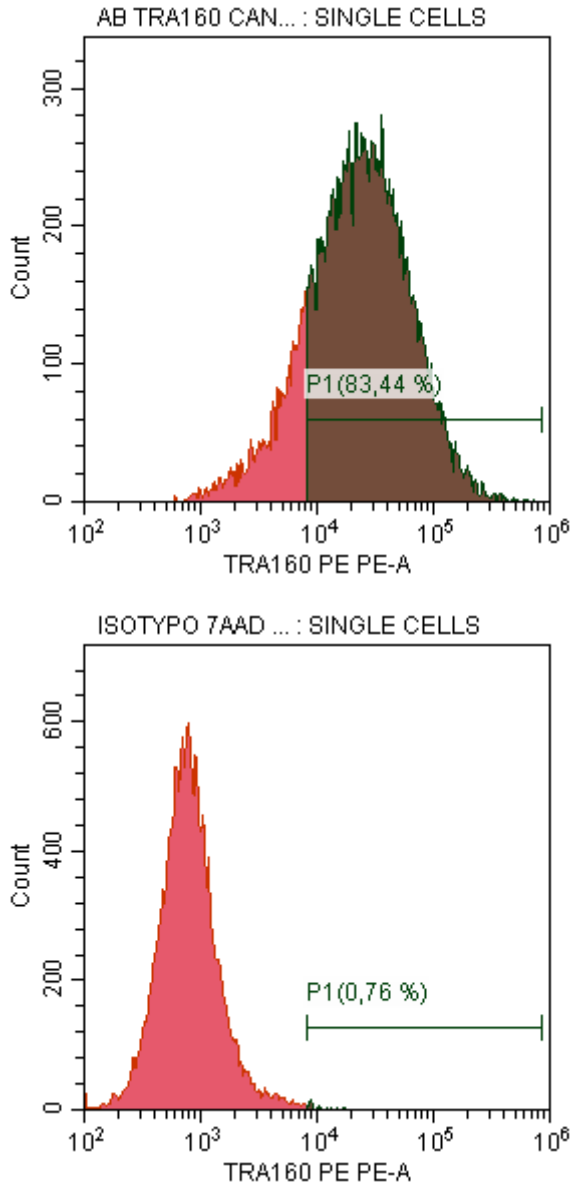
Citometría pluripotencia ESi064-A

Marcador TRA1-60 (ISOTYPO como control negativo)



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Lab Resource: Stem Cell Line

Generation of human induced pluripotent stem cell (iPSC) line from an unaffected female carrier of mutation in SACSIN gene

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A B S T R A C T

The human iPSC cell line, CARS-FiPS4F1 (ESi064-A), derived from dermal fibroblast from the apparently healthy carrier of the mutation of the gene SACSIN, was generated by non-integrative reprogramming technology using OCT3/4, SOX2, CMYC and KLF4 reprogramming factors. The pluripotency was assessed by immunocytochemistry and RT-PCR. This iPSC line can be used as control for Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) disease.

Resource table.

Unique stem cell identifier	ESi064-A
Alternative name(s) of stem cell line	CARS-FiPS4F1
Institution	Research Center Principe Felipe, Eduardo Primo Yufera 3, Valencia, Spain
Contact information of distributor	Slaven Erceg, serceg@cipf.es
Type of cell line	iPSC
Origin	Human skin cells
Additional origin info	Sex: female Age: 29 Ethnicity: Not known
Cell source	Human fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus. Oct4, Sox2, cMyc, Klf4
Genetic modification	NO
Type of modification	N/A
Associated disease	N/A
Gene/locus	Gene: SACSIN gene (SACS) Locus: 13q12.12 Mutation: heterozygote c.11374C > T (p.R3792X)
Method of modification	N/A
Name of transgene or resistance	N/A

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Inducible/constitutive system	N/A
Data archived/stock date	11/05/2017
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: RS:S-081/18)

Resource utility

Human induced pluripotent stem cells (hiPSC) from the healthy unaffected carrier could serve as control for hiPSC derived from ARSACs patient. These cells can be differentiated toward affected cells to better understand molecular mechanisms of disease and pathophysiology.

Resource details

Skin punch biopsy was taken from a 29- year-old healthy female individual which *SACS* gene was sequenced due to family relationship to persons that have been diagnosed for autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) harboring the c.11374C > T (p.R3792X) mutation in heterozygosis. Primary fibroblast cell line was established. The generation of the human induced pluripotent stem cell (hiPSC) line, CARS-FiPS4F1 (registered as ESi064-A at www.hPSCreg.com), was carried out using non-integrative Sendai virus containing the human reprogramming factors, Oct3/4, Sox2, C-Myc, Klf4 (Takahashi et al., 2007) following instructions by manufacturer. After 30 days generated colonies displayed a typical ES-like morphology (polygonal shape; refractive edges, high nuclear/cytoplasmic ratio) and growth behaviour. DNA sequencing analysis of CARS-FiPS4F1 confirmed the mutation in one allele in *SACS* (Fig. 1A). The clearance of the virus and the exogenous reprogramming factor genes was confirmed by RT-PCR after twelve cell culture passages (Fig. 1B). The genetic fingerprinting was performed with CARS-FiPS4F1 hiPSC line and proved its genetic identity to parental fibroblasts (available with the author). The selected line karyotypicaly showed normal karyotype (46, XY) at low passages (passage9) (Fig. 1C) and medium passage number (passage 30). Genetic and functional assays were performed to determine the quality of the CARS-FiPS4F1 line. Pluripotency was assessed by immunocytochemistry to pluripotency markers OCT-4, SOX2, NANOG and SSEA-4 and flow cytometry for SSEA-4 pluripotency marker (Fig. 1D). The alkaline phosphatase 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. 1D). The expression of endogenous plutipotency genes was detected by RT-PCR (Fig. 1E). To test the ability of the hiPSC line to generate derivates of three germ layers in vitro, the hiPSC were differentiated into the three germ layers using an embryoid body based assay. Spontaneous differentiated cells were immunostained for differentiation markers such as TUJ1 for ectoderm, SMA for mesoderm and positive FOXA2 and Alfa-Feto protein (aFP) for endoderm (Fig. 1F). The mycoplasma was regularly checked without positive results (Supplementary Fig. 1).

Materials and methods

Reprogramming patient's fibroblasts

The hiPSC were derived from patient's fibroblasts using Sendai virus (Cyto Tune- iPS 2.0 reprogramming Kit, Life Technologies) according to manufacturer instructions. hiPSCs were grown on irradiated (45Gy) human foreskin fibroblasts (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. Cells were mechanically passaged every 6–8 days.

In vitro differentiation assay

For in vitro differentiation assay the colonies from a fully confluent 6-well plate were cut mechanically and cultured in suspension to form embryoid bodies in hiPSCs media without bGFG. After 7 days in suspension, embryoid bodies were transferred into 0.1% gelatin-coated plates and cultured in same medium for additional 7–10 days to allow spontaneous differentiation. Then the cells were fixed and immunostained to detect cells from the three germ layers.

Karyotype analysis

The hiPSCs were adapted to feeder-free cell culture on Matrigel (BD, #354277) coated plates using mTeSR1 medium. Passages were performed using Dispase (STEMCELL Technologies, #07913), every 5–7 days. 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, Hilden, Germany) in the presence of RNAse (Roche). Fingerprinting analyses was performed using 5 microsatellite markers (D19S572, D2S159, D14S972, D8S601and D9S1853) and analyzed on an Abi PRISM 3130 Genetic Analyzer using GeneMapper (Thermo Fisher) by Biobanco de Sistema Sanitario Público, Granada, Spain.

Mutation sequencing

Genomic DNA from fibroblasts and hiPSCs was isolated using the QIAamp DNA Blood mini kit (Qiagen). Primers used for amplification and directed sequencing of *SACS* around the mutation site c.9938delC Sense: 5′- GCAGAACATCTCCTTCAGGA -3′, Antisense: 5′- CCGCTATG TAAGCATTGGAAA-3′ (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 PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH, Germany) using Applied Biosystems Veriti Thermal Cycler. The expression level of pluripotency markers was analyzed using the primers described in Table 2. Fibroblasts and hESC H9 (WiCell) were used as negative and positive control respectively.

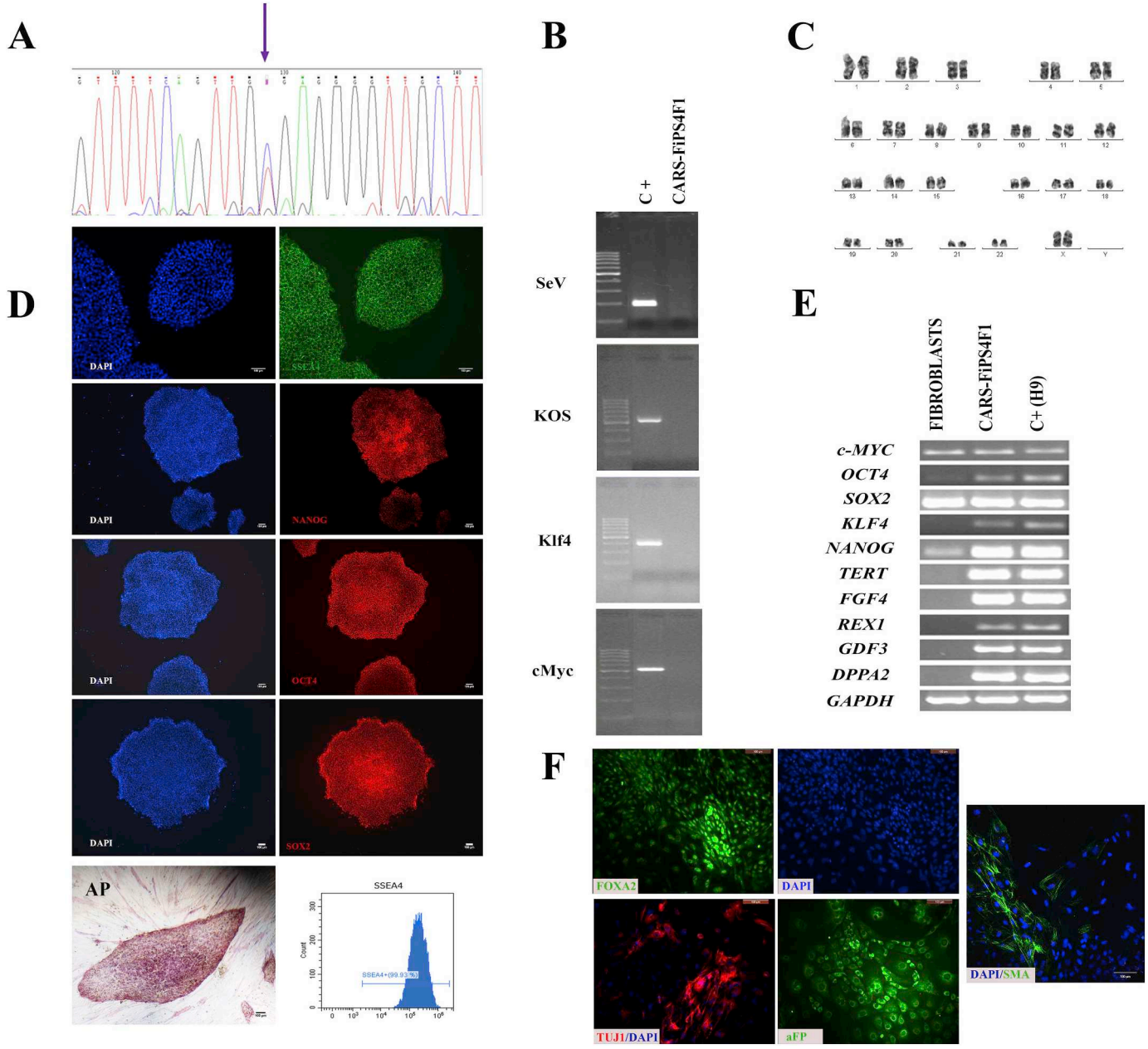

Fig. 1. Characterization of CARS-FiPS4F1 line. A. DNA electropherograms showing the heterozygous mutation c.11374C > T in SACS gene in CARS-FiPS4F1 line of a healthy carrier. B. RT-PCR analysis of SeV genome and transgenes in hiPSCs and positive control (C+). C. Representative metaphase of normal human karyotype (46, XX). D. Immunocytochemistry for pluripotency markers SSEA-4, NANOG, OCT4, SOX2. Nuclei were stained with DAPI. Lower panel: Alkaline phosphatase (AP) staining of CARS-FiPS4F1 colonies (left). Flow cytometry of surface pluripotency markers SSEA-4 (right). E. RT-PCR analysis of pluripotency markers. F. Immunocytochemistry for ectodermal (TUJ1), endodermal (aFP, FOXA2) and mesodermal (SMA) markers. Nuclei were stained with DAPI.

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 antibody was added and incubated for 20 min at RT. IgG3, kappa isotype (STEMCELL technologies # 60073PE.1) was used as negative control. The cells were

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	*Shown by immunocytochemistry*
Phenotype	Immunocytochemisty	*Positive staining/expression of pluripotency markers: Oct4, Nanog, Sox2, SSEA4*	Fig. 1*panel D*
	Flow cytometry	*SSEA4 99,9%*	Fig. 1*panel D*
Genotype	Karyotype (G-banding) and resolution	*46XX, Resolution 450–500*	Fig. 1*panel C*
Identity	STR analysis	*5 loci analyzed, all matching*	*Available with authors*
Mutation analysis (IF APPLICABLE)	Sequencing	*c.9938delC (p.G3313QfsX11)*	Fig. 1*panel A*
	Southern Blot OR WGS	*N/A*	*N/A*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by luminescence. Negative*	*Supplementary Fig. 1*
Differentiation potential	Embryoid body formation	*Positive TUJ1 ectodermal staining, positive SMA mesodermal staining and positive AFP and FOXA2 endodermal staining.*	Fig. 1*panel F*
Donor screening (OPTIONAL)	N/A	*N/A*	N/A
Genotype additional info (OPTIONAL)	N/A	*N/A*	N/A

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker	*Rabbit anti-Nanog*	*1:400*	*Cell Signaling Technology Cat# D73G4*
Pluripotency Marker	*Rabbit anti-Oct4*	*1:400*	*Cell Signaling Technology Cat# C30A3*
Pluripotency Marker	*Rabbit anti-Sox2*	*1:400*	*Cell Signaling Technology Cat# D6D9*
Pluripotency Marker	*Rabbit anti-SSEA4*	*1:100*	*BD Pharmigen Cat# 560073*
Pluripotency Marker	*Mouse anti human SSEA4-PE*	*1:800*	*STEMCELL Technologies Cat #60062PE.1*
Differentiation Marker	*Mouse anti-FoxA2*	*1:100*	*R&D Cat# AF2400*
Differentiation Marker	*Mouse anti-SMA*	*1:200*	*Abcam Cat# ab11570*
Differentiation Marker	*Mouse anti-AFP*	*1:100*	*R&D Cat# MAB1368*
Differentiation Marker	*Mouse anti-BTubulin (Tuj1)*	*1:500*	*Neuromics Cat# MO15013*

Primers

	Target	Forward/Reverse primer (5′-3′)
Pluripotency Marker (qPCR)	*OCT4*	AAGCCCTCATTTCACCAGG CTTGGAAGCTTAGCCAGGTC
Pluripotency Marker (qPCR)	*NANOG*	*CCAAATTCTCCTGCCAGTGAC* CACGTGGTTTCCAAACAAGAAA
Pluripotency Marker (qPCR)	*SOX2*	TCACATGTCCCAGCACTACC CCCATTTCCCTCGTTTTTCT
Pluripotency Marker (qPCR)	*TERT*	TGGCTGCGTGGTGAACTTG GCGGTTGAAGGTGAGACTGG
Pluripotency Marker (qPCR)	*FGF4*	CTACAACGCCTACGAGTCCTACA GTTGCACCAGAAAAGTCAGAGTTG
Pluripotency Marker (qPCR)	*REX1*	CAGATCCTAAACAGCTCGCAGAAT GCGTACGCAAATTAAAGTCCAGA
Pluripotency Marker (qPCR)	*GDF3*	CTTATGCTACGTAAAGGAGCTGGG GTGCCAACCCAGGTCCCGGAAGTT
Pluripotency Marker (qPCR)	*DPPA2*	CCGTCCCCGCAATCTCCTTCCATC ATGATGCCAACATGGCTCCCGGTG
House-keeping gene (qPCR)	*GAPDH*	ATCGTGGAAGGACTCATGACCACA CCCTGTTGCTGTAGCCAAATTCGT
Sendai virus detection	*SeV*	GGATCACTAGGTGATATCGAGC ACCAGACAAGAGTTTAAGAGATATGTATC
Transgenes detection	*KOS*	ATGCACCGCTACGACGTGAGCGC ACCTTGACAATCCTGATGTGG
Transgenes detection	*c-Myc*	TAACTGACTAGCAGGCTTGTCG TCCACATACAGTCCTGGATGATGATG
Transgenes detection	*Klf4*	TTCCTGCATGCCAGAGGAGCCC AATGTATCGAAGGTGCTCAA

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).

hiPSC Nomenclature

The generated hiPSC line was named following Spanish National Stem Cell Bank recommendations. The line is registered on https://hpscreg.eu/ as ESi043-A line.

Funding

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.10.016.

References

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, 861–872.

	HDF	CARS-FiPS4F1
D19S572	129/129	129/129
D2S159	178/178	178/178
D14S972	199/199	199/199
D8S601	223/225	223/225
D9S1853	252/261	252/261

Mycoplasma test by MycoAlert PLUS™ **30/09/2017**	
Sample	**Read B/Read A**
Positive control	3,537
Negative control	0,347
CARS-FiPS4F1	0,789