:21163;RRID:Addgene_21163) was a gift from James Thomson. pLentiCMV GFP Hygro (656-4) was a gift from Eric Campeau and Paul Kaufman (Addgene plasmid # 17446;http://n2t.net/addgene:17446;RRID:Addgene_17446). 7 µg of plasmid were transfected individually into Lenti-X 293T cells (Takara Bio), grown at 80% confluence to complete lentivirus packaging using a fourth-generation Lenti-X lentiviral packaging system (Takara Bio), according to the manufacturer's instructions. The viral-containing medium was collected at 48 and 72 hours post-transfection. The collected medium was combined and filtered using a 0.45 uM filter. The filtered viral supernatant was concentrated from the media at a 1:100 ratio in serum-free DMEM using Lenti-X Concentrator (Takara Bio). Afterwards, it was aliquoted and stored at -80 °C until further analysis.

Qualitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The total RNA was extracted using NucleoSpin RNA Virus Kit (Macherey-Nagel) and one-step PCR, which was carried out using Quant-X One-Step qRT-PCR TB Breen kit (Takara Bio), according to the manufacturer's instructions. All experiments were performed on an ABI StepOnePlus Real-Time PCR (Thermo Fisher Scientific) and the PCR products were quantified fluorometrically using ROX Reference Dye (Takara Bio). The RNA content was calculated based on a standard curve of known RNA dilution.

Cell culture

Human Dermal Fibroblast 1 (HDF1) was from a 27-year-old female and Human Dermal Fibroblast 2 (HDF2) was from a 34-year-old male, both healthy donors with no known medical conditions. The samples were collected by punch biopsy and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 4 mM GlutaMAX 3.7 g/L sodium bicarbonate and 1mM Sodium Pyruvate (Gibco) supplemented with 10% FBS (Gibco).

BJ neonatal foreskin fibroblasts (ATCC, CRL-2522) were used for both iPSCs generation and to prepare the feeder cells. To produce feeder cells, 1×10^6 BJ fibroblasts were seeded on gelatine-coated 100 mm culture dishes. BJ cells were incubated in 10 µg/mL final concentration of Mitomycin-C (HiMedia) for 3 hours before replacing with fresh fibroblast medium.

The fibroblast growth medium was replaced with an embryonic stem cell (ES) medium after seeding the reprogrammed cells on the feeder cells. The ES medium consists of DMEM/F-12 with 2.5 mM GlutaMAX (Gibco) supplemented with 20% Knock-out (KO) serum replacement (Gibco), 1% penicillin-streptomycin (Sigma), 1% non-essential amino acids (NEAA), (Gibco), 55 µM 2-mercaptoethanol (Gibco), and 4 ng/mL Human FGF-basic recombinant protein (bFGF) (Gibco). iPSCs grown in a feeder-free system were grown in mTeSR Plus medium (Stemcell Technologies). 0.1% gelatine (Stemcell Technologies) and VitronectinXF (Stemcell Technologies) were used as a growth substrate for iPSCs growth on the feeder and feeder-free systems, respectively.

GENERATION OF iPSC LINES

Reprogramming was performed with the combination of lentiviruses as previously described by Nethercott, Brick and Schwartz (2011). Briefly, BJ cells were plated to achieve approximately 1×10^5 cells in a 12-well plate and were cultured in fibroblast medium on the day of transduction. Lentiviruses were combined to form a cocktail containing each virus preparation at a multiplicity of infection (MOI) of 5. Transduction efficiency was monitored by transducing a separate identical culture with GFP lentivirus at MOI of 5. Transduction with a lentiviral-containing medium was carried out over 24 hours. Subsequently, the spent medium was replaced daily with fibroblast medium. On day 5, following transduction, each transduced well was passaged at a 1:2 ratio and plated onto a 100 mm dish containing prepared mitomycin-C inactivated BJ fibroblasts. On day 6, the fibroblast medium was changed to ES medium supplemented with 4 ng/mL of bFGF. ES medium was replaced daily until colonies were ready to be picked. The iPSC colonies were picked and expanded by clonal expansion. Briefly, the selected clones were manually cut into small pieces, removing differentiated cells and large clumps using a heat-pulled glass pipette, where they were transferred to a feeder-free culture system. 10 mM of Y27632 (Rho kinase inhibitor) (ATCC) was added to the culture medium overnight after every passage. The medium was replaced every alternate day. The iPSC clones were cryopreserved in KO serum replacement supplemented with 20% DMSO (Sigma) after passage 5.

CHARACTERIZATION OF iPSCs MORPHOLOGY

The expanded iPSCs were cultured on Vitronectin-coated plates in mTeSR Plus medium. The morphology of the iPSC clones was compared to the previous description and images of iPSCs and ES cells (Liao et al. 2008; Nagasaka et al. 2017; Takahashi & Yamanaka 2006; Takahashi et al. 2007). Immunofluorescent images were taken using an Axio Imager 2 (Zeiss) fluorescent microscope. Image analysis and quantification were carried out using ImageJ software.

ALKALINE PHOSPHATASE AND IMMUNOCYTOCHEMICAL ANALYSIS OF THE STEM CELL MARKERS

For alkaline phosphatase staining, expanded iPSCs colonies in the feeder system were incubated in Alkaline Phosphatase Live Stain (Molecular Probes) for 20 to 30 minutes as per the manufacturer's protocol. The alkaline phosphatase stain was removed and the cultures were washed with DMEM/F-12 medium twice for 5 min each. Alkaline phosphatase activity was visualized under a fluorescent microscope with the standard FITC filter. The iPSCs were seeded onto Vitronectin-coated chamber slides and cultured for 2-3 days for colonies to adhere and expand. The slides were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min, blocked with 10% normal horse serum (Vector Laboratories) for 1 hour and incubated in Oct4 (1:200, Cell Signalling,) and TRA-1-81 (1:200, Cell Signalling) or Sox2 (1:200, Cell Signalling) and TRA-1-60 (1:200, Cell Signalling) overnight at 4 °C. The slides were washed and incubated with horse anti-mouse IgG (H+L) DyLight® 549 (1:500, Vector laboratories) and horse anti-rabbit IgG (H+L) DyLight® 488 (1:500, Vector Laboratories) secondary antibodies for 1 hour at room temperature. The slides were washed and mounted with Vectashield mounting medium with DAPI (Vector laboratories). The images were taken on an Imager M2 Zeiss Fluorescent microscope. Image analysis was carried out using Image J software.

For flow cytometry analysis, the iPSCs were detached using Accutase (Gibco) solution and resuspended into a single cell suspension. The cells were fixed with 4% PFA (BD Biosciences) for 20 min and permeablized with 1% triton-X solution (BD Bioscience) before incubation in conjugated antibody PerCP-Cy 5.5 Mouse anti-Oct3/4 (1:20, BD Biosciences), Alexa Fluor 647 Rat anti-SSEA-4 (1:20, BD Biosciences) and PE Rat anti-SSEA-1 (1:20, BD Biosciences) for 30 min. The cells were analysed using FACSCanto (BD Bioscience) flow cytometer and the data were analysed using FACS Diva software.

EMBRYOID BODY FORMATION

The iPSCs growing on Vitronectin-coated plates were detached using Versene (Gibco) for 5 min until the colonies became loose. Versene was removed and the cell colonies were gently dislodged and collected using a pasture pipette. The suspension was gently triturated to break the cell colonies and cell density was calculated using a haematocytometer. The cells were pelleted and resuspended to obtain approximately 3×10^6 cells per mL. Fifteen uL drops of cell suspension were plated on an upturned 100 mm culture dish. Twenty mL of PBS were added to the dish to avoid the cell suspensions from drying out. Cells in hanging drops were cultured for 3 days before collecting the formed EB and replating them

into chamber slides. The EBs were allowed to adhere and spontaneously differentiate in the EB medium (ES medium without bFGF supplementation). The EB medium was replaced every alternate day. Differentiated cells were fixed on day 28 and analysed with fluorescent microscopy after staining with anti-smooth muscle actin (SMA) (1:100, ABCAM), anti-alpha fetoprotein (AFP) (1:100, ABCAM) and anti-BIII tubulin (TUJ1) (1:100, ABCAM) and secondary staining as described.

NEURAL DIFFERENTIATION

Differentiation of iPSCs into neural stem cells (NSC) was carried out using a defined small molecule direct differentiation protocol as previously described (Bell et al. 2019). The iPSCs colonies at 80% confluence were harvested and seeded at 50,000 cells/cm^2 in a Vitronectin-coated 6-well plate supplemented with ROCK inhibitor at 10 µg/mL. On day 1 of directed differentiation, the feeder-free ES medium was replaced with a Neural induction medium and was changed every alternate day. The iPSCs were differentiated into neural stem cells as previously described. The cells were plated at a high density on Vitronectin plates in mTeSR Plus medium before replacing it with Neural Induction Medium (Gibco) following the manufacturer's protocol. On day 7, homogenous neural stem cells (P0) were harvested using Accutase for 5 min at 37 °C and replated onto Geltrex (Invitrogen)-coated plates. P1 neural stem cells were expanded in Neural Expansion Medium (Gibco) and were stained for neural marker Nestin (1:100, Invitrogen), stem cell marker, SOX2 (1:200, CellSignalling) and pluripotent cell marker, OCT4 (1:200, CellSignalling). The expanded neural stem cells were cryopreserved in a neural expansion medium supplemented with 10% DMSO. For neural maturation medium, neural stem cells were cultured in Neurobasal medium supplemented with ascorbic acid (Sigma), penicillin-streptomycin (Gibco), B27 Supplement (50X) (Gibco) and CultureOne Supplement (100X) (Gibco). The media was changed every 3 days by removing half of the spent medium and replacing it with fresh medium.

RESULTS

FOURTH-GENERATION LENTIVIRAL PACKAGING SYSTEM PRODUCED HIGH-TITRE LENTIVIRUSES WITH HIGH TRANSDUCTION EFFICIENCY

To efficiently generate iPSCs, the first aim of this study was to generate a high-titre lentivirus. Lentiviruses were generated by co-transfecting the fourth-generation lentiviral packaging system, containing 5 separate packaging plasmids with Self-Inactivating (SIN) bicistronic vectors as listed in Table 1 in LentiX-293T cells. The supernatant containing concentrated virus was collected at 48 and 74 hours post-transfection and was titred by qRT-PCR, which produced between 2 -7 × 10^9 viral particles/mL (Table 1).

To assess the transduction efficiency with the fourth-generation system in producing lentivirus vectors, fibroblasts were transduced with the lentivirus carrying fluorescent marker GFP (LV-GFP) at a multiplicity of infection (MOI) of 5 (Figure 1(a)). The transduction efficiency of all fibroblast lines was between 69% and 87% (Figure 1(b)).

TABLE 1. Bicistronic plasmids containing transcription factors and marker protein green fluorescent protein (GFP) used for transfection

Plasmid ID	Gene	Viral Titre (copies/mL)	Source	Reference
pSIN4-EF2-O2S	OCT4, SOX2	6.14 × 10^9	Addgene (#21162)	
pSIN4-EF2-N2L	NANOG, LIN28	2.91 × 10^9	Addgene (#21163)	Yu et al. (2009)
pSIN4-CMV-K2M	KLF4, C-MYC	3.5 × 10^9	Addgene (#21164)	
pLenti_CMV_GFP	GFP	5.6 × 10^9	Addgene (#17446)	Campeau et al. (2009)

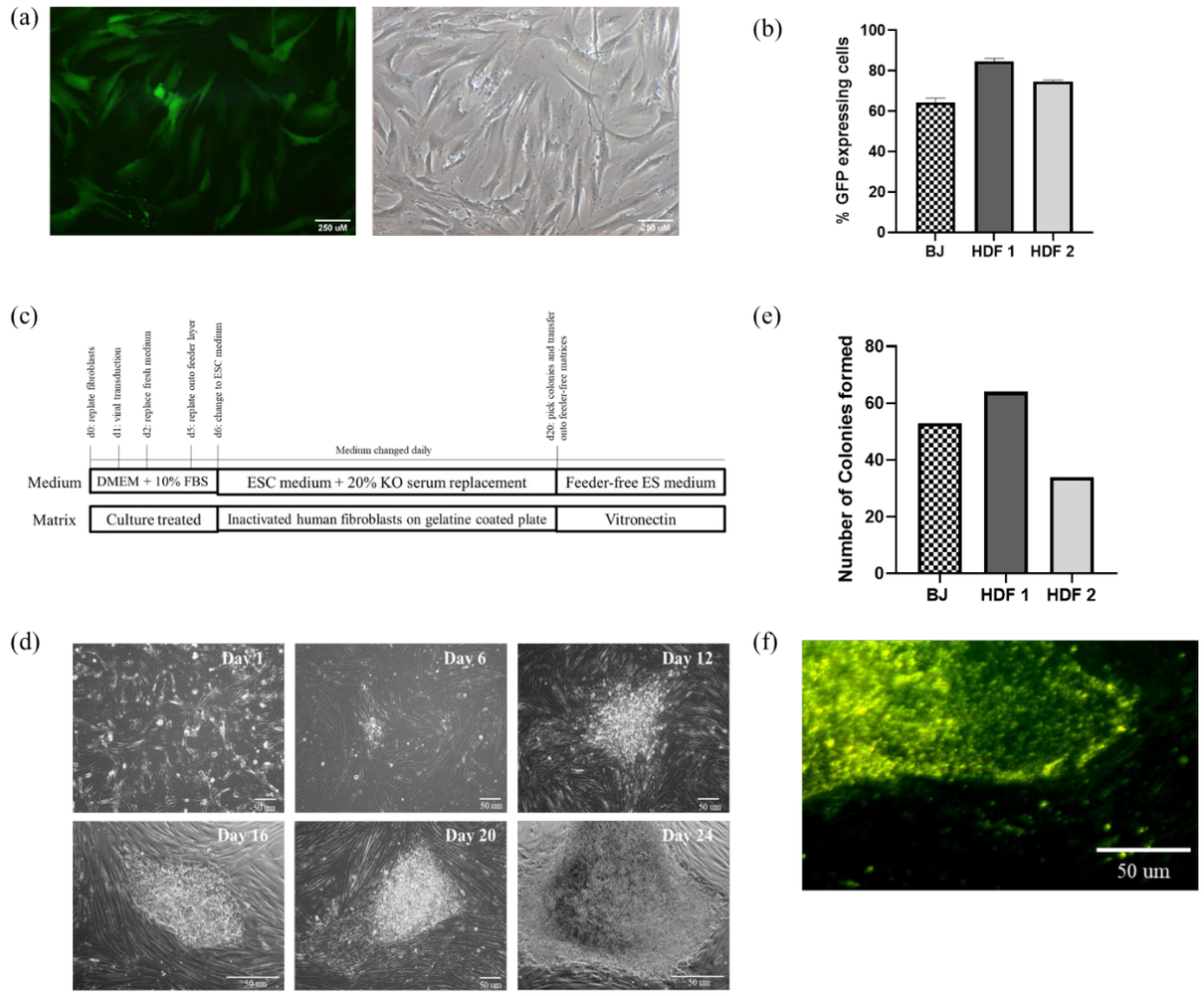

FIGURE 1. Transduction of fibroblasts and generation of iPSC colonies. (a) Expression of GFP in BJ fibroblasts transduced with LV-GFP at MOI 5, (b) Percentage of cells expressing GFP in all primary fibroblast lines compared to BJ fibroblasts at MOI 5, (c) Schematic of protocols for generation of iPSCs, (d) Morphological changes seen in transduced fibroblasts at day 1, 6, 12 16, 20 and 24 following transductions, (e) Resulting number of dense cell colonies formed after 20 days post-transduction with lentivirus carrying transcription factors at MOI 5, (f) Alkaline phosphatase live staining of formed colonies showing enhanced fluorescence of dense cell colonies compared to surrounding feeder layer cells

REPROGRAMMING OF HUMAN DERMAL FIBROBLAST INTO iPSCs BY LENTIVIRAL TRANSDUCTION PRODUCED EMBRYONIC STEM-CELL LIKE COLONIES

Two human dermal fibroblast cell lines isolated from healthy donors, and one commercially available fibroblast cell line, BJ, originating from neonatal foreskin fibroblasts were used for the generation of iPSCs. An overview of the reprogramming protocol is shown in Figure 1(c).

Morphological changes were observed from day 6 post-transduction. Large changes in cell morphology were seen from day 12 post-transduction as elongated narrow fibroblast cells changed into small, juxtaposed colonies, resembling ES cells (Figure 1(d)). The colonies appeared to increase in confluency and individual cells were unable to be distinguished.

On day 24 of culture, between 34 and 64 colonies had visibly formed over the fibroblast feeder layer with reprogramming efficiencies between 0.06 and

0.13% (Figure 1(e)). The colonies strongly expressed fluorescent alkaline phosphatase staining compared to the surrounding fibroblast feeder layer (Figure 1(f)). A minimum of 20 clones were selected from each fibroblast line and expanded prior to cryopreservation. One clone from each fibroblast cell line was subjected to characterization. The expanded clones were identified as iPSCs.

CHARACTERIZATION OF FIBROBLAST-DERIVED iPSCs AND PLURIPOTENCY

The fibroblast-derived iPSCs generated were able to robustly proliferate in ES cell culture conditions. The morphology of the expanded cells derived from each cell line following expansion on feeder-free Vitronectin-coated plates exhibited tightly packed cell colonies with large nuclei to cytoplasmic ratio, a similar morphology to human ES cells (Figure 2(a)). The morphology of the derived iPSCs retained until passage 20. All fibroblast-derived iPSCs clones expressed pluripotency markers, Oct4A, Sox2, TRA-1-81 and TRA-1-61, which was detected by fluorescent microscopy (Figure 2(b)). Flow cytometry analysis of BJ-derived iPSCs showed approximately 97.8% of cells expressing Oct3/4, 100% of cells were positive for SSEA4, and 0.2% of cells expressing SSEA1. In contrast, the flow

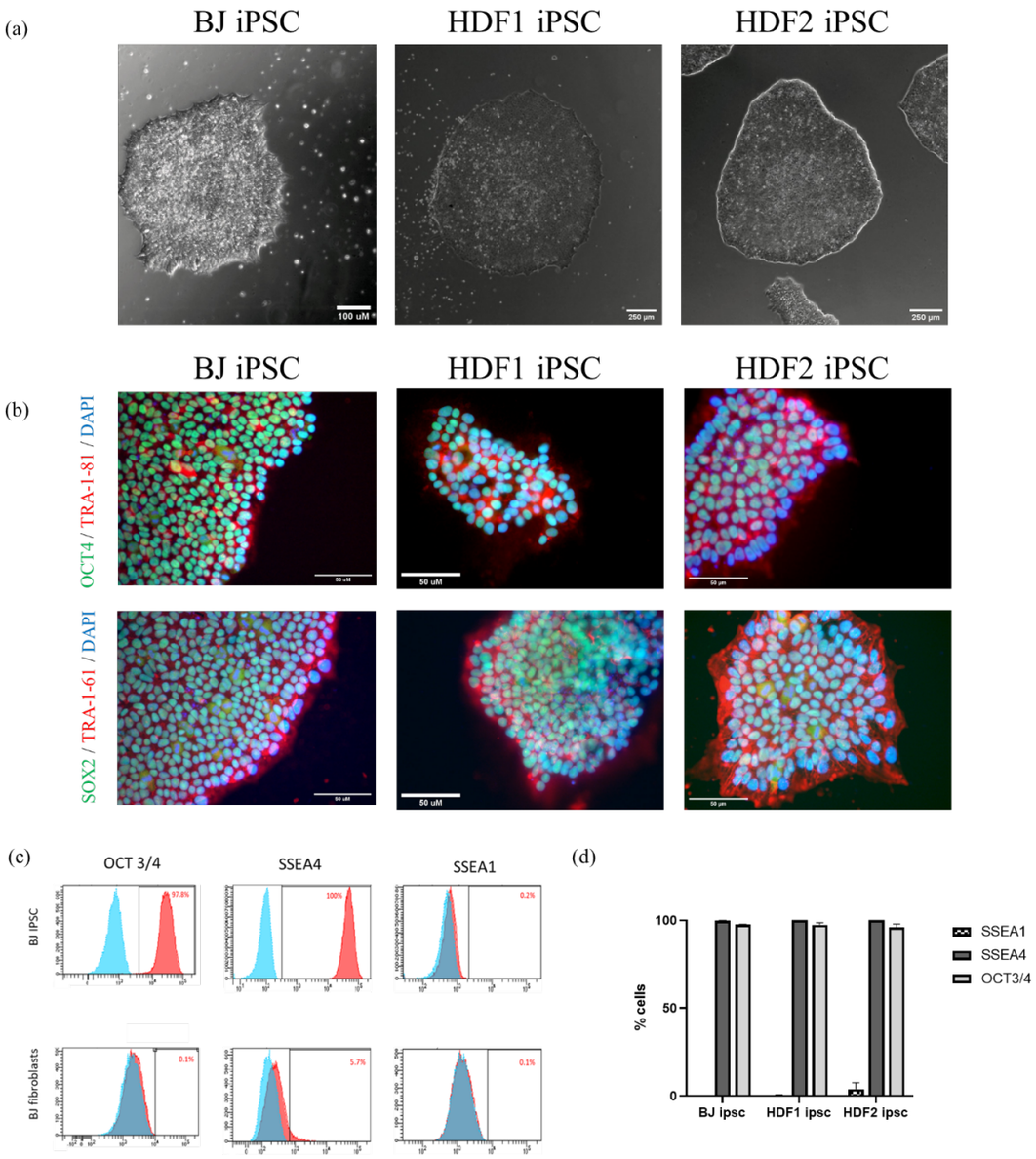

FIGURE 2. Characterization of generated iPSCs. (a) Morphology of cell colonies expanded on Vitronectin coated surface in a feeder-free system (b) Immunostaining of generated colonies with pluripotent markers Oct44, TRA-1-81, Sox2 and TRA-1-61 under fluorescent microscopy. Scale bar represents 50 µm. (c) Histogram following flow cytometry analysis of BJ iPSCs compared to primary BJ fibroblasts (d) Expression of SSEA1, SSEA4 and Oct3/4 in expanded iPSC colonies from all tested cell lines

cytometry characterization of the parental BJ fibroblast cells showed less than 6% of cells expressed Oct3/4, SSEA4, and SSEA1 (Figure 2(c)). The flow cytometry analysis of HDF1 and HDF2 iPSCs-derived clones exhibited over 90% expression of TRA-1-81 and SSEA4 and <5% expression of SSEA1 (Figure 2(d)).

CHARACTERIZATION OF DIFFERENTIAL POTENTIAL

To further demonstrate the pluripotency of the iPSCs *in vitro*, the iPSCs were cultivated to form embryoid bodies (EBs). The iPSCs were grown in individual hanging drops on an upturned lid of a 100 mm plate to form cell aggregates. The cell aggregates were plated on Vitronectin-coated chamber slides and were allowed to adhere and undergo spontaneous differentiation. An overview of the protocol for the spontaneous differentiation of EBs is shown in Figure 3(a). Following 28 days of culture in EB medium, phase-contrast microscopy showed a mixed morphology of cells, including neuronal-like cells, epithelium cells and cobblestone-like cells originating from each EB

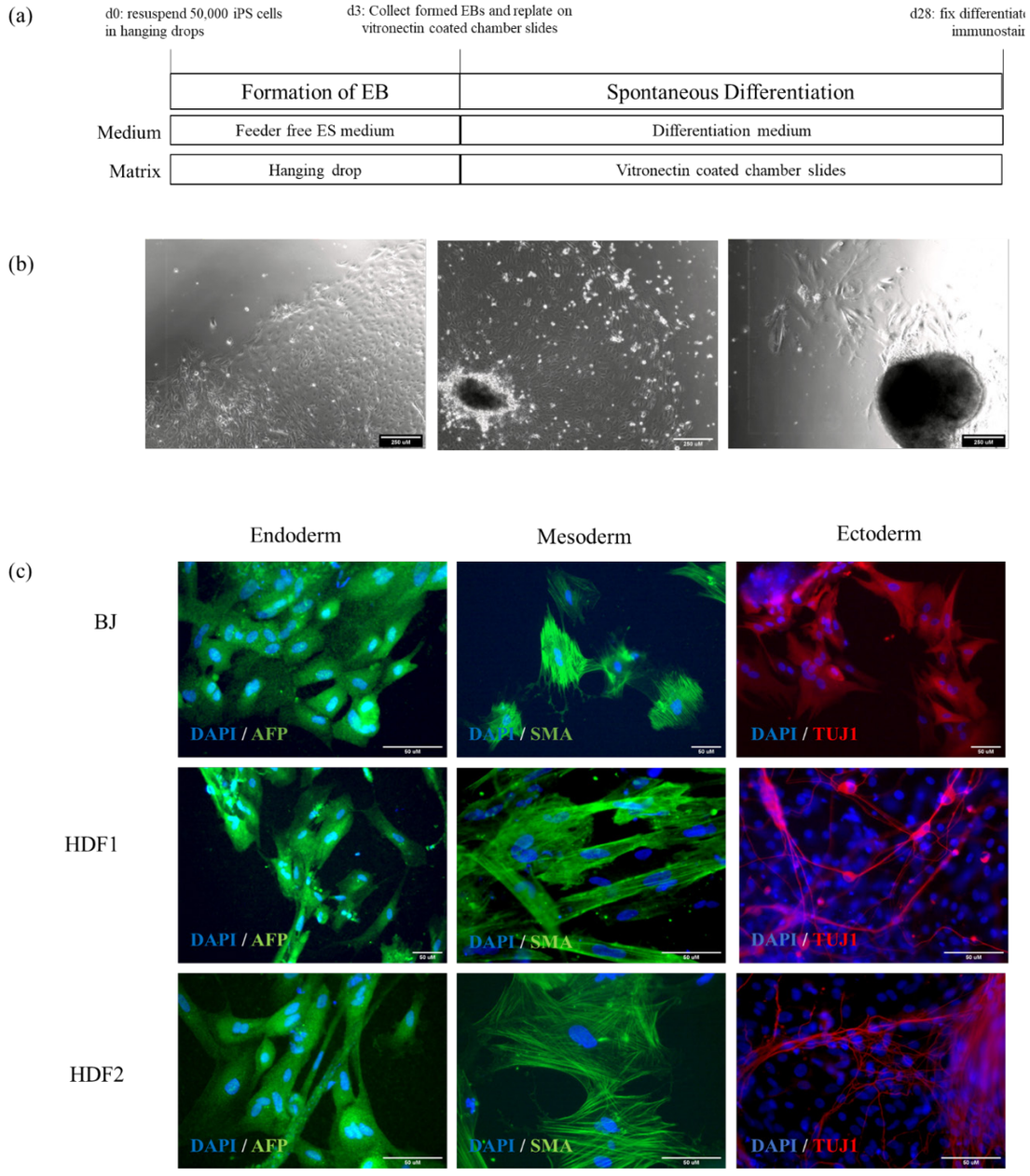

FIGURE 3. Formation of embryoid body for characterization of pluripotent potential, (a) Schematic of protocol for embryoid body formation and spontaneous differentiation, (b) Morphology of resulting populations following 28 days of spontaneous differentiation on Vitronectin coated surface. Scale bar represents 250 µm, (c) Characterization of cells generated by spontaneous differentiation of iPSCs by immunofluorescent staining. AFP is used as marker of Endoderm cell, a-SMA as a marker of mesoderm and BIII-tubulin (TUJ1) as a marker of Ectoderm. Scale bar represents 50 µm

(Figure 3(b)). Protein detection by immunofluorescent staining demonstrated that distinct populations in the cells generated after 28 days expressed the endoderm marker, alpha-fetoprotein (AFP), the mesoderm marker, alpha-smooth muscle actin (a-SMA), and the ectoderm marker, BIII-tubulin (TUJ1) (Figure 3(c)).

iPSCs DIFFERENTIATION AND EFFICIENT PRODUCTION OF NEURAL STEM CELLS AND NEURONS

To obtain high efficiency in neural differentiation, extensive standardization was carried out to obtain high-quality neural stem cells as outlined in Figure 4(a). Using enzymatic dissociation of iPSCs, smaller cell clumps were seeded at a density of 50,000 cells/cm^2. When the seeded cells reached a confluency of 25%, the cells were grown in Neural Induction Medium (NIM). Cells maintained in NIM reached 100% confluency on day 7 (Figure 4(b)).

The morphology of the iPSCs shifted from having large prominent nuclei to less visible nuclei and formed more tightly packed cell clumps (Figure 4(b)). Flat heterogenous mixed cell populations surrounding tightly packed colonies were observed after the first passage. Selective dissociation of cells produced homogenous cell morphology in subsequent passages.

Immunofluorescent staining of the cells detected the presence of Nestin and Sox2 in most cells (Figure 5(a)). The presence of Oct4-positive cells was also detected (Figure 5(b)). Quantification of neural stem cell markers in all three differentiated iPSC lines showed

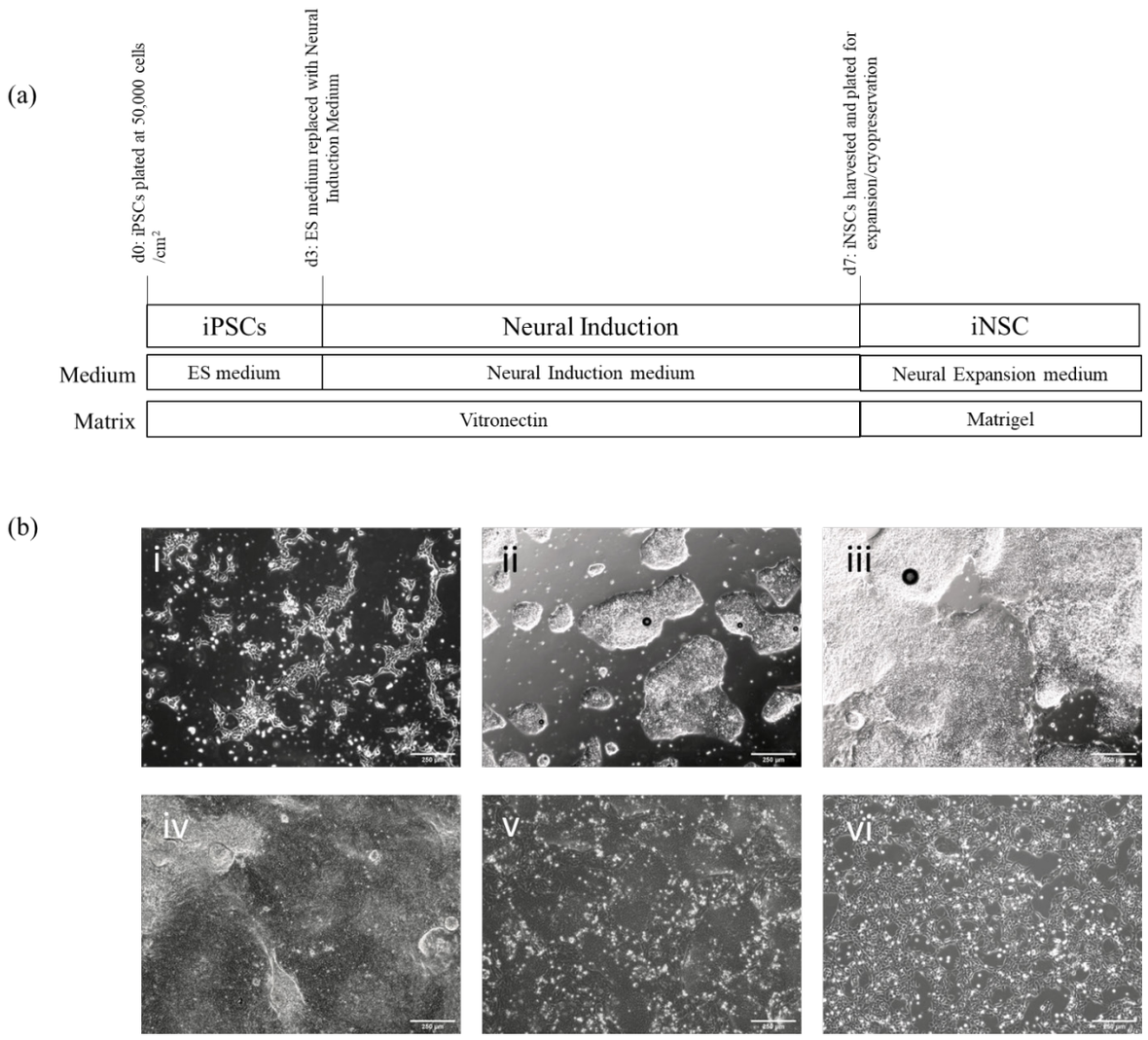

FIGURE 4. Generation of neural stem cells (NSCs). (a) Schematic of protocol for iPSC derived NSCs, (b) Morphology of iPSCs during neural induction. (i) iPSCs at day 1 of splitting with 20-25% confluence. (ii-iv): The morphology of cells at days 3, 5, and 7 after neural induction. (v-vi): iPSC derived NSCs at passage 1 (v) showing heterogenous mixed cell morphology and passage 2 (vi) following selective dissociation and removal of non-neural stem cell population. Scale bar represents 250 µm

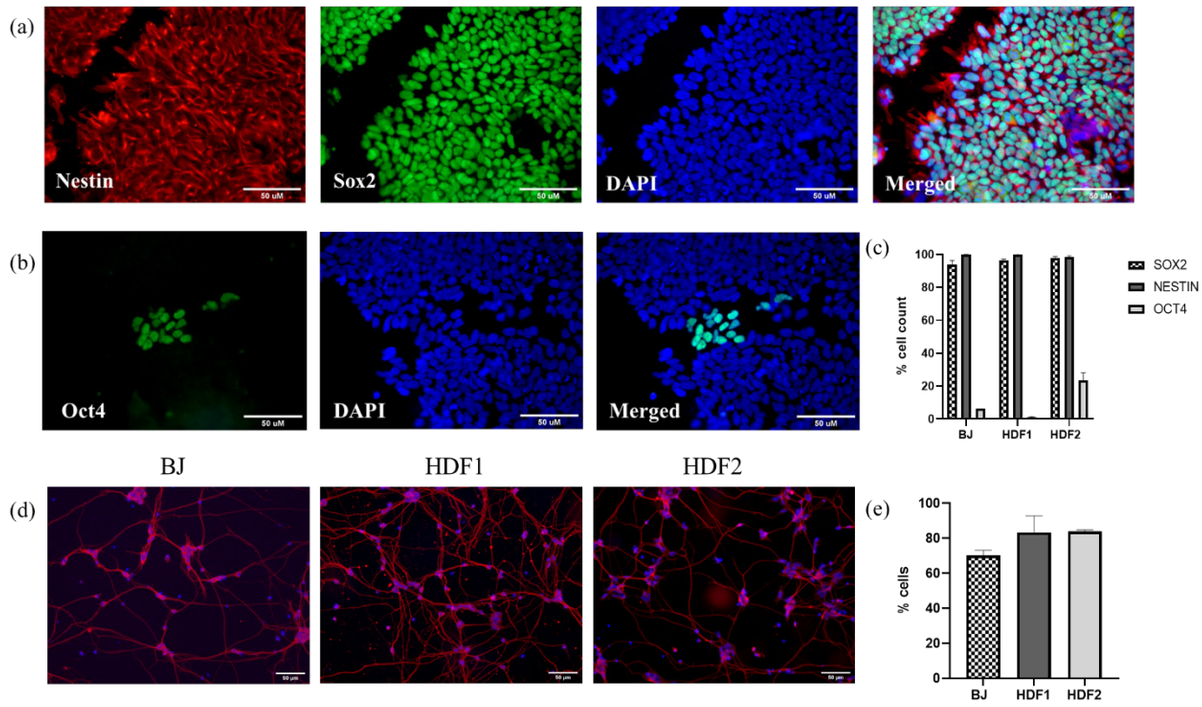

FIGURE 5. Characterization of neural stem cells (NSCs) and neurons by immunostaining (a) Detection of pluripotency markers Sox2 and NSC marker Nestin in iPSC derived NSCs, (b) Detection of Oct4 present in low numbers in derived NSC population, (c) Quantification of pluripotency and NSC markers in generated iPSC derived NSC lines, (d) Detection of neural marker BIII-tubulin (TUJ1) in iPSC derived neurons, (e) Differentiation efficiency of iPSCs derived NSC into neurons. Cell nuclei were counterstained with nuclear DNA marker DAPI (blue). Scale bar represents 50 µm

more than 90% expression of Nestin and Sox2 in all cells. Oct4 expression was less than 5% in BJ and HDF1 differentiated iPSCs, whereas 20% of Oct4 expression was found in HDF2 differentiated iPSCs (Figure 5(c)). The differentiated cells will be identified as neural stem cells.

To assess whether neural stem cells were able to differentiate into neurons, neural stem cells were grown in a neural maturation medium for 7 days before being subjected to morphological analysis. The resulting cells from all three cell lines expressed BIII-tubulin (TUJ1) with extrusions coming off the cell body with branching, resembling neurites (Figure 5(d)). The expression of BIII-tubulin (TUJ1) was more than 70% in all three cell lines (Figure 5(e)).

DISCUSSION

In this study, the lentiviral reprogramming strategy was used to produce iPSCs. To minimise risks associated with lentiviral production, we utilized a fourth-generation lentiviral packaging system. The commonly used third-generation lentiviral vectors encode only three out of the nine HIV-1 proteins expressed in separate plasmids (Ferreira, Cabral & Coroadinha 2021). The fourth-generation packaging plasmid improves this vector design by further separating the viral sequences into five separate plasmids (Wu et al. 2000b). This effectively increases the number of recombination events required to generate replication-competent viral particles, increasing safety during lentiviral production.

Self-inactivating (SIN) based transfer vectors were selected to generate the lentiviruses. The use of transfer vectors containing self-inactivating (SIN) modifications of HIV-based lentiviruses may prevent vector DNA mobilization and recombination (Pauwels et al. 2009). The combination of fourth-generation lentiviral packaging with SIN-transfer vectors was aimed at improving the safety of lentiviral production. However, the selection of 'safer' vectors for lentiviral

production should not affect vector performance. This study has shown that this combination was able to produce high-viral titres. The ability to safely produce high titres of lentivirus potentiates the translation of lentiviral reprogramming and iPSC generation for clinical applications.

The study then continued to test the efficiency and reproducibility of the reprogramming method. Human dermal fibroblasts were selected as the starting material as they are readily available, able to withstand cryopreservation well, and are easy to handle. BJ cells were selected as the reprogramming control as it is a neonatal foreskin fibroblast cell line that was previously shown to have a high reprogramming ability compared to other primary human dermal fibroblast lines (Takahashi et al. 2007). The iPSCs have successfully been generated from various cell sources, such as blood cells (Yang et al. 2018), keratinocytes (Aasen et al. 2008), and urine epithelial cells (Zhou et al. 2011). Despite these advancements, generating iPSCs from fibroblasts remains widely relevant.

This study has shown that the efficiency of transduction in primary fibroblasts was similar to BJ cells. However, the level of transduction with GFP did not reflect the number of resulting colonies. As transcription factors on separate vector plasmids were used during lentiviral production, only the number of viruses that are transduced was able to be controlled but not the level of genomic integration of each gene into the DNA of the target cells as well as the expression of those genes.

The iPSCs were successfully created from as little as one or two transcription factors (Kim et al. 2008). However, this is dependent on the starting cell type as progenitor cells already express some level of pluripotency genes. In adult somatic cells, Oct4, Sox2, Klf4 and c-Myc are the most widely used combination known as the Yamanaka factors (Takahashi & Yamanaka 2006). The transcription factors chosen for generating iPSCs in this study were based on the findings by Thomson's laboratory that showed Oct4, Sox2, Nanog and Lin28 (OSNL) were sufficient to reprogram human somatic cells into iPSCs (Yu et al. 2007). According to Liao et al. (2018), the efficiency of reprogramming was improved ten times when Klf4 and c-Myc were combined with the OSNL genes (Liao et al. 2008). The combination of transcription factors used has previously been used with the second-generation lentiviral packaging system (Nethercott, Brick & Schwartz 2011). The combination of the fourth-generation lentiviral packaging system with the same set of transcription factors used in this study is capable of producing high-titre lentiviruses.

The use of lentiviral transduction of six transcription factors raises the concern of increased genomic integration. However, the use of lentiviruses has proven to be an efficient strategy for gene transfer. Foot-print free viral methods have much lower reprogramming efficiencies in human fibroblasts, 0.0002% with adenoviruses (Zhou & Freed 2009) and 1% with Sendai viruses (Malik & Rao 2013). Lentiviral and retroviral reprogramming methods cause random integration of foreign genes into the DNA of the cell, increasing the risk for tumour formation and insertional mutagenesis (Yoshida & Yamanaka 2017). Reducing the number of transcription factors used will not eliminate genomic integration and risk for tumourigenicity as long as an integrative system is being used. Consequently, the priority is to utilize a method that has high reprogramming efficiency.

Transduction with the six transcription factors in adult fibroblasts resulted in early and efficient colony formation. However, the number of colonies calculated in this experiment was based on the morphology and visible colonies, not on any specific ES stain, such as alkaline phosphatase as previously used in other studies (Štefková, Procházková & Pacherník 2015). Our protocol was carried out in a single 100 mm dish to allow consistency in feeding and monitoring of emerging iPSC clones. Consideration of the cost of carrying out live staining in a 100 mm dish and the inability to visualize the fluorescence of the entire plate limited our analysis. Ideally, a separate culture would have to be created in a separate plate and grown simultaneously with the specific purpose of colony staining and quantification. However, considering the cost of maintaining culture media and supplements over 20 days had to be considered.

Emerging iPSCs clones were cultured on feeder cells generated from BJ cells. BJ fibroblasts were inactivated with mitomycin-C to create a xeno-free co-culture system. The use of human fibroblasts was to avoid cross-contaminating the human primary cells with different species, such as mice. Human placenta, uterine endometrium, postnatal fibroblasts, and neonatal fibroblasts are some of the established cell types used for maintaining undifferentiated ES or iPSCs in culture (Richards 2003; Teotia et al. 2016). The use of a feeder layer co-culture system is to provide a constant source of growth factors to the developing iPSCs and provide a substrate for growth (Teotia et al. 2016). However, the

use of human fibroblasts as a feeder layer has shown to be less effective than using mouse embryonic fibroblasts (Richards 2003; Willmann et al. 2013). Mouse embryonic fibroblast feeder layers have been shown to support the production and growth of larger colonies (Willmann et al. 2013). Using human fibroblasts as a source of feeder cells in the generation of iPSCs was found to be sufficient to support the development of high-quality iPSCs.

Colonies were picked on days 20-27 of cultures based on the confluency of the colonies to avoid differentiation in overlapping cells. These colonies were further expanded in a feeder-free culture system on Vitronectin-coated plates. The resulting colonies that were expanded resembled ES cells as described in previous studies (Nagasaka et al. 2017; Takahashi & Yamanaka 2006). These colonies were able to maintain the morphology of ES cells over prolonged cultures. The study also aimed to assess the expression of key nuclear and surface markers of pluripotency. The derived iPSC lines strongly expressed Oct4, Sox2, and Nanog. More importantly, the study had shown that the iPSCs endogenously expressed TRA-1-81, TRA-1-61, and SSEA4. The expression of pluripotency was quantified through flow cytometry and found a high expression of pluripotency proteins and low expression of early differentiation protein, SSEA1, indicating high-quality iPSCs. The iPSC lines generated from all three fibroblasts matched the immunophenotypic characterization criteria of iPSCs used for clinical applications (Andrews et al. 2015; Sullivan et al. 2018) with >90% of iPSCs being positive for nuclear and surface markers of pluripotency. *In vitro* differentiation using a 28-day protocol to induce spontaneous differentiation following EB formation was able to produce cell types that are representative of three germ layers; endoderm, mesoderm and ectoderm, which is sufficient to demonstrate the pluripotent capacity of the generated iPSCs (Sullivan et al. 2018).

This study has demonstrated that the iPSC line can be differentiated into neural lineages. Previously, the ability of iPSCs to differentiate was affected by the number of reprogramming factors used with less efficiency of differentiation seen in iPSCs generated using lesser reprogrammed factors (Löhle et al. 2012). However, Zhou et al. (2018) showed that the iPSCs generated from either integrative viral methods or non-integrative non-viral methods did not affect the ability of the cells to differentiate. Despite using six transcription factors with an integrative viral reprogramming method, high-quality neural stem cells were able to be generated. Nestin is an intermediate filament protein expressed by neural stem cells (Wislet-Gendebien et al. 2003). Sox2 plays an important role in maintaining pluripotency and is expressed in iPSCs as well as neural stem cells (Avilion et al. 2003). The expression of these two proteins indicates that the iPSCs have differentiated into the neural lineage while still maintaining their stemness. However, the directed differentiation method using chemically defined media is not 100% efficient. This is seen by the presence of Oct4-positive cells still present in the NSC cultures. Further purification may be necessary for further downstream applications. Neural stem cells from all three generated iPSC lines were able to undergo further differentiation and express pan-neuronal marker, BIII-tubulin (TUJ1), in 60 to 80% of the cells, indicating moderate to high differentiation efficiency. However, to investigate if neurons generated are indeed phenotypically and functionally mature, further testing using protein biomarkers such synaptophysin and MAP2 accompanied by functional assays such as measurement of neuron action potential may provide further insight into ability to generate mature neurons.

The characterization of two primary dermal fibroblasts compared to the control cell line, BJ fibroblasts, by lentiviral transduction of six transcription factors has shown that the protocol from this study is robust, reproducible, and has high efficiency. The use of human-derived feeder cells supports the development of iPSCs. This study has shown that the generated iPSCs have the potential to be used in further downstream applications involving neuronal cells. However, due to the separation of transcription factors used in this integrative method, caution should be taken as random integration of the transcription factors may create heterogeneous iPSCs despite originating from the same source (Li et al. 2015). This heterogeneity could potentially affect cell function (Aradi et al. 2004), hence, it is recommended that the generated iPSCs be expanded by individual clones with multiple derived iPSC lines from the same donor to compare clone-to-clone heterogeneity prior to the selection of iPSCs for further downstream research.

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