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Original Article

Differentiation of blood T cells: Reprogramming human induced pluripotent stem cells into neuronal cells

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Abstract

Background: Human induced pluripotent stem cells (iPSCs) morphologically and functionally resemble human embryonic stem cells, which presents the opportunity to use patient-specific somatic cells for disease modeling and drug screening. In order to take one step closer to clinical applications, it is important to generate iPSCs through a less invasive approach and from any accessible tissue, including peripheral blood. Meanwhile, how to differentiate blood cell-derived iPSCs into neuron-like cells is still unclear.
Methods: We utilized Epstein−Barr nuclear antigen-1-based episomal vectors, a nonviral system that can reprogram somatic cells into iPSCs in both feeder-dependent and feeder-free conditions, to generate iPSCs from T cells via electroporation and then induce them into neuronal cells.
Results: We successfully isolated sufficient T cells from 20 mL peripheral blood of the donors and reprogrammed these T cells into iPSCs within 4 weeks. These iPSCs could be stably passaged to at least 50 passages, and exhibited the abilities of pluripotency and multiple-lineage differentiation. Notably, under the medium induction for 21 days, these T-cell-derived iPSCs could be differentiated into Nestin (neural progenitor marker)-, GFAP (glial cell marker)-, and MAP2 (neuron cell marker)-positive cells detected by immunofluorescence methods.
Conclusion: We have developed a safer method to generate integration-free and nonviral human iPSCs from adult somatic cells. This induction method will be useful for the derivation of human integration-free iPSCs and will also be applicable to the generation of iPSCs-derived neuronal cells for drug screening or therapeutics in the near future.

Keywords: electroporation; Epstein-Barr nuclear antigen-1; human-induced pluripotent stem cells; nonviral induced pluripotent stem cells; T cells

1. Introduction

Reprogramming human differentiated cells into induced pluripotent stem cells (iPSCs) could potentially provide new vistas in using patient-specific cells with a perfect genetic match for disease modeling, pathophysiology studies, drug screening, and cell-based therapies.[1] iPSCs have been generated from many types of somatic cells by induction of viral vectors expressing defined reprogramming factors since 2007.[2,3] The genome-integrating viral vectors may produce insertional mutations or even result in increasing the risk of tumor formation.[4,5] Unfortunately, the use of viral vectors raises serious clinical safety concerns, which have been tragically demonstrated in several clinical gene therapy trials.[6,7] To address these issues, many alternatives, such as nonviral and genomic integration-

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free methods that are potentially safer, have been recently reported, for example, using electroporation of nonintegrating plasmids, small molecule modulators, recombinant proteins, minicircle DNA vectors, and synthetic messenger RNA.[8−10]

So far, fibroblasts have been the most commonly used cells in the generation of iPSCs. The use of fibroblasts is still suboptimal for large-scale clinical application, due to the invasive skin biopsies and the time-wasting process for establishing stable cell lines from primary tissue.[11] Generation of human iPSCs from blood mononuclear cells (MNCs) has been widely accepted as a more convenient, less invasive, and almost unlimited resource for cell reprogramming, compared to dermal fibroblasts.[12] In general, establishing a primary cell culture from skin biopsy takes several weeks. Additionally, large numbers of frozen blood samples are stored in biorepositories worldwide.[13,14] Many studies have successfully generated iPSCs from human immature MNCs expressing specific-markers, such as CD34.[14] The immature MNCs, isolated from umbilical cord blood, peripheral blood, and bone marrow, were reprogrammed by transduction of four (Oct4, Sox2, c-Myc, and Klf4) or fewer reprogramming genes via retroviral system.[13,15] Since T cells are the most abundant cells in peripheral blood MNCs (PBMNCs), Seki et al isolated PBMNCs and stimulated the expansion of T cells with an anti-CD3 antibody and IL-2.[12] However, the low efficiency of these methods of generating iPSC, and reprogramming factor-encoded oncogenes such as c-Myc was noted.[16] Recently, Epstein−Barr nuclear antigen-1 (EBNA-1)-based episomal plasmid was one of the methods used for deriving non-integrating iPSCs.[17] This system may allow modification of the reprogramming factors and enable relatively high and long-term expression of the reprogramming factors.[17,18]

Accumulating studies have shown that a wide range of donor cells, gene-delivery vehicles, and combinations of reprogramming factors can be used for generating iPSCs. Among them, minimally invasive and maximally safe methods provide significant practical advantages for generating human iPSCs. In this study, we isolated T cells from donors and subsequently reprogrammed the obtained T cells into iPSCs. Using the electroporation method, blood T cells from adult humans have been reprogrammed into iPSCs with integration-free EBNA-1 episomal vectors. Importantly, these iPSCs could be differentiated into neuron cells under the 21 days of medium-based induction. The platform of T-cell-based iPSCs provided an alternative cell source and strategy for integration-free iPSC generation without viral infection. Notably, the neuronal cells differentiated from T-cell-based iPSCs may be employed as an expandable platform for investigating pathogenesis, *in-vitro* drug screening and therapeutics for neurodegeneration in the near future.

2. Methods

2.1. T cell isolation

This study was carried out in compliance with the Helsinki Declaration. Peripheral blood mono-nuclear cells (PBMCs) were isolated from six donors whose written informed consents were obtained in accordance to the guidelines of the Institutional Review Board (Taipei Veterans General Hospital). This study was approved by our Institutional Review Board (VGHIRB 2014-05-002C). The patients were all documented as deceased prior to our data review. In brief, one ratio of blood sample was layered on one ratio of Ficoll−-Plaque Plus, and the pellet (400*g*, 30 minutes at 20°C) and buffy coat were collected, washed twice with phosphate buffered saline (PBS), and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma−Aldrich Corporation, St. Louis, MO, USA) with 100 IU/mL of penicillin, 100 μg/mL of streptomycin and 10% v/v fetal bovine serum (PAA, Pasching, Austria).

2.2. T cell stimulation

T cells were expanded in freshly prepared AIM-V medium (Invitrogen, Carlsbad, CA, USA) supplemented with penicillin/streptomycin/glutamine (Invitrogen) plus 300 IU/mL rhIL2 (Peprotech) and 10 ng/mL soluble anti-CD3 antibody (OKT3 clone; eBioscience).[19]

2.3. Human iPSC generation and culture

To generate integration-free iPSCs, T cells were nucleofected with 3 μg expression plasmid mixture using AmaxaTM human T Cell NucleofectorTM Kit (Lonza). In each nucleofection, 2×10^6 cells T cells were treated by 0.83 μg PCXLE-hOCT3/4-shp53, 0.83 μg PCXLE-hSK, 0.83μg pCXLE-hUL, and 0.5 μg pCXWB-EBNA1. The plasmids were all obtained from Addgene (https://www.addgene.org/): pCXLE-hOCT3/4-shp53-F (plasmid 27077); pCXLE-hSK (plasmid 27078); pCXLE-hUL (plasmid 27080); and pCXWB-EBNA1 (plasmid 37624). Cells were then cultured, and freshly thawed inactivated mouse embryonic fibroblasts (MEFs) feeder cells were added into each well every 10−14 days. The number of alkaline phosphate-positive iPSC colonies was counted at 3−4 weeks interval after nucleofection. Undifferentiated iPSCs were maintained on inactivated MEFs (50,000 cells/cm^2) in human embryonic stem cell (ESC) medium: DMEM/F12 (Gibco) supplemented with 20% KnockOut serum replacer (KSR; Invitrogen), 0.1mM nonessential amino acids (Invitrogen), 1mM L-glutamine, 0.1mM β-mercaptoethanol, 10 ng/mL recombinant human basic fibroblast growth factor, and antibiotics (Gibco). To prevent cell contamination by MEFs, these iPSCs were transferred to feeder-free/serum-free culture in HESF V2 medium (Cell Science & Technology) without KSR supplementation, as described previously.[20]

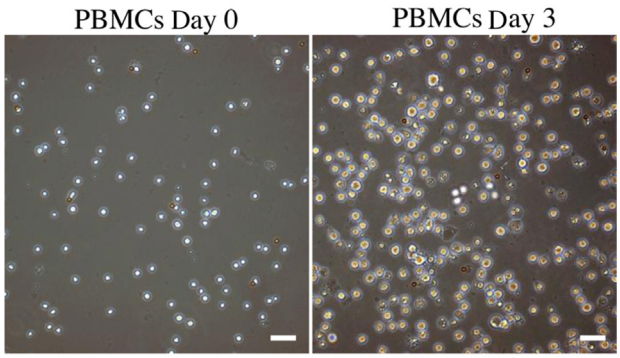
2.4. Quantitative reverse transcription polymerase chain reaction

SYBR-green was used for real-time reverse transcription polymerase chain reaction (RT-PCR) detection. Briefly, total RNA (1 μg) of each sample was reverse-transcribed in 20 μL

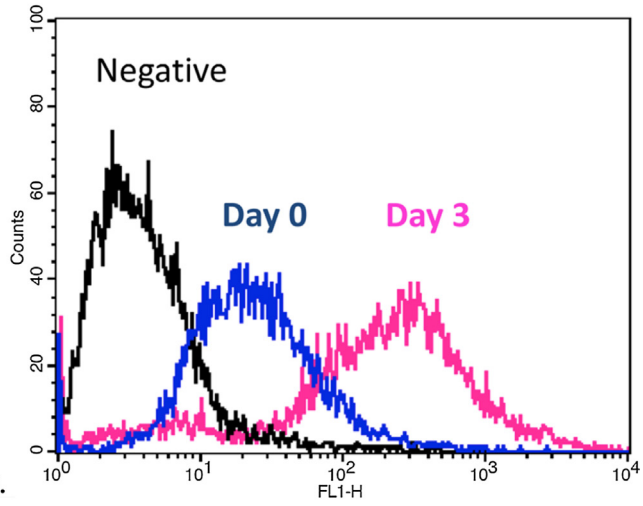
A.

Case No.	Age (y)	T cell No. / 20 ml blood	Established lines	Neuron differentiation
Case 1	23	$5.24*10^7$	3	Successful
Case 2	36	$3.21*10^8$	2	Successful
Case 3	33	$9.82*10^7$	2	Successful
Case 4	27	$8.47*10^7$	3	Successful
Case 5	26	$3.12*10^7$	2	Successful
Case 6	38	$3.26*10^7$	1	Successful

B.



C.



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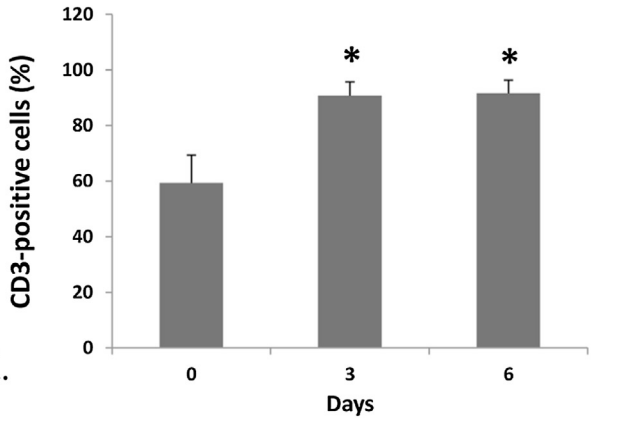

Fig. 1. (A) Clinical characteristics of donors' isolation and characterization of CD3+ T cells. (B) After 3 days in expansion/selection medium, PBMCs were highly proliferative. Phase contrast microscopy, magnification ×200. (C) Flow cytometric analysis of PBMCs gated on the $CD3^+$ cell population. (D) Quantitative analysis of $CD3^+$ positive cells in PBMCs ($n = 6$) in each group. Data are presented as mean ± standard deviation of three independent experiments. *$p < 0.05$ versus Day 0.

using 0.5 μg of oligo-dT and 200 U Superscript II RT (Invitrogen). Amplification was carried out in a total volume of 20 μL containing 0.5μM of each primer, 4mM $MgCl_2$, 2 μL LightCycler–FastStart DNA Master SYBR green I (Roche Diagnostics GmbH, Mannheim, Germany), and 2 μL of 1:10 diluted cDNA. All reactions were performed in triplicate. The transcript levels of genes were standardized to the corresponding GADPH level, and for each candidate gene, mRNA levels relative to the highest candidate gene level were estimated in percentages.

2.5. Immunofluorescence

The living cells and spheres were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 5% normal goat serum-PBS. Cells were incubated with primary antibodies, washed three times in PBS, and then incubated with goat antimouse or secondary antibodies conjugated with Fluorescein isothiocyanate (FITC) (green) or Phycoerythrin (PE) (red). The 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain (blue). Images were obtained using fluorescent microscopy and a digital camera. The antibodies that were used are listed in Table S1.

2.6. Induced differentiation of T-iPSCs into neuron-like cells

There were 1×10^5 iPSCs at the fifth to eighth passage that were treated with a serum-free modified neurogenic selection medium for 2 weeks. The serum-free medium for the selection of neural precursor cells contained DMEM/F12 1:1 (Gibco/BRL) and was supplemented with 0.6% glucose, 25 μg/mL insulin, 100 μg/mL transferrin, 20nM progesterone, 60μM putrescine, 30nM selenium chloride, 2mM glutamine, 3mM sodium bicarbonate, 5mM HEPES, 2 μg/mL heparin, 20 ng/mL epidermal growth factor, and 20 ng/mL basic fibroblast growth factor (all from Sigma). For further neural differentiation, epidermal growth factor was removed from the medium, and the medium was supplemented with 20 ng/mL SHH (Sonic hedgehog) (R&D), 10 ng/mL BDNF (Brain-derived neurotrophic factor) (R&D), and with all–trans-retinoic acid (100nM) for another 7 days.[21]

2.7. Statistical analysis

The results are represented as mean ± standard deviation. Statistical analyses were performed using *t* test for comparing two groups. One-way or two-way analysis of variance, and Bonferroni test were used to detect differences among three or more groups. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Isolation and characterization of T cells from peripheral blood

As shown in Fig. 1A, peripheral blood was obtained from six donors ranging in age from 23 years to 38 years. PBMNCs mainly consist of T lymphocytes, but also include B lymphocytes, monocytes, and macrophages. We further culture the isolated PBMNCs by plate-bound anti-CD3 monoclonal antibody and IL-2 for T cell expansion. Anti-CD3 antibody modulates the TCR-CD3 complex to induce T cell proliferation and activation, whereas IL-2 also activates general T cell signaling pathways and eventually promotes cytokine transcription, cell survival, cell-cycle entry, and growth. Our data show that the number of cells in the cultured PBMCs was apparently increased (Fig. 1B). In order to characterize these cells, the expression of CD3 protein (the complex of TCR proteins on the surface of T cells) was used as a T cell–specific marker. At Day 3 of culture with anti-CD3 monoclonal antibody and IL-2, CD3-positive cells had increased up to ~90% of cultured PBMCs (Figs. 1C and 1D).

3.2. Characterization of iPSC generation from T cells

The development of novel approaches for generating integration-free iPSCs has eliminated integrating virus-associated genotoxicity in clinical applications. In this study, we used EBNA1-based episomal vectors, a nonviral system that can reprogram somatic cells into iPSCs in both feeder-dependent and -free conditions, to generated iPSCs from T cells via electroporation (Fig. 2A). The ESC-like colonies began to emerge 28 days after the plasmid transfection and were selected for expansion. Established clones formed flat and compacted colonies, showed high nucleus-to-cytoplasm ratios, stained positive for alkaline phosphate, and exhibited morphology indistinguishable from that of human ESCs (Figs. 2B and 2C). As shown by immunofluorescence, selected clones exhibited the stemness signature and revealed the

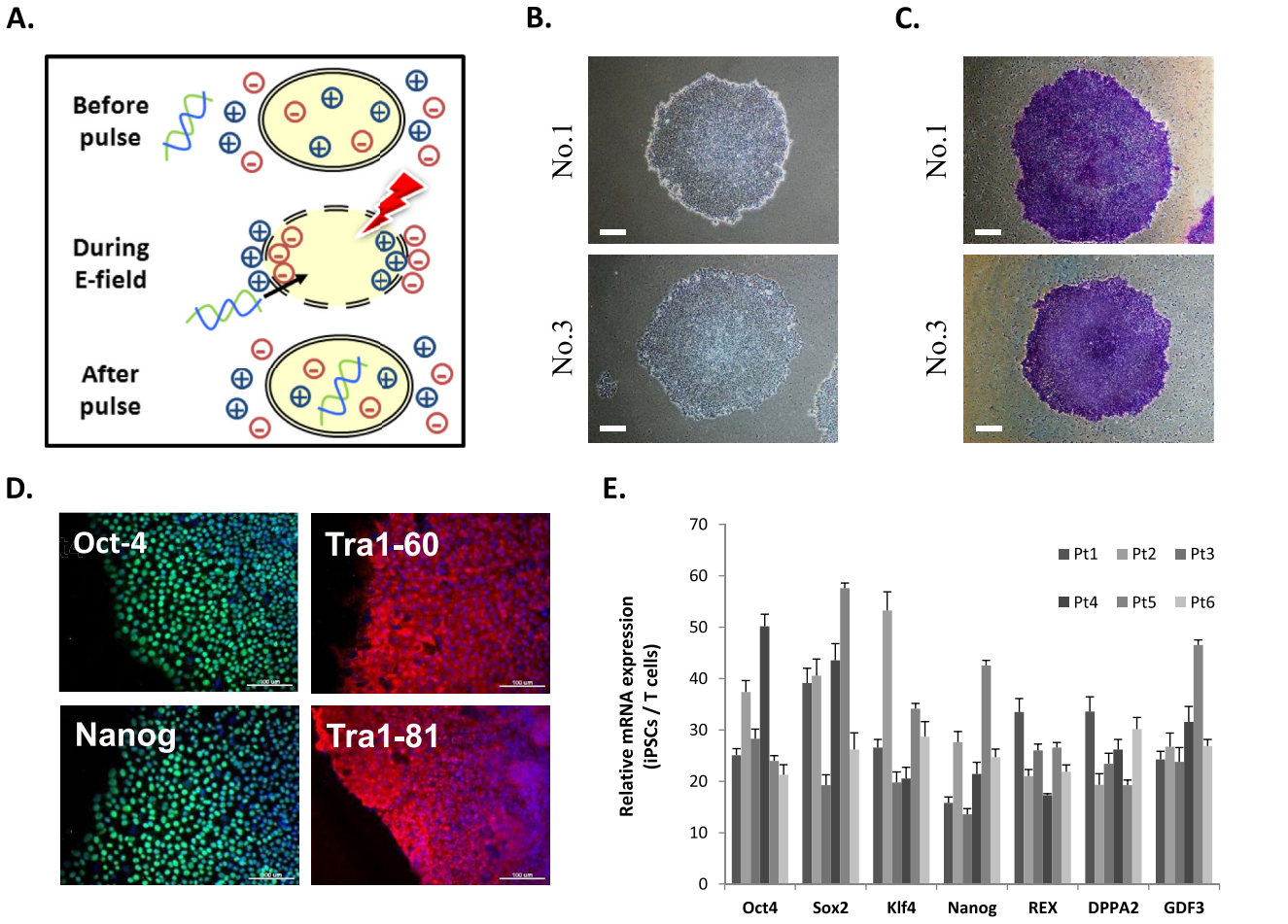

Fig. 2. Generation and characterization of iPSCs derived from patients with AMD and unaffected control. (A) Schematic diagram of the reprogramming with episomal plasmids via electroporation. (B) Phase-contrast photomicrograph and (C) alkaline phosphatase activity of undifferentiated T-iPSCs. Scale bar = 100 μm. Data presented are derived from donor No. 1 and No. 3. (D) Immunofluorescence staining demonstrated the expression of pluripotency markers (OCT4, TRA-1-61, NANOG, and TRA-1-81) in undifferentiated T-iPSCs. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) (blue). Scale bar = 200 μm. (E) The qRT-PCR results indicated an ESC-like gene expression pattern in representative colonies of iPSCs. T cells were used as a negative control. ESC = embryonic stem cell; iPSCs = human induced pluripotent stem cells; qRT-PCR = quantitative reverse transcription polymerase chain reaction.

strong expression of Oct4, Nanog, Tra-1-60, and Tra-1-81 in these T-cell derived iPSCs (Fig. 2D). RT-PCR also showed that both Ctrl- and AMD-iPSCs expressed various stemness genes, such as Oct4, Sox2, klf4, Nanog, REX, DPPA2, and GDF3, identical to those observed in H9 human ESC lines (Fig. 2E). Taken collectively, these results demonstrate that fully reprogrammed iPSCs with pluripotent propensity can be effectively derived from T cells.

3.3. Differentiation of human T cell-derived iPSCs into neural progenitor cells

We followed previously established methods for differentiating iPSCs into neural progenitors. Both the iPSCs and ESCs were separated from the feeder fibroblasts before differentiation.[22] Fig. 3A shows representative morphological features of neurospheres following attachment, with extensive neurite outgrowth at the periphery of neurospheres. Immunostain demonstrated that the differentiated cells consisted of mature neurons. Three weeks after the neural induction, T-iPSC-derived neurons (T-iPSC-Neus) formed extensive networks and showed positively stained Nestin, as well as the mature neuronal marker MAP2 (Fig. 3B). Quantitative real-time RT-PCR analysis revealed that the expression of neuron-specific markers, including Nestin, GFAP, MAP2, and NCAM1 were initially upregulated in T-iPSC-Neus after induction of neural differentiation for 10 days and 20 days (Figs. 3C and 3D). The mRNA expressions of these neuron-specific markers were statistically increased during the course of differentiation. Multidimensional scaling (Fig. 3E) further showed that the gene expression pattern of T-iPSC-Neus was closer to the gene signature of ESC-neurons. Altogether, these data support that T-iPSCs could present pluripotent potential and differentiate into neuron-like cells.

4. Discussion

Recently, patient-specific neural cells derived from human somatic cells have been revolutionizing the study of neurodegeneration diseases and holding great hope for future

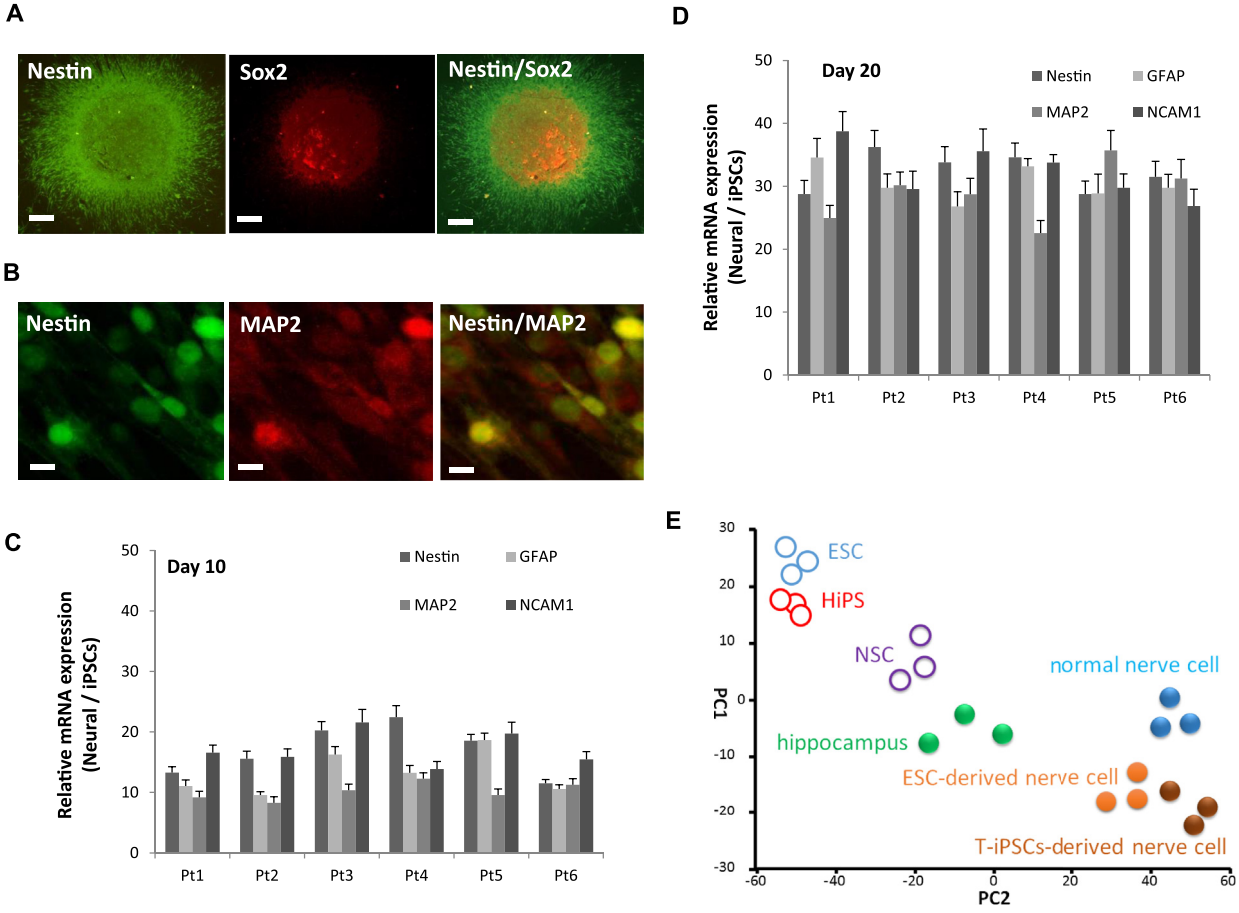

Fig. 3. Differentiation of T-iPSC into neural cells. (A) Immunofluorescence staining for Nestin and Sox2 in neurospheres. Scale bar = 100 μm. (B) Immunofluorescence staining for neuronal marker Nestin and MAP2 demonstrated morphology and neurons formed extensive networks in T-iPSCs-Neus. Scale bar = 200 μm. (C, D) Expression of neural-specific genes during the differentiation course of T-iPSC-Neus for 10 days and 20 days. (E) Multidimensional scaling analysis showed that the expression pattern of T-iPSCs-Neus was closer to the gene signature of ESC-Neus. Principal component analysis (PC1: vertical axis; PC2: horizontal axis; Unit: D-chip unit) was used to measure the expression profiling using bioinformatics methods. ESC = embryonic stem cell; iPSCs = human induced pluripotent stem cells; SOX2 = Ssex determining region Y-box 2; GFAP = glial fibrillary acidic protein; MAPs = microtubule-associated proteins; NCAM = neural cell adhesion molecule.

Table 1
Comparison of human iPSCs derived from different somatic cell types.

Cell type	Fibroblast	Dental pulp cell	T cell
Source	Skin biopsy	Teeth	Peripheral blood
Invasive	High	High	Low
Length of isolation	3–4 wk	3–4 wk	3 d
Length of reprogramming	3 wk	3–4 wk	3–4 wk
Efficiency of reprogramming	0.02%	0.01%	< 0.01%

iPSCs = human induced pluripotent stem cells.

personalized medical remedies. The generation of iPSCs must be carried out using readily accessible sources of somatic cells from patients for reprogramming, for instance, skin fibroblasts, hair keratinocytes, epithelial cells found in urine, and dental pulp.[23–26] The majority of iPSC studies have focused on taking fibroblasts as the somatic cell source, the most invasive yet the least suitable approach. In our previous study, we demonstrated that dental pulps cells from deciduous and permanent teeth could be reprogrammed into iPSCs without c-Myc. Moreover, these iPSCs were capable of differentiating into neuron-like cells. Although dental pulp cells from teeth provide another source of cells for reprogramming, collecting dental pulp cells from teeth could be a limiting factor for scaled production. Reciprocally, PBMNCs have been widely accepted as a more convenient and almost unlimited resource for cell reprogramming, due to the ease of obtaining patient samples.[11,12] Several studies have established iPSCs from PBMNCs, and successfully differentiated PBMNCs-derived iPSCs into mesenchymal stems cells, cardiomyocytes, and hepatocytes.[27–29] It has been well documented that the generation of human iPSCs from blood MNCs offers several advantages over other cell sources (Table 1). It is more convenient and less invasive to obtain peripheral blood than dermal fibroblasts and dental pulp. This new approach saves several weeks of cell preparation because T cells are not required to establish a primary cell culture like skin biopsy. We successfully isolated sufficient T cells from only 20 mL peripheral blood of the donors and reprogrammed these T cells into iPSCs. These iPSCs could be stably passaged to at least 50 passages and retained their pluripotency and ability for tridermal differentiation. Subsequently, we differentiated these iPSCs into neuron-like cells. These features enabled the use of these patient-specific neuron-like cells as a malleable platform for investigating the mechanisms of diseases and drug screening *in vitro*.

To make human iPSCs amenable to clinical applications, several challenges remain to be addressed. Previously devised strategies for production of iPSCs have so far been mainly through retroviral vectors and constitutive lentiviral systems. These viral systems hampered the clinical use of iPSCs, due to genetic variability that is caused by random integration of multiple pro-viral copies.[4,5] Therefore, it is necessary to pursue nonintegration approaches.[30] In this study, we generated patient-specific iPSCs via integration-free episomal vectors instead of integrating viral vectors. EBNA1 is a multifunctional, dimeric viral protein associated with Epstein–Barr virus. These episomal vectors based on the EBNA1 have been proven to generate iPSCs very efficiently without the risk of transgenic sequences being inserted into the target cell genome. Our data demonstrate that the proliferation of T cells could be activated by the combination of IL-2 and anti-CD3 antibodies. iPSC induction was greatly enhanced when we used the extra EBNA1 vector and the Y4 combination in the T-cell stimulating medium. Encouragingly, this method represented one step closer to the clinical application of iPSC-based cell therapy.

In this study, we developed a novel method for generating iPSCs from PMNCs, which is highly convenient and far less invasive, compared to the traditional means. Only a few milliliters of peripheral blood are sufficient for the generation of iPSCs. Accordingly, our report provides a very practical and extremely efficient way to generate patient-specific iPSCs. This will be of great potential for the derivation of human integration-free iPSCs, and will also be applicable to the generation of clinical-grade iPSCs hereafter.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jcma.2015.03.007.

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