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Lab Resource: Stem Cell Line

Generation and characterization of the human iPSC line PBMC1-iPS4F1 from adult peripheral blood mononuclear cells

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ABSTRACT

Here we describe the generation and characterization of the human induced pluripotent stem cell (iPSC) line PBMC1-iPS4F1 from peripheral blood mononuclear cells from a healthy female with Spanish background. We used heat sensitive, non-integrative Sendai viruses containing the reprogramming factors Oct3/4, Sox2, Klf4 and c-Myc, whose expression was silenced in the established iPSC line. Characterization of the PBMC1-iPS4F1 cell line included analysis of typical pluripotency-associated factors at mRNA and protein level, alkaline phosphatase enzymatic activity, and *in vivo* and *in vitro* differentiation studies.

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Resource Table: PBMC1-iPS4F1 cell line.

Name of stem cell construct	CytoTune® 2.0 Sendai Reprogramming Kit (Life Technologies, Invitrogen)
Institution	Gene regulation, Stem Cells and Development Group, GENYO — Centre for Genomics and Oncological Research, Pfizer/Universidad de Granada/ Junta de Andalucía, PTS Granada
Person who created resource	Rosa Montes, Veronica Ramos, Pedro J. Real
Contact person and email	Rosa Montes, rosa.montes@genyo.es; Veronica Ramos-Mejia, veronica.ramos@genyo.es; Pedro J. Real, pedro.real@genyo.es
Date archived/stock date	Mar 20, 2015
Origin	Healthy human female peripheral blood mononuclear cells
Type of resource	Biological reagent: human induced pluripotent stem cell (iPSC) line
Sub-type	Cell line
Key transcription factors	Oct3/4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed
Link to related literature (direct URL links and full references)	
Information in public databases	http://www.isciii.es/ISCIII/es/contenidos/fd-el-instituto/fd-organizacion/fd-estructura-directiva/fd-subdireccion-general-investigacion-terapia-celular-medicina-regenerativa/fd-centros-unidades/fd-banco-nacional-lineas-celulares/fd-lineas-celulares-disponibles/lineas-de-celulas-iPS.shtml

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Resource details

We generated the PBMC1-iPS4F1 cell line using the reprogramming factors Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007) (Yu et al., 2007) included in the CytoTune®-iPS 2.0 Reprogramming System (Life Technologies, Invitrogen), which is based on a modified, non-transmissible form of Sendai virus (SeV). PBMC1-iPS4F1 colonies displayed a typical round shape morphology with small, tightly packed cells with a high nucleus/cytoplasm ratio and prominent nucleoli (Fig. 1A), and they were positive for alkaline phosphatase activity (Fig. 1B). We confirmed the absence of exogenous reprogramming transgenes by RT-PCR after 8 passages and two heat treatments (6 days at 39 °C) to eliminate temperature sensitive mutant SeV (Fig. 1C).

The PBMC1-iPS4F1 cell line has been adapted to feeder-free culture conditions and displays a normal karyotype (46, XX) after extensive mechanical and enzymatic transfers (Fig. 1D). The iPSC line was also characterized using a genetic fingerprinting assay by microsatellite analysis.

The endogenous expression of the pluripotency associated transcription factors Oct3/4, Sox2, Rex1 and Nanog was evaluated by RT-PCR (Fig. 2A). We also confirmed the protein expression levels of the pluripotency markers Oct3/4, SSEA3, SSEA4, Tra1-60 and Tra1-81 by flow cytometry analysis (Fig. 2B).

The *in vivo* differentiation capacity of the PBMC1-iPS4F1 cell line was demonstrated by teratoma formation. The teratomas showed expression of representative markers of the three germ layers (Glial Fibrilar Acid Protein/GFAP for ectoderm, Vimentin for mesoderm, and Cytokeratin/CK AE1-AE3 for endoderm) (Fig. 3A). In addition, *in vitro* differentiation capacity was tested by spontaneous embryoid body (EB) differentiation. The cells inside the EBs were able to differentiate to the three germ layers, demonstrated by the expression of representative markers of each layer (β-III-tubulin for ectoderm, Vimentin for mesoderm and CK AE1-AE3 for endoderm) (Fig. 3B). We also routinely monitor the PBMC1-iPS4F1 cell line to confirm the absence of Mycoplasma contamination.

Materials and methods

Generation of vector free PBMC1-iPS4F1 cell line from PBMCs

A blood sample was obtained from a healthy 39-years old woman with a Spanish genetic background accordingly with the Andalusian Ethics Review Board for Cellular Reprogramming requirements and with Spanish and EU legislation. The blood sample was diluted 1:2 (vol:vol) in PBS and centrifuged with Ficoll Paque™ PLUS (GE Healthcare) to separate and isolate peripheral blood mononuclear cells (PBMCs). Isolated PMBCs were cultured for 4 days in PBMC medium consisting in StemSpan™ SFEM (StemCell Technologies) supplemented with 100 ng/ml hSCF, 100 ng/ml hFLT3L, 20 ng/ml hTPO, 10 ng/ml G-CSF and 2 ng/ml hIL3 (Peprotech). The PBMCs were then transferred to a 12-well fibronectin coated plate (BD BioCoat™) and exposed overnight to SeV particles (CytoTune®-iPS 2.0 Reprogramming kit, Life Technologies, Invitrogen) at MOI:3 in the presence of 8 μg/ml Polybrene (Sigma-Aldrich) and 10 μM Y-27632 (Sigma-Aldrich). Subsequently, the SeV particles were removed by centrifugation and the transduced cells were grown for 3 days in PBMC medium. Then, the cells were transferred onto irradiated human mesenchymal stem cells (hMSC, Inbiobank) (Montes et al., 2009) and cultivated one day with PBMC medium. On the second day, the cells were cultivated in 1:1 (vol:vol) PBMC medium and pluripotent stem cell (PSC) medium (KO-DMEM supplemented with 20% knockout serum replacement (both from Life Technologies), 8 ng/ml bFGF2 (Milteny Biotec), 10 μM Y-27632 (Sigma-Aldrich), 1 mM glutamine, 1% MEM non-essential amino acids and 0.1 mM 2-mercaptoethanol (all from Life Technologies). From the third day onwards, the cells were cultured in PSC medium. Seven days after transduction, emerging iPSC colonies were picked individually and expanded for characterization.

Phosphatase alkaline analysis

PBMC1-iPS4F1 cells were seeded on 12-well Matrigel™ coated plates. After 5 days in culture, we tested the phosphatase alkaline enzymatic activity using Alkaline phosphatase detection kit (Merck-Millipore) following manufacturer's instructions (Fig. 1B).

Analysis of expression of exogenous reprogramming factors

Silencing of the exogenous reprogramming factors was analyzed by RT-PCR following manufacturer's instructions (CytoTune®-iPS 2.0 Reprogramming kit, Life Technologies, Invitrogen). Total mRNA was isolated using RNeasy Plus Mini Kit (Qiagen). 2 μg of total RNA were used to synthesize cDNA by First-Strand cDNA Synthesis kit (GE Healthcare) and the resulting cDNA was diluted in water at a concentration of 10 ng/μl. 20 ng were used for PCR amplification (Taq DNA Polymerase, Qiagen). Initially, RT-PCR analysis revealed Klf4 SeV elimination and maintenance of the temperature sensitive mutants c-Myc and KOS (Klf4, Oct3/4, Sox2) SeVs (data not shown). After two heat treatments

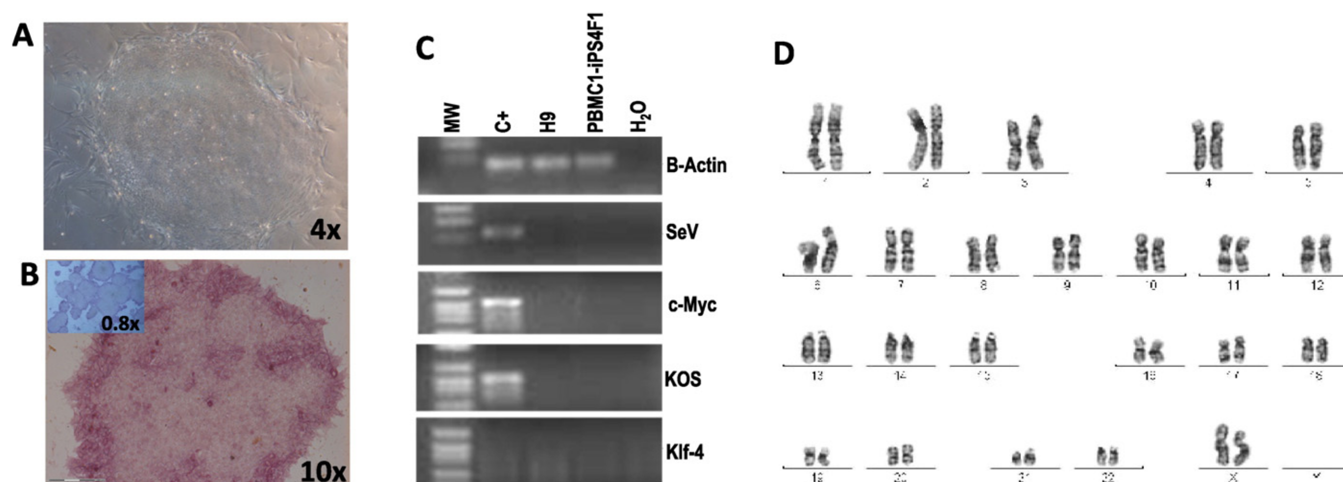

Fig. 1. Characterization of PBMC1-iPS4F1 cell line (A) PBMC1-iPS4F1 cell line displays a typical round shape colony morphology with small, tightly packed cells. (B) Alkaline phosphatase enzymatic activity. (C) Exogenous reprogramming factors and SeV vector silencing was confirmed by RT-PCR. H9 cell line was used as negative control. (D) Typical GTG-banding showing the normal karyotype displayed by PBMC1-iPS4F1 cell line.

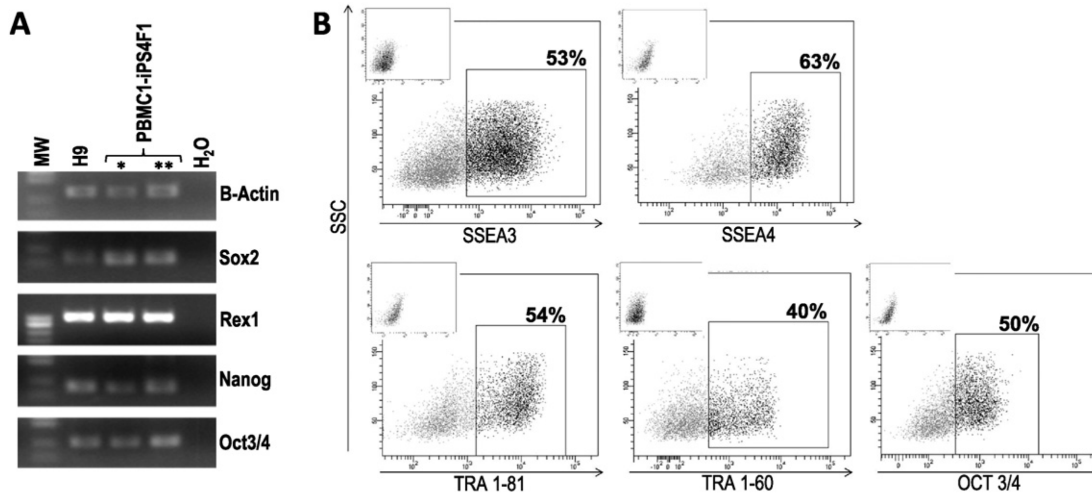

Fig. 2. Expression of pluripotency-associated markers in PBMC1-iPS4F1. (A) Endogenous pluripotency markers Sox2, Rex1, Nanog and Oct3/4 were confirmed by RT-PCR. (B) Expression of pluripotency-associated markers SSEA3, SSEA4, TRA1-81, TRA 1-60 and OCT3/4 at protein level by FACS analysis. The inset shows the staining using the corresponding irrelevant isotype-matched antibody.

(39 °C for 6 days), the PBMC1-iPS4F1 cell line at passage 8 (p8) showed the complete removal of all thermo sensitive SeVs by RT-PCR (Fig. 1C).

Pluripotent stem cell markers expression

RT-PCR

cDNA from total mRNA was synthesized as previously described and used for RT-PCR. Endogenous pluripotency-associated transcription factors (Sox2, Rex1, Nanog and Oct3/4) and β-Actin genes were amplified using the primers detailed in Table 1. cDNA from H9 cell line (WA09-Wicell) was used in the reaction to validate pluripotency markers expression in PBMC1-iPS4F1 cell line (Fig. 2A) (Ramos-Mejia et al., 2012).

Flow cytometry analysis

To check pluripotency-associated markers expression at protein level, FACS analyses were performed. PBMC1-iPS4F1 colonies were dissociated by incubation with TrypLE Express (Life Technologies) for 5 min. Then, the cells were suspended in FACS buffer (5% FBS 2 mM EDTA in PBS) at a concentration of 1–2 × 10^5 cells per 100 μl and incubated with the specific primary antibody for 30 min at 4 °C. We analyzed the pluripotency-associated surface markers SSEA3 (PE, BioScience), SSEA4 (Alexa Fluor® 647, BD Pharmingen), Tra1-60 (PE, BioScience) and Tra1-81 (Alexa Fluor® 647, BD Pharmingen) and the intracellular marker Oct3/4 (BD BioScience). An irrelevant isotype-match antibody was always used as a negative control. To analyze Oct3/4, sequential incubations with fixation and permeabilization solutions were necessary (A and B Fix & Perm Solutions, Invitrogen). After Oct3/4 primary antibody incubation, the cells were incubated with FITC-conjugated secondary antibody (BD BioScience). Then, the cells were washed with FACS buffer and stained with 7-aminoactinomycin D (7-AAD) (BD Bioscience) for 5 min at RT. Stained cells were analyzed using a FACS Canto and FACS Diva™ software program (BD Bioscience) (Fig. 2B) (Ramos-Mejia et al., 2010).

Three germ layer differentiation capacity

In vivo differentiation of the iPSC line

We used an *in vivo* teratoma formation assay to test the capacity of the PBMC1-iPS4F1 cell line to differentiate to the three germ layers. Feeder-free iPSCs colonies were dissociated with collagenase IV (Gibco) to obtain a single cell suspension. 2–5 × 10^6 cells per mouse were injected subcutaneously into dorsal flanks of SCID mice (NOD/LtSz-scid interleukin-2R$\gamma^{-/-}$ mice, The Jackson Laboratory).

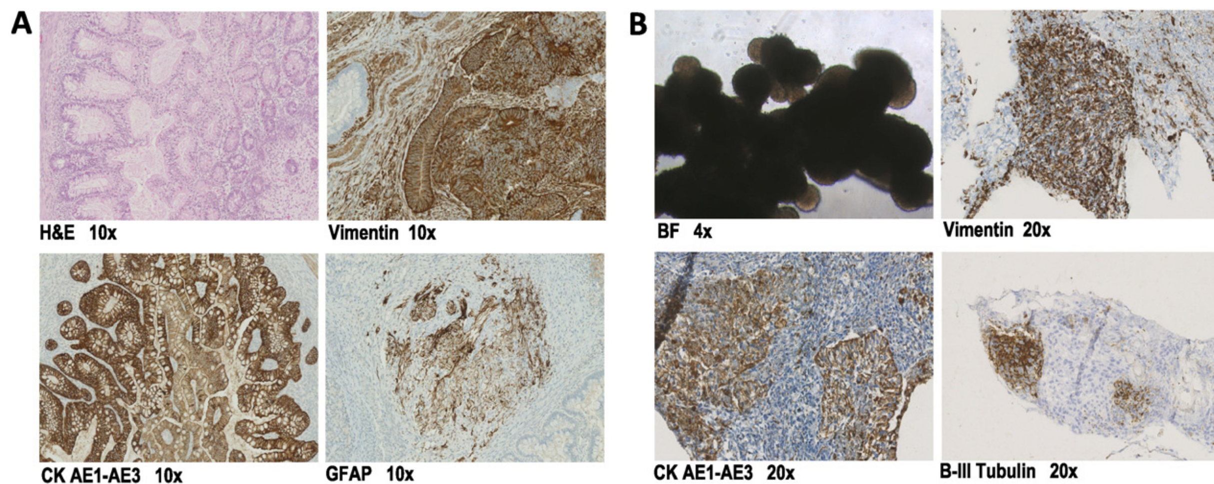

Fig. 3. Three germ layers differentiation testing of PBMC1-iPS4F1. (A) *In vivo* differentiation test by teratoma formation assay. The pictures show hematoxylin/eosin staining (H&E) and immunohistochemistry analysis of mesoderm (Vimentin), endoderm (CK AE1-AE3) and ectoderm (GFAP). (B) *In vitro* differentiation study by EB formation. EBs were grown for 21 days (see brightfield picture, BF) and analyzed for mesodermal (Vimentin), endodermal (CK AE1-AE3) and ectodermal (β-III Tubulin) markers expression.

Table 1
Primers sets used for pluripotency-associated genes expression by RT-PCR.

Gene	Primer sequence	Product size
B-Actin	Forward: CTGGAACGGTGAAGGTGACA Reverse: AAGGGACTTCCTGTAACAATGCA	165 bp
Oct4	Forward: AGTGAGAGGCAACCTGGAGA Reverse: ACACTCGGACCACATCCTTC	110 bp
Sox2	Forward: TCAGGAGTTGTCAAGGCAGAGAAG Reverse: CTCAGTCCTAGTCTTAAAGAGGCAGC	80 bp
Rex1	Forward: CAGATCCTAAACAGCTCGCAGAAT Reverse: GCGTACGCAAATTAAAGTCCAGA	306 bp
Nanog	Forward: TGCAGTTCCAGCCAAATTCTC Reverse: CCTAGTGGTCTGCTGTATTACATTAAGG	96 bp

Teratoma growth was determined by palpation at ~12 weeks post-injection and mice were euthanized at ~15 weeks post-injection. Teratomas were fixed and embedded in paraffin. The sections were stained with hematoxylin/eosin or by immunocytochemistry to detect the expression of GFAP (ectoderm), Vimentin (mesoderm) and CK AE1-EA3 (endoderm) as previously described (Gutierrez-Aranda et al., 2010) (Fig. 3A). The Animal Care Ethics Committee of the University of Granada approved all animal protocols.

In vitro differentiation of the iPSC line

The *in vitro* pluripotency capacity of the PBM1-iPS4F1 cell line was tested by spontaneous EB differentiation. One to two days before reaching confluence, 1 ml of Matrigel 1:6 (diluted in KO-DMEM) was added to the culture. The confluent iPSC culture was washed with PBS and incubated with collagenase IV (Gibco) for 1 min. Then, colonies were rinsed, scrapped and cultured in ultra-low attachment wells (Sigma-Aldrich) to induce EB formation. EBs were cultured for 21 days with PSC medium without bFGF2 for spontaneous differentiation to the three germ layers. Medium changes were performed every 2–3 days. At the end of differentiation, EBs were centrifugated and the pellet was fixed and embedded in paraffin. Sections were immunostained to analyze the germ layer associated markers: β-III-tubulin for ectoderm, Vimentin for mesoderm and CK AE1-EA3 for endoderm (Fig. 3B).

Verification and authentication

Demonstration of intact genome of the PBMC1-iPS4F1. Chromosomal analysis was performed by GTG-banding analysis at the Andalusian Public Health System Biobank, Spain, according to the International System Cytogenetics Nomenclature recommendations (Shaffer, 2012) (Catalina et al., 2007). As shown in Fig. 1D, PBMC1-iPS4F1 cell line displays a normal karyotype (46, XX).

PBMC1-iPS4F1 cell line identity. HLA analysis was employed for cell identification. HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 loci were typed (A*03:02, *31:01; B*15:01, *51:01; C*03:03, *15:02; DRB1*07:01, *15:01; DQB1*02:02, *06:02).

Author disclosure statement

There are no competing financial interests in this study.

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References

Catalina, P., Cobo, F., Cortes, J.L., Nieto, A.I., Cabrera, C., Montes, R., Concha, A., Menendez, P., 2007. Conventional and molecular cytogenetic diagnostic methods in stem cell research: a concise review. Cell Biol. Int. 31 (9), 861–869.

Gutierrez-Aranda, I., Ramos-Mejia, V., Bueno, C., Munoz-Lopez, M., Real, P.J., Macia, A., Sanchez, L., Ligero, G., Garcia-Parez, J.L., Menendez, P., 2010. Human induced pluripotent stem cells develop teratoma more efficiently and faster than human embryonic stem cells regardless the site of injection. Stem Cells 28 (9), 1568–1570.

Montes, R., Ligero, G., Sanchez, L., Catalina, P., de la Cueva, T., Nieto, A., Melen, G.J., Rubio, R., Garcia-Castro, J., Bueno, C., Menendez, P., 2009. Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-beta and IGF-II. Cell Res. 19 (6), 698–709.

Ramos-Mejia, V., Melen, G.J., Sanchez, L., Gutierrez-Aranda, I., Ligero, G., Cortes, J.L., Real, P.J., Bueno, C., Menendez, P., 2010. Nodal/Activin signaling predicts human pluripotent stem cell lines prone to differentiate toward the hematopoietic lineage. Mol. Ther. 18 (12), 2173–2181.

Ramos-Mejia, V., Montes, R., Bueno, C., Ayllon, V., Real, P.J., Rodriguez, R., Menendez, P., 2012. Residual expression of the reprogramming factors prevents differentiation of iPSC generated from human fibroblasts and cord blood CD34+ progenitors. PLoS One 7 (4), e35824.

Shaffer, L.G.e., 2012. ISCN 2013: an International System for Human Cytogenetic Nomenclature (2013). Karger, Switzerland.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861–872.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., Thomson, J.A., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318 (5858), 1917–1920.