Stem Cell Research 28 (2018) 157–160

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Stem Cell Line

Generation of a human iPSC line by mRNA reprogramming

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ARTICLE INFO

Article history:
Received 17 January 2018
Received in revised form 5 February 2018
Accepted 12 February 2018
Available online 20 February 2018

ABSTRACT

The human iPSC cell line, derived from foreskin fibroblasts was generated by non-integrative, non-viral reprogramming technology using OCT4, SOX2, KLF4, LIN28, c-MYC mRNAs.

Resource Table

Unique stem cell line identifier	ESi062-A
Alternative name(s) of stem cell line	Ctrl2-FiPS5F2
Institution	Research Center Principe Felipe, Eduardo Primo Yufera 3, Valencia, Spain
Contact information of distributor	Dunja Lukovic dlukovic@cipf.es
Type of cell line	iPSC
Origin	Human
Additional origin info	Age:4 Sex: Male
Cell source	Foreskin fibroblasts
Clonality	Clonal
Method of reprogramming	mRNA reprogramming
Genetic modification	No
Type of modification	n/a
Associated disease	n/a
Gene/locus	n/a
Method of modification	n/a
Name of transgene or resistance	n/a
Inducible/constitutive system	n/a
Date archived/stock date	January 16, 2017
Cell line repository/bank	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
Ethical approval	Ethics Review Board-competent authority approval obtained by the Valencian Authority for Stem Cell Research (Approval number: S:177-15)

Resource utility

Human induced pluripotent stem cells (hiPSCs) hold great potential in regenerative medicine as they can be coaxed toward any cell type of the human body (Takahashi et al., 2007). An inherent concern with DNA – based reprogramming strategies is the insertional mutagenesis and the retention of the transgenes in the genome. The generation of cells by non-integrative, non-viral approach, using reprogramming factor mRNAs provides a cell source suitable for clinical applications, such as disease modelling, drug discovery, and regenerative medicine.

Resource details

We have generated new hiPSC line Ctrl2-FiPS5F2 (registered as ESi062-A at Human pluripotent stem cell registry, http://hPSCreg.eu) by non-integrative, non-viral approach, by introducing reprogramming factor mRNAs. The human foreskin fibroblasts (HFF, ATCC CRL-2429) were expanded at low passages and reprogramed using mRNA Reprogramming Kit (Stemgent). Individual colonies were picked and subcultured into individual cell lines after 25–30 days and analyzed at cellular and genetic level to confirm successful reprogramming. After 30 days generated colonies displayed a typical human embryonic stem cell (hESC) colony-like morphology with refractive edges as seen by bright field (BF) and phase contrast (PC) microscopy (Fig. 1A, scale bar 200 μm) and the cells had high nuclear/cytoplasmic ratio. Pluripotency was additionally assessed by immunocytochemistry to pluripotency markers OCT-4, SOX2, NANOG, SSEA-4 and TRA-1-81 (Fig. 1A, Scale bar 200 μm) and flow cytometry for SSEA-4 pluripotency

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https://doi.org/10.1016/j.scr.2018.02.011

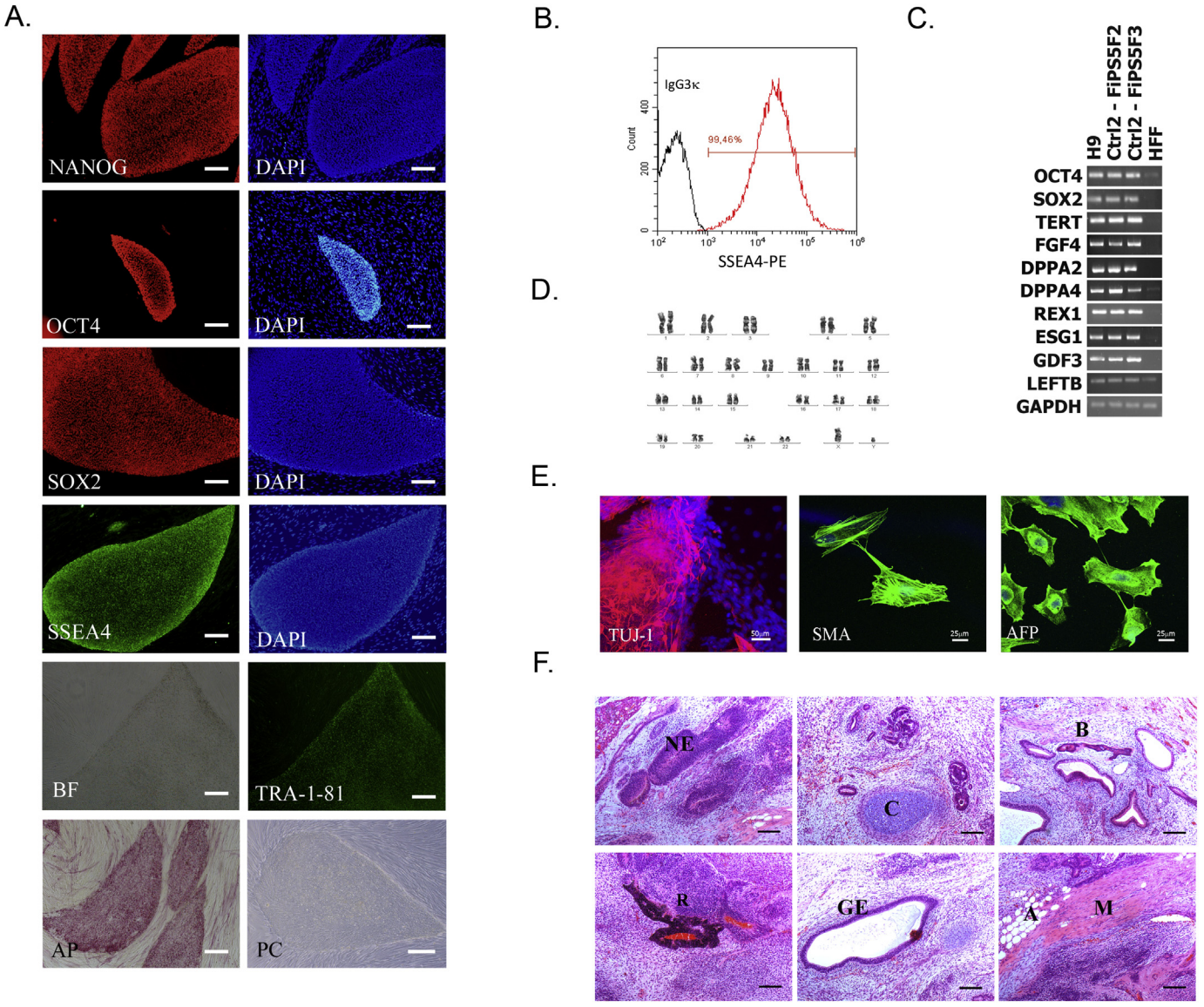

Fig. 1.

marker (Fig. 1B). The alkaline phosphatase (AP) is known to be more active in hiPSCs and the colorimetric assay depicting its activity confirmed that the selected hiPSC colonies are indeed pluripotent (Fig. 1A, scale bar 200 μm). The expression of endogenous pluripotency genes was detected by RT-PCR using hESC H9 as positive- and HFF as negative control (Fig. 1C). The genetic fingerprinting was performed and proved its genetic identity to parental fibroblasts (available with the authors). The selected line was karyotypically normal at passage 10 (Fig. 1D) and 30 (not shown). The mycoplasma was regularly checked without positive results (Supplementary Fig. 1). To test the ability of the hiPSC line to generate derivates of three germ layers *in vitro*, the embryoid body based assay was performed. Spontaneous differentiated cells were immunostained for differentiation markers such as β-III tubulin (TUJ-1) for ectoderm, smooth muscle actin (SMA) for mesoderm and AFP for endoderm (Fig. 1E). To test the ability of generated hiPSC line to generate derivatives of three germ layers *in vivo*, the hiPSCs were transplanted subcutaneously into the immunodeficient (SCID) mice. Eight weeks after injection, tumor was formed and extracted. Histological sections showed that the tumor contained derivatives of all three germ layers including gut-like tissues (gut endoderm, GE), neural epithelium (NE) and retina (R) (ectoderm) and striated muscle (M), bone (B), cartilage (C) and adipose tissue (A) (mesoderm) (Fig. 1F, scale bar 100 μm).

Materials and methods

Reprogramming fibroblasts

Foreskin fibroblasts were reprogrammed using mRNA Reprogramming Kit (Stemgent) according to manufacturer instructions. hiPSCs were grown on irradiated (45Gy) HFF (ATCC CRL 2429) in hiPSCs medium containing KO DMEM, KSR 20%, Glutamax 2 mM, non-essential amino acids 0.1 mM, β-mercaptoethanol 0.23 mM, basic FGF 10 ng/mL, penicillin/streptomycin, at 37 °C/5% CO_2. Cells were mechanically passaged every 6–8 days at 1:3–1:6 split ratio.

In vitro differentiation assay

The iPSC colonies were cut mechanically and cultured in suspension to form embryoid bodies in hiPSC medium without bFGF. After 7 days in suspension, embryoid bodies were transferred into 0.1% gelatin-coated plates and cultured for additional 7–10 days to allow spontaneous

differentiation with alternate day medium changes. Then the cells were fixed and immunostained to detect cells from the three germ layers.

In vivo differentiation assay

The colonies from 4 wells of a 6 well plate (80–100 colonies) were cut mechanically and resuspended in 200 μL of hiPSC medium and 10 μL of Matrigel (BD, #354277). The suspension was injected into SCID nude 5 week-old mice subcutaneously in the back. After about 8 weeks, the teratomas of 1 cm in diameter were excised and processed as described (Lukovic et al., 2017).

Karyotype analysis

The hiPSCs were adapted to feeder-free cell culture on Matrigel (BD, #354277) coated plates using mTeSR1 medium. Matrigel was diluted in DMEM/F-12 according to the dilution factor specified on the certificate of analysis and incubated for 1 h/RT at 1 mL/well of a 6-well plate. Passages were performed using Dispase (STEMCELL Technologies, #07913), every 5–7 days at 1:10–1:15 split ratio. The karyotype was analyzed by G-banding at 400–550 band resolution, 30 metaphases analyzed (Service of Biobanco de Sistema Sanitario Público, Granada, Spain).

Fingerprinting

gDNA from fibroblasts and hiPSCs was extracted using QIAamp DNA Blood mini kit (Qiagen) in the presence of RNAse (Roche). Fingerprinting analyses was performed using Promega kit 10 microsatellite markers (TH01, TPOX, vWA, CSF1PO, D16S539, D7S820, D13S317, D5S818 y D21S11, Amelogenin) and analyzed on Abi PRISM 3130 using GeneMapper (Thermo Fisher) by Biobanco de Sistema Sanitario Público, Granada, Spain (Table 1).

Detection of pluripotency markers by RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and treated with DNase I to remove any genomic DNA contamination. QuantiTect Reverse Transcription Kit (Qiagen) was used to carry out cDNA synthesis from 1 μg of total RNA according to the manufacturer's instructions. The expression level of pluripotency markers was analyzed using the primers described in Table 2. HFF were used as negative control while hESC H9 (WiCell) was used as positive control.

The PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH, Luckenwalde, Germany) using Applied Biosystems Veriti Thermal Cycler with following steps: denaturation 94 °C; 15 s, annealing 50–65 °C; 30 s, extension 72 °C; 45 s, for 35 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as a control housekeeping gene. Thereafter, PCR products were analyzed on 2% agarose gels.

Immunocytochemistry

Cells were washed in PBS and fixed in 4% PFA for 15 min at room temperature (RT). Fixed cells were washed twice in PBS and placed in blocking solution (3% serum, 0.5% Triton-X100 in PBS) for 1 h at RT. Cells were then incubated overnight at 4 °C with primary antibodies. The following day, cells were washed three times in PBS and incubated with an appropriate secondary antibody at RT for 1 h. Thereafter, cells were stained with DAPI (1:1000) at RT during 5 min, washed three times in PBS and visualized on Leica DM600 fluorescent microscope equipped with Leica DC500 camera. Samples grown on coverslips were mounted using Vectashield.

Flow cytometry

hiPSCs were dissociated using Accutase (Innovative Cell Technologies) for 2–4 min at RT, centrifuged at 300 rcf for 5 min and resuspended in PBS + 2% FBS. Anti-human SSEA-4, PE conjugated, antibody was added at 1:800 dilution and incubated for 20 min at RT. IgG3 kappa isotype was used at as negative control at the same final concentration ($3{,}12 \times 10^{-5}$mg/mL). The cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter) and data analyzed by CytExpert 2.0 software.

Alkaline phosphatase staining

Alkaline phosphatase staining was carried out using Alkaline Phosphatase Staining Kit II (Stemgent, MA).

Mycoplasma detection

The presence of mycoplasma was tested regularly measuring enzyme activity *via* luciferase (MycoAlert™ PLUS Mycoplasma Detection Kit, Lonza).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Bright field (BF) microscopy Fluorescence microscopy
Phenotype	Immunocytochemisty	Positive staining/expression of pluripotency markers: Oct4, Nanog, Sox2, SSEA4, TRA-1-81	Fig. 1 panel A
	Cytometry	SSEA4 99%	Fig. 1 panel B
Genotype	Karyotype (G-banding) and resolution	46XY, Resolution 450–500	Fig. 1 panel D
Identity	Microsatellite PCR (mPCR)	Not performed	
	STR analysis	10 loci analyzed, all matching	Available with authors
Mutation analysis (IF APPLICABLE)	Sequencing	n/a	
	Southern Blot OR WGS	No	
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Negative	Supplementary Fig. 1
Differentiation potential	Embryoid body formation	Positive TUJ-1 for ectoderm, positive SMA mesodermal staining and positive AFP endodermal staining	Fig. 1 panel E
	Teratoma assay	Teratomas showed the presence of gut endoderm, neural epithelium and retina (ectoderm) and striated muscle, bone, cartilage and adipose tissue (mesoderm)	Fig. 1 Panel F
Donor screening (OPTIONAL)	N/A	N/A	N/A
Genotype additional info (OPTIONAL)			

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-Nanog	1:400	Cell Signaling Technology Cat# D73G4
Pluripotency markers	Rabbit anti-Oct4	1:400	Cell Signaling Technology Cat# C30A3
Pluripotency markers	Rabbit anti-Sox2	1:400	Cell Signaling Technology Cat# D6D9
Pluripotency markers	Mouse anti-SSEA4	1:100	BD Pharmigen Cat# 560073
pluripotency markers	Mouse anti human SSEA4-PE	1:800	STEMCELL Technologies Cat# 60062PE.1
Isotype control	Mouse IgG3 kappa-PE	1:6400	STEMCELL Technologies Cat# 60073PE.1
Pluripotency markers	Mouse anti-TRA-1-81	1:100	StainAlive, Stemgent Cat# 09-0069
Ectoderm marker	Mouse anti-TUJ-1	1:500	Abcam 7751
Mesoderm marker	Mouse anti-SMA	1:300	Sigma A5228
Endoderm marker	Mouse anti-AFP	1:20	RD MAB 1368
Secondary antibody	Anti-mouse	1:500	Invitrogen A11001
Secondary antibody	Anti-rabbit	1:500	Invitrogen A11002

Primers		
	Target	Forward/Reverse primer (5′-3′)
Pluripotency markers (qPCR)	OCT4	AAGCCCTCATTTCACCAGG CTTGGAAGCTTAGCCAGGTC
Pluripotency markers (qPCR)	SOX2	TCACATGTCCCAGCACTACC CCCATTTCCCTCGTTTTTCT
Pluripotency markers (qPCR)	TERT	TGGCTGCGTGGTGAACTTG GCGGTTGAAGGTGAGACTGG
Pluripotency markers (qPCR)	FGF4	CTACAACGCCTACGAGTCCTACA GTTGCACCAGAAAAGTCAGAGTTG
Pluripotency markers (qPCR)	REX1	CAGATCCTAAACAGCTCGCAGAAT GCGTACGCAAATTAAAGTCCAGA
Pluripotency markers (qPCR)	GDF3	CTTATGCTACGTAAAGGAGCTGGG GTGCCAACCCAGGTCCCGGAAGTT
Pluripotency markers (qPCR)	DPPA2	CCGTCCCCGCAATCTCCTTCCATC ATGATGCCAACATGGCTCCCGGTG
Pluripotency markers (qPCR)	DPPA4	GGAGCCGCCTGCCCTGGAAAATTC TTTTTCCTGATATTCTATTCCCAT
Pluripotency MARKERS (qPCR)	LEFTB	CTTGGGGACTATGGAGCTCAGGGCGAC CATGGGCAGCGAGTCAGTCTCCGAGG
Pluripotency markers (qPCR)	ESG1	ATATCCCGCCGTGGGTGAAAGTTC ACTCAGCCATGGACTGGAGCATCC
House-keeping gene (qPCR)	GAPDH	TGCACCACCAACTGCTTAGC GGCATGGACTGTGGTCATGAG

hiPSC nomenclature

The generated hiPSC line was named following Spanish National Stem Cell Bank recommendations. The line is registered at https://hpscreg.eu/ as ESi062-A line.

Funding

This work was supported by Institute of Health Carlos III/FEDER (PI16/00409 (DL), CP10/00579 (SE), PI14/02209 (SE), Platform of Biomolecular and Bioinformatics Resources of the Institute of Health Carlos III, PRB^2, PT13/0001/0042).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.02.011.

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