Generation of Human Induced Pluripotent Stem Cells with Non-Integrating Episomal Vectors and Xeno-Free Culture System

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Abstract: The generation of induced Pluripotent Stem Cells (iPSCs) enable people to have the opportunity to use patient-specific somatic cells which radically advance the field of regenerative medicine including disease modeling and drug discovery. The use of lentiviruses in the process of reprogramming human somatic cells into induced Pluripotent Stem (iPS) cells could produce tumorigenic insertional mutations and residual or reactivation of transgene expression during iPSC differentiation which could limit their therapeutic usefulness and affect lineage choice and the unctionality of iPSC derivatives. In addition, the exposure of iPSCs to animal-derived products may cause potential xenopathogenic transmission and immune rejection, these problems should be avoided and urgently needed to be resolved before the clinical application. Here, researchers report a practical protocol enabling efficient iPSC induction from Human Adult Dermal Fibroblasts cells (HADFs) under xeno-free, virus-free, condition and without oncogene c-MYC using a combination of plasmids encoding OCT3/4, SOX2, KLF4, L-MYC, LIN28 and shRNA for TP53. Using the approach described in this study, researchers can generated 20 hiPS cell clones from 2×10^5 Human Adult Dermal Fibroblasts cells (HADFs). Meanwhile, researchers developed a culture system in which human vitronectin, a secreted glycoprotein that is rich in the extracellular matrix and blood, replaced conventionally used mouse feeder cells to generate iPS cells, we not only can maintain the long-term culture of iPSCs but also efficiently generate xeno-free iPSCs using vitronectin as an extracellular matrix, this induction method will promote the derivation of patient-specific integration-free and xeno-free iPSCs and would also be very useful to generation the clinical-grade iPSCs in the future.

Key words: Human induced pluripotent stem cells, xeno-free, virus-free, vitronectin biomaterial-based culture, reprogramming, plasmid

INTRODUCTION

Human induced Pluripotent Stem Cells (iPSCs), similar to human Embryonic Stem Cells (ESCs) are able to proliferate unlimitedly and provides an opportunity to develop and use patient-specific somatic cells like neurons, cardiomyocytes and hepatocyte-like cells which are otherwise difficult to obtain (Takahashi *et al.*, 2007; Yu *et al.*, 2007). Cell transplantation is a promising therapy for repairing structure and function of patients with neurologic disease (Xiong *et al.*, 2013). The transplantation of iPS cells-derived neural precursor cells or neurospheres play an important role in the nerve regeneration of neurodegenerative diseases, spinal cord injury, peripheral nerve injury and brain stroke. Human iPS can be generated from a patient's own cells and have great potential in personalized regenerative medicine, drug discovery and disease modeling avoiding concerns associated with immune rejection and circumventing of ethical issues. However, initial methods for iPSC generation used genome-integrating retroviral or lentiviral vectors which could produce insertional mutations and residual of transgene expression in the process of iPSC differentiation, this could affect the functionality of iPSC derivatives and hamper their further clinical applications. To promote the application of iPS technology, it is necessary to establish and optimize a practical approach to generate iPS cells under a feeder-free, virus-free and

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serum-free condition. To overcome these obstacles, Seki *et al.* (2010) used the Sendai virus, a non integrating virus vector as a tool to generate iPSC. When the cells were cultured at the permissive temperature (37°C), the temperature-sensitive mutations of these vectors in RNA genome allowed Sendai virus amplification in the cells and maintained the transgene expression. After the iPSCs were established, the Sendai virus vectors could be elimitated by culturing the cells at the 38°C, a non-permissive temperature. However, this method still needs an infectious virus and requires special care to handle these viruses in Biosafety level, meanwhile, modify reprogramming factors and vector sequences is very difficult in conventional laboratories because of the lack of the equipment and facilities when it encodes oncogenic genes like c-MYC. Direct delivery of proteins or RNA requirs repeated delivery of the reprogramming factors and intensive labor. Modifying Sendai virus vectors or preparing synthesized RNA are technically demanding.

A number of reports have shown that episomal plasmids could also be used for the deviation of non-integrating iPSCs (Yu *et al.*, 2009). In this study, researchers used an efficient combination of plasmids which encode OCT3/4, SOX2, KLF4, L-MYC, LIN28 and a shRNA for TP53 to generate iPSCs (Okita *et al.*, 2011). IPSC generation is markedly enhanced by p53 suppression (Hong *et al.*, 2009) and non-transforming L-Myc is more potent and safe than c-MYc during human iPSC generation (Nakagawa *et al.*, 2010).

Human iPSCs were usually cultured using Mouse Embryonic Fibroblasts (MEF) as the feeder cells, it creates a high risk for immune rejection after cell transplantation due to, most likely due to the presence of nonhuman cell surface proteins and circulating antibodies within the human body. Furthermore, animal-derived products (such as animal serum) in the culture medium and Extra Cellular Matrix (ECM) can be a source to transmit nonhuman pathogens to humans. In contrast to the rapid progress in non-chromosomal integrating methods to generate iPSCs, it is still in its infancy to generate the clinically applicable iPSCs in a biologically safe culture condition (Rodriguez-Piza *et al.*, 2010; Sugii *et al.*, 2010; Chen *et al.*, 2011; Wang *et al.*, 2012). Researchers herein develop a simple and highly efficient method of iPSC induction from HADFs using these plasmid vectors with xeno-free and feeder cell-free culture system. Researchers were able to establish about 20 iPSCs from 2×10^5 HADFs. The resulting human iPS cells are free of DNA integration, show typical human ESC morphology and high nucleus to cytoplasm ratios, immunofluorescence and Representative FACS analysis demonstrate that these cells express pluripotent markers, quantitative PCR

analysis shows that the iPSC clones express endogenous pluripotent stem cell genes including OCT4, NANOG, SOX2 and LIN28. Furthermore, they can form teratomas in immunodeficient animals. Thus, researchers establish a simple and efficient method to generate patient-specific iPSCs and this would be applicable for the generation of clinical-grade iPSCs in the future.

MATERIALS AND METHODS

Collection and expansion of HADFs: HADFs were purchased from Lifeline Cell Technology and cultured in FibroLife Serum-Free Medium (Lifeline Cell Technology No.: LL-0001, FibroLife Basal Medium+HAS 500 μg mL^{-1}+ Linoleic Acid 0.6 μM+Lecithin 0.6 μg mL^{-1}+ rh FGF basic 5 ng mL^{-1}+ rh EGF/TGF 5 ng mL^{-1}+ L-Glutamine 7.5 mM+ Hydrocortisone Hemisuccinate 1.0 μg mL^{-1}+Ascorbic Acid 50 μg mL^{-1}+rh Insulin 5 μg mL^{-1}). The cells passaged >4 times were used for iPS cell generation.

iPS cell generation: Briefly, 1×10^6 HADFs at early passages were individualized by trypsin treatment (Trypsin/EDTA Xeno-Free, 100 mL, No. CM-0046) and electroporated with indicated episomal (plasmids episomal plasmids from Addgene: pCXLE-hOCT3/4-shp53-F (plasmid 27077) pCXLE-hSK (plasmid 27078) pCXLE-hUL (plasmid 27080) pCXLE-EGFP (plasmid 27082). Using Amaxa Nucleofector II and Basic Fibroblasts Nucleofector kit (25 RCT) (Lonza AG Cat No. VPI-1002) for primary mammalian fibroblasts cells, program U-023 (LONZA). The electroporated HADFs were seeded onto vitronectin (truncated recombinant human (VTN-N) (5 μg mL^{-1}, BD Biosciences) pre-coated P6 wells. In each electroporation, 2.5 μg pCXLE-hOCT3/4-shp53-F, 2.5 μg pCXLE-hSK, 2.5 μg pCXLE-hUL and 2.5 μg pCXLE-EGFP were used. Change medium to the fibro life serum-free medium when the cell influences are up to 60-70%, the medium are replaced by PSCeasy iPSCs medium (Modified Essential 8 medium) (Chen *et al.*, 2011) (PSCeasy No. CA1001500, DMEM/F12, L-ascorbic acid-2-phosphate magnesium (64 mg L^{-1}), sodium selenium (14 μg L^{-1}), bFGF (100 ng mL^{-1}), insulin (19.4 mg L^{-1}), NaHCO$_3$ (543 mg L^{-1}) and transferrin (10.7 mg L^{-1}), TGFβ1 (2 μg L^{-1}). PSCeasy iPSCs medium was changed daily during generation. The iPS cell colonies were picked at around days 20-30 and cultured in modified E8 medium on vitronectin. On the day of colony picking, researchers added an inhibitor for Rho kinase Y-27632 (10 μM Selleckchem, Houston, TX, USA); to promote the survival of the transferred colony (Silva *et al.*, 2008; Lin *et al.*, 2009; Lai *et al.*, 2010). The culture medium was changed daily. The iPS cells were passaged with 0.5 mM EDTA (Sigma, dissolved in PBS (Gibco).

Alkaline phosphatase activity: Pluripotent cells in culture on MEFs were fixed with 4% paraformaldehyde for 1-2 min at room temperature. The cells were then rinsed with TBST (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20) and incubated for 15 min at room temperature with a mixture of Fast Blue Violet, Naphtol AS-BI phosphate solution and water (2:1:1) from the Alkaline Phosphatase Detection kit (SiDanSai, ShangHai, China) according to the manufacturer's protocol. Following staining, the cells were rinsed with TBST and covered with Phosphate-Buffered Saline (PBS).

Teratoma formation: For the teratoma assay, the human induced Pluripotent Stem Cell (hiPSC) colonies were collected with 0.5 mM EDTA treatment and injected into the testes and muscle of a NOD/SCID mouse. Roughly 2×10^6 hiPSCs from a 6 cm dish were injected into a NOD/SCID mouse. After 6-8 weeks, the teratomas formed and were dissected was excised and fixed in 10% formalin overnight. The sample were subjected to hematoxylin and eosin staining for histological analysis under a microscope.

Flow cytometry analysis and quantitative PCR: For flow cytometry analysis, cultured cells were dissociated using a PBS-based cell dissociation buffer (Thermo), washed with 0.25% BSA in PBS and stained 1 h with antibodies directed against OCT3/4-PE, SSEA4-PE, TRA1-60-PE or TRA1-81-PE (OCT3/4 1 µg; SSEA4 0.5 µg; TRA1-60 1 µg; TRA1-81 1 µg) at 4°C. Flow cytometry analysis was performed using the FACSCalibur (BD Biosciences). Quantitative PCR reactions were carried out with SYBR Green/Flourescein qPCR Master Mix (2X) (Fermentas). The cDNA from human hESC was used as a relative standard. For each sample, 1 mL of diluted cDNA (1:10) was added as template in PCR reactions. The expression of genes of interest was normalized to that of β-actin in all samples (Table 1).

Immunofluorescence: For immunofluorescence, cells were rinsed with Phosphate-Buffered Solution (PBS) and fixed using 4% paraformaldehyde in PBS for 10 min at room temperature. Incubating the cells for 30 min in a solution containing 0.3% Triton-X 100 and 3% normal donkey serum to perform blocking and permeabilization. Then, cells were incubated with primary antibodies overnight at 4°C and then with the appropriate secondary antibodies. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Images were obtained using a fluorescent microscope. Primary antibodies included the hES-cell specific proteins OCT4, SOX2, SSEA4, TRA-1-60, TRA-1-80 and Nanog (1:400, Abcam). Secondary antibodies were goat anti-rabbit IgG-PE and goat anti-mouse IgG-PE (1:1000, Santa Cruz).

RESULTS AND DISCUSSION

Generation of integration-free, feeder-free and serum-free human induced pluripotent stem cells: Lentiviruses was previously used to generate human iPS cells from Human foreskin fibroblasts through the method that four plasmid constructs coding for OCT4, NANOG, SOX2 and LIN28 were co-transfected (Yu *et al.*, 2009; Si-Tayeb *et al.*, 2010). Here, researchers report a simple and practical protocol enabling efficient iPSC induction from Human Adult Dermal Fibroblasts cells (HADFs) under xeno-free, virus-free, condition and without oncogene c-MYC using a combination of plasmids encoding OCT3/4, SOX2, KLF4, L-MYC, LIN28 and shRNA for TP53. Researchers called this combination as plasmids Y4. As the first step, researchers tried to use the combination of plasmids Y4 to generate iPSCs from Human Adult Dermal Fibroblasts cells (HADFs) isolated from human skin with electrotransfection. The frozen Human Adult Dermal Fibroblasts cells (HADFs) were cultured for a few days with FibroLife Serum-Free Medium. The Y4 mixture was then transduced by electroporation. To test the episomal system in HADFs, researchers transfected an episomal vector encoding EGFP into these cells through electroporation. Researchers showed that EGFP expression efficiency was remarkable, about 50% cells were positive for EGFP expression at 1 week, the signal quickly decreased thereafter and only 5% of cells were fluorescent 4 weeks after electroporation (Fig. 1). The electroporated HADFs (Fig. 2a) were seeded onto vitronectin (BD Biosciences) pre-coated P6 wells. The cells were recovered in the FibroLife Serum-Free Medium for a few days, the medium are replaced by PSCeasy iPSCs medium (Modified Essential 8 medium) until the cell influences are up to 60-70%. The medium was then changed every days. On the day of colony picking (approximate days 20-30), researchers added an inhibitor for Rho kinase Y-27632 to promote the survival of the transferred colony. The ESC-like colonies began to

Table 1: Primers for PCR

Genes	Primer (5'-3')	Size (bp)
HOMO OCT4	OCT4 F 5'-CGAAAGAGAAAGCGAACCAG-3'	157
	OCT4 R 5'-GCCGGTTACAGAACCACACT-3'	
HOMO NANOG	NANOG F 5'-CAAAGGCAAACAACCCACTT-3'	158
	NANOG R 5'-TCTGCTGAGGGCTGAGGTAT-3'	
HOMO SOX2	SOX2 F 5'-AATAGCATGGCGAGCGGGGTC-3'	235
	SOX2 R 5'-TCTGCGAGCTGGTCATGGAG-3'	
HOMO LIN28	LIN28 F 5'-CAGTGGAGTTCACCTTTAAG-3'	133
	LIN28 R 5'-CCTTTTGATCTGCGCTTCTG-3'	
Human *β-actin*	β-actin f1379:5'-AGCGAGCATCCCCCAAAGTT-3'	284
	β-actin r1663:5'-GGGCACGAAGGCTCATCATT-3'	

Phase   Fluorescence

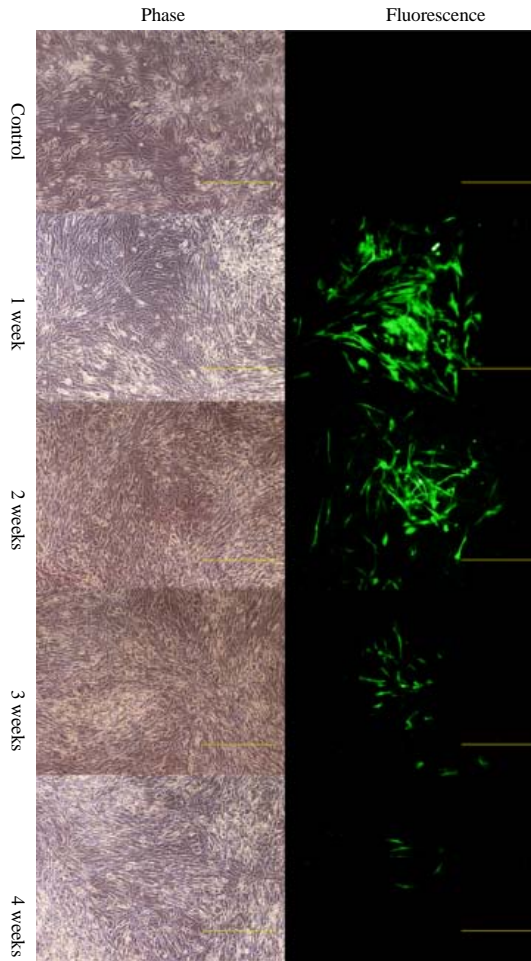Control

1 week

2 weeks

3 weeks

4 weeks

Fig. 1: Expression of EGFP fluorescence from episomal vector at 1-4 weeks after transduction. The signal quickly decreased in 4 weeks after electroporation. Scale bars, 500 µm

emerge on 14th-18th days (Fig. 2c and d) after the plasmid transfection and were selected for expansion. The reprogramming efficiency was relatively high (up to 0.01%). All experiments were repeated five times.

Characterizing iPSCs generated with the feeder-free and serum-free episomal reprogramming method: Established clones formed flat and compacted colonies and showed high nucleus to cytoplasm ratios and prominent nucleoli which were typical morphology of human ESCs (Fig. 2e and f). They display alkaline phosphatase activity positive (Fig. 3a and b) which is a phenotypic characteristic of undifferentiated ESCs and iPSCs. To identify whether these iPSCs had the characteristics of typical hESCs, researchers further examined the expression of typical human ESC-specific

antigens pluripotent markers through immunofluorescence. As demonstrated in Fig. 3c, OCT4, SSEA-4, SOX2, TRA-1-60, TRA-1-81, NANOG were continuously presented on the iPSCs examined. In addition, FACS analyses revealed that ≥82% of cells expressed OCT4, SSEA4, TRA1-60 and TRA1-81 (Fig. 4a). Quantitative RT-PCR analysis revealed that these clones expressed endogenous pluripotent stem cell genes such as OCT4, NANOG, SOX2 and LIN28, human ES cell line H1 was used as control (Fig. 4b). When injected into immunodeficient mice, the clones developed into teratomas consisting of various types of cells of all three germ layers, like neurons, gut-like epithelium, cartilage (Fig. 4c). Therefore, the Y4 mixture can induce iPSCs from Human Adult Dermal Fibroblasts cells (HADFs).

Human PSCs such as hESCs and iPSCs, hold great potentiality in cell replacement therapy to treat a variety of incurable diseases such as aging, trauma or birth defects. However, current human iPS production techniques have several obstacles that restrict their clinical application. For example, the clones of iPS cells which were generated by most reprogramming procedures and the extent of reprogramming can be heterogeneous. In addition to the heterogeneity associated with reprogramming using lentiviruses are still the most commonly used reprogramming protocols which can integrate into the host cell's genome and are potentially mutagenic. The choice of using lentiviruses to reprogram is partly historical. Initial studies showed that transducing exogenous factors (OCT4, SOX2, NANOG and LIN28 or OCT3/4, SOX2, KLF4 and C-MYC) into genomic DNA was sufficient to reprogram human somatic cells by using the method of lenti-viruses (Takahashi *et al.*, 2007; Yu *et al.*, 2007). This will allow transient expression of reprogramming factors until endogenous regulators of pluripotency take over and expression of the viral cDNAs will be repressed by methylation. Although, some researchs suggested that it would be challenging to reprogram with the non-integrating approaches due to the need for extended expression of introduced cDNAs, Tadtfeld *et al.* (2008) demonstrated that genomic integration was not essential using adenoviruses to supply cDNAs encoding reprogramming factors can successfully reprogram mouse fibroblasts.

For the clinical application of iPSCs, it is necessary to establish a xeno-free culture system to avert the potential risk of xenopathogen transmission or immune rejection caused by non-human immunogenic molecules (Martin *et al.*, 2005; Cerdan *et al.*, 2006; Heiskanen *et al.*, 2007; Sakamoto *et al.*, 2007; Hisamatsu-Sakamoto *et al.*, 2008). There are a variety of primary and immortalized human cells that have been tested as feeder cells for
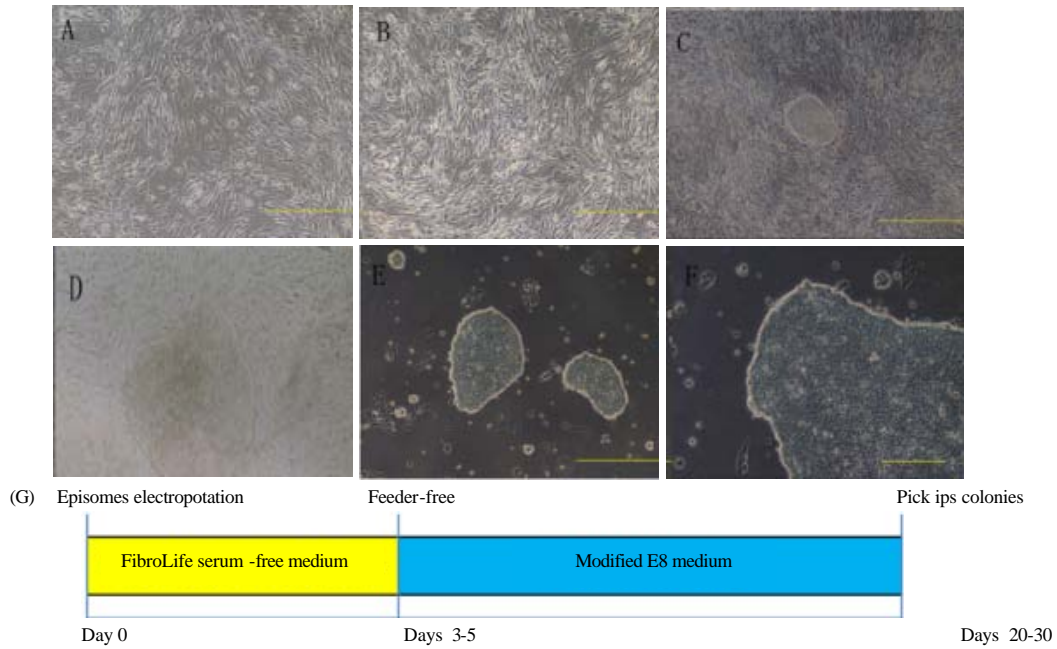
Fig. 2: Generation of human iPS cells (A-D). Emerging iPS cell colony generating by the optimized method in Modified E8 medium from HADFs at different time points. A) 2nd day; B) 7th day; C) 14th day; D) 20th day. Scale bars, 500 μm; E) Human iPS cells maintained under feeder-free conditions. Scale bars, 500 μm; F) Human iPS cells maintained under feeder-free conditions. Scale bars, 200 μm and G) Schematic representation of iPS cell generation
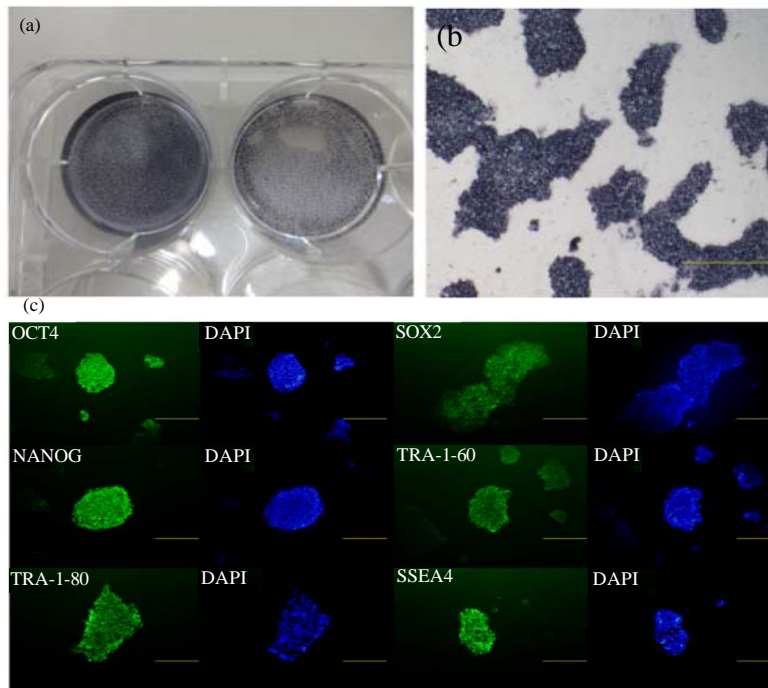

Fig. 3: a) Example of a P6 wells dish stained for ALP at a density of 5×10^5 cells per wells. Many ALP-positive colonies are visible; b) ALP staining of iPSC colonies. Scale bars, 500 μm and c) Immunofluorescence for OCT4, SOX2, SSEA4, TRA-1-60, TRA-1-80 and NANOG. Scale bars, 200 μm
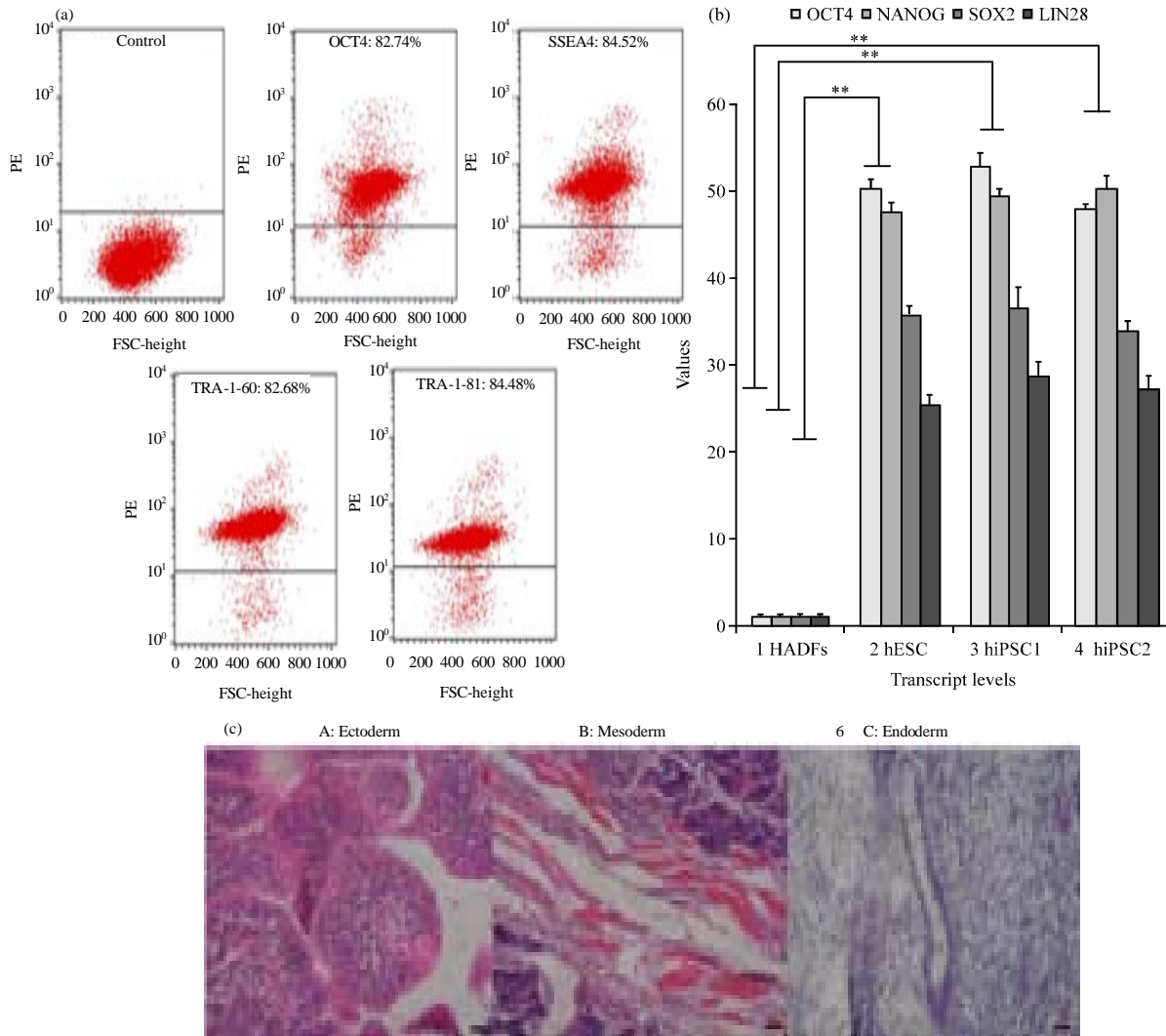
Fig. 4: a) Representative FACS analysis demonstrating that ≥82% of hiPS cells in culture (passage 8-10) express markers of pluripotency including OCT4, SSEA4, TRA1-60 and TRA1-81; b) Quantitative PCR analysis of the endogenous OCT4, NANOG, SOX2 and LIN28 expression in iPSC clones. Data shown are mean±SEM (n = 3). Transcript levels were normalized to β-actin levels. hESC: Human ES Cell line. hiPSC1: Human induced Pluripotent Stem Cells clone#1. hiPSC2: Human induced Pluripotent Stem Cells clone#2. The p-value is referred to HADFs. **indicates p<0.01 and c) Teratomas were obtained from all iPSC clones; A) Neural tissue (ectoderm); B) Muscle (mesoderm) and C) Gut epithelium (endoderm). Scale bars, 50 μm

culturing hiPSCs (Richards *et al.*, 2002, 2003; Amit *et al.*, 2003; Cheng *et al.*, 2003; Lee *et al.*, 2004, 2005; Wang *et al.*, 2005; Unger *et al.*, 2009; Hongisto *et al.*, 2012). However, these human feeder cells have several limitations, they include a time consuming and laborious procedure, the shortage of primary cell sources, difficulty in scaling up, ethical concerns and inconsistent outcomes from batch to batch variations. In addition, animal-derived materials in the culture medium and Extracellular Matrix (ECM) can transmit nonhuman pathogens to humans. If these potentially harmful risks could be avoided, it would facilitate the clinical application of iPSCs. To obtain non-integrating iPS cells from Human Adult Dermal Fibroblasts cells (HADFs), researchers chose episomal system to deliver the reprogramming factors.

Because involvement of oncogene c-MYC during reprogramming might increase the risk of genomic toxicity, researchers use L-MYC to substitute c-MYC, researchers prepared vector combinations which contained five

factors (OCT3/4, SOX2, KLF4, L-MYC, LIN28) in three epi-somal plasmids and an additional TP53 (also known as p53) shRNA in one of the three plasmids. To avoid the aforementioned concerns associated with the human feeder cells, researchers set out to establish an efficient feeder-free culture condition and serum-free medium that does not contain any animal-originated ingredients.

Human vitronectin is a secreted glycoprotein that is rich in the extracellular matrix and blood, it has been shown to maintain hESC attachment as an important component of the extracellular matrix through interaction with a variety of isotypes of integrin, especially $\alpha V\beta 5$ integrin (Braam *et al.*, 2008; Rowland *et al.*, 2009). Thus, the xeno-free culture system used human plasma-derived vitronectin as an extracellular matrix.

In this report, researchers describe here a feasible and highly practical protoco to generate iPS cells from HADFs in a totally defined condition that is feeder-free, virus-free, serum-free and without using c-MYC. Researchers have now generated about 20 iPS cell lines from 2×10^5 HADFs, the efficiency of human iPS cell reprogramming by plasmid transfection (1 iPS cell from 10,000 fibroblasts) was 0.01% which is similar (0.01%) when reprogramming is performed with lenti-viruses. Generation of non-viral and feeder-free iPS cell bank is an important step towards the further clinical applications.

Employing Human Adult Dermal Fibroblasts cells (HADFs) for non-viral iPS cell generation has several advantages. Firstly, it is a totally non-invasive and easy process that allowing direct reprogramming of human somatic cells into human iPS cells and avoiding exogenous DNA without the need for extensive experimental procedures. While several other procedures have been reported that integrating DNA-free iPS cells had been produced, most of the procedures require some specialized knowledge of virology or biochemistry. Secondly, the efficiencies of most reported non-viral approaches are very low, the method showed the reprogramming efficiency around 0.01% which was similar to that of lenti-viruses method. This is particularly important for using non-viral approaches to generate iPS cells. Thirdly, the episomal method enable the iPSCs to completely lose episomal vectors undergoing multiple passages, this will unlikely present a problem for the applications in future, since it is essential for iPSCs to undergo multiple passages to be rid of epigenetic memory of the donor cells (Polo *et al.*, 2010) and to be fully characterized for downstream applications. Lastly, the whole serum-free and feeder-free reprogramming process makes it practicable to generate clinical non-viral human iPS cells under current Good Manufacture Practice (cGMP) conditions in future.

CONCLUSION

Researchers have demonstrated that simple transient transfection of plasmid DNA encoding reprogramming actors is sufficient to generate human iPS cells from Human Adult Dermal Fibroblasts (HADF) with non-integrating episomal vectors and without using serum, feeders and c-MYC. This approach is simple, highly accessible and could expand the use of iPS cells in the study of human disease, regenerative medicine and drug discovery as well as increase the availability of safer iPS cells in clinical cell therapy.

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REFERENCES

Amit, M., V. Margulets, H. Segev, K. Shariki and I. Laevsky *et al.*, 2003. Human feeder layers for human embryonic stem cells. Biol. Reprod., 68: 2150-2156.

Braam, S.R., L. Zeinstra, S. Litjens, D. Ward-van Oostwaard and S. van den Brink *et al.*, 2008. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via $\alpha V\beta 5$ integrin. Stem Cells, 26: 2257-2265.

Cerdan, C., S.C. Bendall, L. Wang, M. Stewart, T. Werbowetski and M. Bhatia, 2006. Complement targeting of nonhuman sialic acid does not mediate cell death of human embryonic stem cells. Nature Med., 12: 1113-1114.

Chen, G., D.R. Gulbranson, Z. Hou, J.M. Bolin and V. Ruotti *et al.*, 2011. Chemically defined conditions for human iPSC derivation and culture. Nat. Methods, 8: 424-429.

Cheng, L., H. Hammond, Z. Ye, X. Zhan and G. Dravid, 2003. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem. Cells, 2: 131-142.

Heiskanen, A., T. Satomaa, S. Tiitinen, A. Laitinen and S. Mannelin *et al.*, 2007. N-Glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells, 25: 197-202.

Hisamatsu-Sakamoto, M., N. Sakamoto and A.S. Rosenberg, 2008. Embryonic stem cells cultured in serum-free medium acquire bovine apolipoprotein B-100 from feeder cell layers and serum replacement medium. Stem Cells, 26: 72-78.

Hong, H., K. Takahashi, T. Ichisaka, T. Aoi and O. Kanagawa *et al.*, 2009. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature, 460: 1132-1135.

Hongisto, H., S. Vuoristo, A. Mikhailova, R. Suuronen, I. Virtanen, T. Otonkoski and H. Skottman, 2012. Laminin-511 expression is associated with the functionality of feeder cells in human embryonic stem cell culture. Stem Cell Res., 8: 97-108.

Lai, K.W.H., J.C.Y. Ho, Y.K. Lee, K.M. Ng and K.W. Au *et al.*, 2010. ROCK inhibition facilitates the generation of human-induced pluripotent stem cells in a defined, feeder- and serum-free system. Cell. Reprogram., 12: 641-653.

Lee, J.B., J.E. Lee, J.H. Park, S.J. Kim, M.K. Kim, S.I. Roh and H.S. Yoon, 2005. Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. Biol. Reprod., 1: 42-49.

Lee, J.B., J.M. Song, J.E. Lee, J.H. Park and S.J. Kim *et al.*, 2004. Available human feeder cells for the maintenance of human embryonic stem cells. Reproduction, 6: 727-735.

Lin, T., R. Ambasudhan, X. Yuan, W. Li and S. Hilcove *et al.*, 2009. A chemical platform for improved induction of human iPSCs. Nature Methods, 6: 805-808.

Martin, M.J., A. Muotri, F. Gaga and A. Varki, 2005. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat. Med., 2: 228-232.

Nakagawa, M., N. Takizawa, M. Narita, T. Ichisaka and S. Yamanaka, 2010. Promotion of direct reprogramming by transformation-deficient Myc. Proc. Natl. Acad. Sci., 107: 14152-14157.

Okita, K., Y. Matsumura, Y. Sato, A. Okada and A. Morizane *et al.*, 2011. A more efficient method to generate integration-free human iPS cells. Nature Methods, 8: 409-412.

Polo, J.M., S. Liu, M.E. Figueroa, W. Kulalert and S. Eminli *et al.*, 2010. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nature Biotechnol., 28: 848-855.

Richards, M., C.Y. Fong, W.K. Chan, P.C. Wong and A. Bongso, 2002. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat. Biotechnol., 20: 933-936.

Richards, M., S. Tan, C.Y. Fong, A. Biswas, W.K. Chan and A. Bongso, 2003. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem. Cells, 5: 546-556.

Rodriguez-Piza, I., Y. Richaud-Patin, R. Vassena, F. Gonzalez and M.J. Barrero *et al.*, 2010. Reprogramming of human fibroblasts to induced pluripotent stem cells under xeno-free conditions. Stem Cells, 28: 36-44.

Rowland, T.J., L.M. Miller, A.J. Blaschke, E.L. Doss and A.J. Bonham *et al.*, 2009. Roles of integrins in human induced pluripotent stem cell growth on Matrigel and vitronectin. Stem Cells Dev., 19: 1231-1240.

Sakamoto, N., K. Tsuji, L.M. Muul, A.M. Lawler and E.F. Petricoin *et al.*, 2007. Bovine apolipoprotein B-100 is a dominant immunogen in therapeutic cell populations cultured in fetal calf serum in mice and humans. Blood, 110: 501-508.

Seki, T., S. Yuasa, M. Oda, T. Egashira and K. Yae *et al.*, 2010. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell, 7: 11-14.

Si-Tayeb, K., F.K. Noto, M. Nagaoka, J. Li and M.A. Battle *et al.*, 2010. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology, 51: 297-305.

Silva, J., O. Barrandon, J. Nichols, J. Kawaguchi, T.W. Theunissen and A. Smith, 2008. Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol., Vol. 6. 10.1371/journal.pbio.0060253.

Sugii, S., Y. Kida, T. Kawamura, J. Suzuki and R. Vassena *et al.*, 2010. Human and mouse adipose-derived cells support feeder-independent induction of pluripotent stem cells. Proc. Natl. Acad. Sci., 107: 3558-3563.

Tadtfeld, M., M. Nagaya, J. Utikal, G. Weir and K. Hochedlinger, 2008. Induced pluripotent stem cells generated without viral integration. Science, 322: 945-949.

Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda and S. Yamanaka, 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 131: 861-872.

Unger, C., S. Gao, M. Cohen, M. Jaconi and R. Bergstrom *et al.*, 2009. Immortalized human skin fibroblast feeder cells support growth and maintenance of both human embryonic and induced pluripotent stem cells. Human Reprod., 24: 2567-2581.

Wang, Q., X. Mou, H. Cao, Q. Meng and Y. Ma *et al.*, 2012. A novel xeno-free and feeder-cell-free system for human pluripotent stem cell culture. Protein Cell, 3: 51-59.

Wang, Q., Z.F. Fang, F. Jin, Y. Lu, H. Gai and H.Z. Sheng, 2005. Derivation and growing human embryonic stem cells on feeders derived from themselves. Stem Cells, 23: 1221-1227.

Xiong, Y.J., B. Yin, L.C. Xiao, Q. Wang and L. Gan *et al.*, 2013. Proliferation and differentiation of neural stem cells co-cultured with cerebral microvascular endothelial cells after oxygen-glucose deprivation. J. Huazhong Univ. Sci. Technol. (Med. Sci.), 33: 63-68.

Yu, J., K. Hu, K. Smuga-Otto, S. Tian, R. Stewart, I.I. Slukvin and J.A. Thomson, 2009. Human induced pluripotent stem cells free of vector and transgene sequences. Science, 324: 797-801.

Yu, J., M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget and J.L. Frane *et al.*, 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science, 318: 1917-1920.