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Generation of three human iPSC lines from PLAN (*PLA2G6*-associated neurodegeneration) patients

Candela Machuca [a,b,c], Marta Correa-Vela [d], Deyanira García-Navas [e], Alejandra Darling [f], Irene Villalón-García [g,h], José Antonio Sánchez-Alcázar [g,h], Belén Pérez-Dueñas [d,i], Slaven Erceg [b,c,k,l], Carmen Espinós [a,b,j,*]

[a] *Unit of Rare Neurodegenerative Diseases, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain*
[b] *Rare Diseases Joint Units, CIPF-IIS La Fe & INCLIVA, Valencia, Spain*
[c] *Stem Cells Therapies in Neurodegenerative Diseases Lab, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain*
[d] *Department of Pediatric Neurology, Hospital Universitari Vall d'Hebron, Vall d'Hebron Institut de Recerca, Barcelona, Spain*
[e] *Department of Pediatric Neurology. Hospital Universitario San Pedro de Alcántara, Cáceres, Spain*
[f] *Unit of Pediatric Movement Disorders, Hospital Sant Joan de Déu, Barcelona, Spain*
[g] *Centro Andaluz de Biología del Desarrollo (CABD-CSIC), Universidad Pablo de Olavide, Seville, Spain*
[h] *Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Instituto de Salud Carlos III (ISCIII), Madrid, Spain*
[i] *Universitat Autònoma de Barcelona, Barcelona, Spain*
[j] *Department of Genetics, Universitat de València, Valencia, Spain*
[k] *National Stem Cell Bank-Valencia Node, Biomolecular and Bioinformatics Resources Platform PRB2, ISCIII, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain*
[l] *Institute of Experimental Medicine, Department of Tissue Cultures and Stem Cells, Czech Academy of Sciences, Prague, Czech Republic*

A B S T R A C T

The human iPSC cell lines, PLANFiPS1-Sv4F-1 (RCPFi004-A), PLANFiPS2-Sv4F-1 (RCPFi005-A), PLANFiPS3-Sv4F-1 RCPFi006-A), derived from dermal fibroblast from three patients suffering PLAN (*PLA2G6*-associated neurodegeneration; MIM 256600) caused by mutations in the *PLA2G6* gene, 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. Differentiation capacity was verified *in vitro*. This iPSC line can be further differentiated toward affected cells to better understand molecular mechanisms of disease and pathophysiology.

Resource Table

Unique stem cell lines identifier	RCPFi004-ARCPFi005-ARCPFi006-A
Alternative names of stem cell lines	Line 1: PLANFiPS1-Sv4F-1 Line 2: PLANFiPS2-Sv4F-1 Line 3: PLANFiPS3-Sv4F-1
Institution	Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain
Contact information of distributor	Carmen Espinós, cespinos@cipf.es
Type of cell lines	iPSC
Origin	Human
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus

(*continued on next column*)

(*continued*)

Unique stem cell lines identifier	RCPFi004-ARCPFi005-ARCPFi006-A
Multiline rationale	e.g. isogenic clones, same disease non-isogenic cell lines, control and disease pair, gene corrected clones, etc.
Gene modification	NO
Type of modification	n/a
Associated disease	*PLA2G6*-associated neurodegeneration (PLAN)
Gene/locus	22q13.1
Method of modification	n/a
Name of transgene or resistance	n/a
Inducible/constitutive system	n/a
Date archived/stock date	n/a
Cell line repository/bank	

(*continued on next page*)

* Corresponding author at: Unit of Rare Neurodegenerative Diseases, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain.
E-mail address: cespinos@cipf.es (C. Espinós).

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(*continued*)

Unique stem cell lines identifier	RCPFi004-ARCPFi005-ARCPFi006-A
Ethical approval	RCPFi004-A, RCPFi005-A, RCPFi006-A, at www.hPSCreg.eu The ethical review board for the donation and use of human cells and tissues of the Instituto de Salud Carlos III (ISCIII; registry no. 2020/1).

1. Resource utility

The generation of human induced pluripotent stem cells (hiPSCs) from the PLAN patients permits the development of disease specific stem cells that can be further differentiated toward affected cells to better understand molecular mechanisms of disease and pathophysiology.

2. Resource details

PLAN (*PLA2G6*-associated neurodegeneration) is a rare disease caused by mutations in *PLA2G6*, which represents the second most common NBIA (neurodegeneration with brain iron accumulation) form (near 20% of NBIA cases). The PLAN phenotypic spectrum comprises INAD (infantile neuroaxonal dystrophy), ANAD (atypical NAD), and PARK14 (*PLA2G6*-related dystonia-parkinsonism). INAD is characterized by early-onset manifestations, while ANAD has an onset at childhood and PARK14 at late adolescence. Main clinical hallmarks may be cerebellar atrophy, psychomotor regression, spasticity, neuroaxonal dystrophy, optic atrophy, dystonia, parkinsonism, and cognitive deterioration. As NBIA condition, the neuroimaging may show T2 hypointensity in the globus pallidus (Hinarejos et al., 2020). PLA2G6 encodes several isoforms of VIA calcium-independent phospholipase A2 (iPLA2β), which plays a role in vital functions such as membrane remodelling, fatty acid oxidation, cell signalling and apoptosis. Mutations responsible for INAD are postulated to impair the phospholipase activity of iPLA2β (Engel et al., 2010).

Primary fibroblast cell lines were established from skin punch biopsies taken from three patients (PLANFiPS1-Sv4F-1, PLANFiPS2-Sv4F-1, PLANFiPS3-Sv4F-1), who suffer from PLAN/INAD caused by the mutations c.2356G > A (p.E786K) in homozygosis [PLANFiPS1-Sv4F-1], c.1010T>A (p.L337Q)/c.1027G>A (p.A343T) [named PLANFiPS2-Sv4F-1], and c.2370T>G (p.Y790*) in homozygosis [PLANFiPS3-Sv4F-1], in the *PLA2G6* gene (NM_003560.4) (Table 1). The generation of the human induced pluripotent stem cells (hiPSCs) lines, named PLANFiPS1-Sv4F-1, PLANFiPS2-Sv4F-1, PLANFiPS3-Sv4F-1 (registered as RCPFi004-A, RCPFi005-A, RCPFi006-A, respectively at www.hPSCreg.eu), was carried out using non-integrative Sendai virus containing the human reprogramming factors, Oct3/4, Sox2, C-Myc, and Klf4 (Takahashi et al., 2007), following the manufacturer's instructions. 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 the human cell lines confirmed the *PLA2G6* mutations in each allele of each index case (Fig. 1A). The clearance of the virus and the exogenous reprogramming factor genes were confirmed by RT-PCR after twelve cell culture passages (Fig. 1B). The genetic fingerprinting was performed with the hiPSC lines and proved its genetic identity to parental fibroblasts (available with the author). The selected lines showed normal karyotypes at medium passage number (passage 20): [46, XX] for PLANFiPS2-Sv4F-1, and [46, XY] for PLANFiPS1-Sv4F-1 and PLANFiPS3-Sv4F-1 (Supplementary Fig. 1A). Genetic and functional assays were performed to determine the quality of the human cell lines. The expression of endogenous plutipotency genes was detected by RT-PCR (Fig. 1C). The alkaline phosphatase is known to be more active in hiPSC and the colorimetric assay depicting its activity confirmed that the selected hiPSC colonies are indeed pluripotent (Fig. 1D). Pluripotency was assessed by immunocytochemistry to pluripotency markers OCT-4, SOX2, NANOG and SSEA-4 and flow cytometry for SSEA-4 pluripotency marker (Fig. 1E). To test the ability of the hiPSCs lines to generate derivate of three germ layers *in vitro*, the hiPSCs 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 for endoderm (Fig. 1F). The mycoplasma was regularly checked without positive results (Supplementary Fig. 1B) (see Table 2).

3. Materials and methods

3.1. Reprogramming patients' fibroblasts

The hiPSCs were derived from patients' fibroblasts using Sendai virus (Cyto Tune- iPS 2.0 reprogramming Kit, Life Technologies) according to manufacturer instructions. hiPSCs were grown on irradiated (45 Gy) 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. The hiPSCs were adapted to feeder-free cell cultured in mTesR1 medium (STEMCELL Technologies, #85850) on hESC-qualified Matrigel (BD, #354277) coated plates at 37 °C/5% CO_2. Passages were performed using Dispase (STEMCELL Technologies, #07913) upon reaching 70–80% confluence (5–7 days) at a 1:10 split ratio.

3.2. Karyotype analysis

The karyotype was analyzed by G-banding at 400–550 band resolution, 30 metaphases analyzed (Biobank of the Andalusian Public Health System, Granada, Spain, and Translational Genetics Group, Hospital Universitari i Politècnic La Fe, Valencia, Spain).

3.3. 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 five STRs (short tandem repeats) markers (*D19S572, D2S159, D14S972, D8S601,* and *D9S1853*) and analyzed on an Abi PRISM 3130 using GeneMapper (Thermo Fisher) by the Biobank of the Andalusian Public Health System (Granada, Spain).

3.4. Mutation screening

Genomic DNA from fibroblasts and hiPSCs was isolated using the QIAamp DNA Blood mini kit (Qiagen). To detect c.1010T>A (p.L337Q) and c.1027G>A (p.A343T), both variants on exon 6, the primers were:

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus*	Disease
PLANFiPS1-Sv4F-1 (RCPFi005-A)	RCPFi004-A	Male	5	Arab	c.2356G>A (p.E786K) in homozygosis	PLAN
PLANFiPS2-Sv4F-1 (RCPFi004-A)	RCPFi005-A	Female	5	Caucasian	c.1010T>A (p.L337Q)/ c.1027G>A (p.A343T)	PLAN
PLANFiPS3-Sv4F-1 (RCPFi006-A)	RCPFi006-A	Male	5	Caucasian	c.2370T>G (p.Y790*) in homozygosis	PLAN

**PLA2G6* gene.

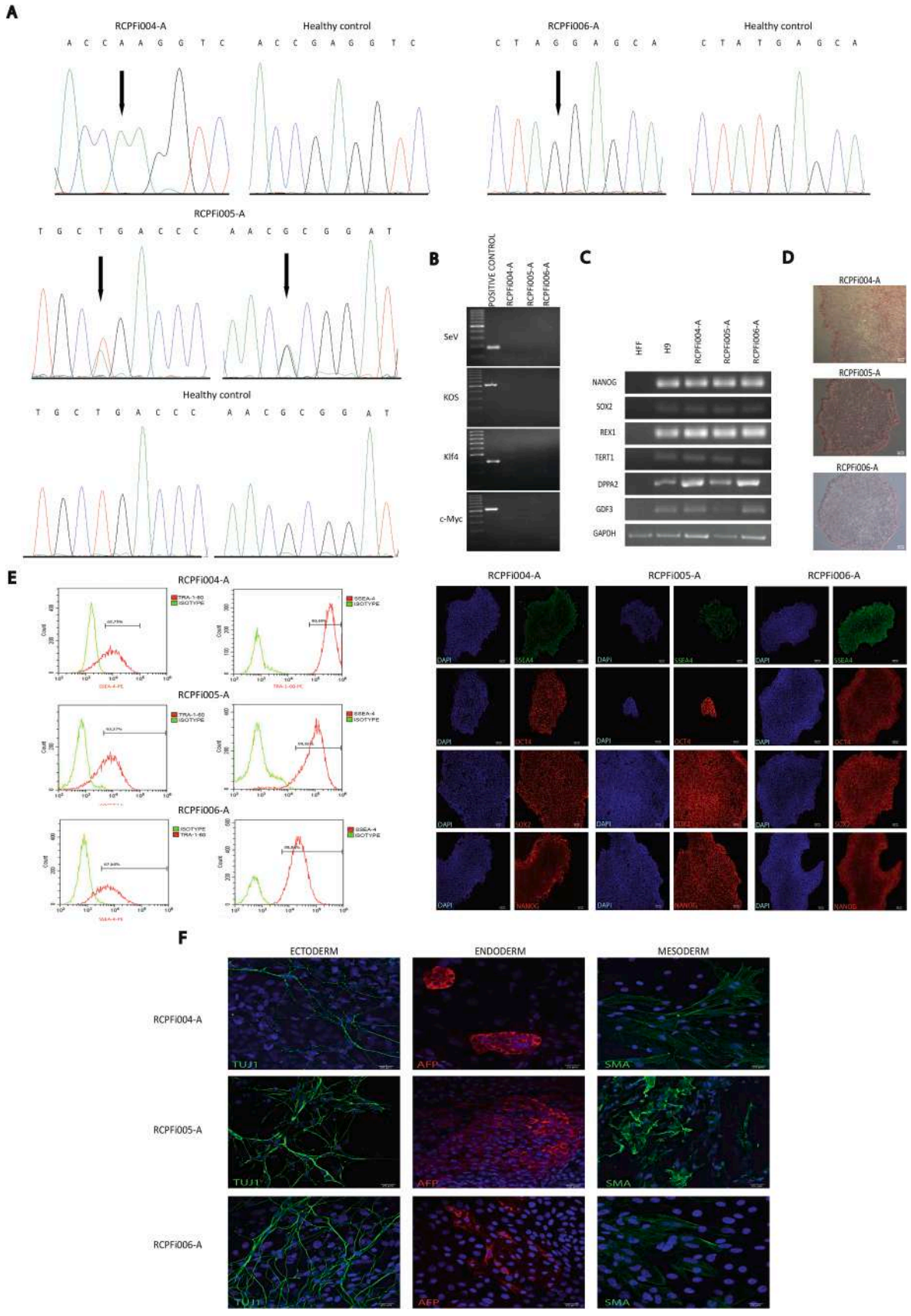

Fig. 1. Characterization of three hiPSC lines from PLAN/INAD patients.

Table 2
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 E
	Cytometry	SSEA4 99%	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	hiPSC-MD11: 46XX hiPSC-MD460 & hiPSC-MD253: 46XY Resolution 450–500	Supplementary Fig. 1 panel A
Identity	STR analysis	10 STRs analyzed	Supplementary Fig. 1
Mutation analysis (IF APPLICABLE)	Sequencing	hiPSC-MD11: compound heterozygous hiPSC-MD460 & hiPSC-MD253: homozygous	Fig. 1 panel A
	Southern Blot OR WGS	No	
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Negative	Supplementary Fig. 1B
Differentiation potential	Embryoid body formation	Positive TUJ1 ectodermal staining, positive SMA mesodermal staining and positive FOXA2 endodermal staining.	Fig. 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3
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# 4903 RRID:AB_10559205
Pluripotency Markers	Rabbit anti-Oct4	1:400	Cell Signaling Technology Cat# 2840 RRID:AB_2167691
Pluripotency Markers	Rabbit anti-Sox2	1:400	Cell Signaling Technology Cat# 3579 RRID:AB_2195767
Pluripotency Markers	Rabbit anti-SSEA4	1:100	BD Biosciences Cat# 560,073 RRID:AB_1645601
Pluripotency Markers	Mouse anti human SSEA4-PE	1:400	STEMCELL Technologies Cat# 60062PE.1 RRID:AB_2721031
Pluripotency Markers	Mouse anti-Human TRA-1–60	1:400	STEMCELL Technologies Cat# 60064PE.1 RRID:AB_2686905
Differentiation Markers	Mouse anti-SMA	1:200	Sigma-Aldrich Cat# A5228 RRID:AB_262054
Differentiation Markers	Rabbit anti-α-fetoprotein (AFP)	1:100	Agilent Cat# A0008 RRID:AB_2650473
Differentiation Markers	Mouse anti-BTubulin (TUJ1)	1:500	Neuromics Cat# MO15013 RRID:AB_2737114
Secondary antibody	Goat anti-mouse IgG	1:500	Thermo Fisher Scientific Cat# A-11001 RRID:AB_2534069
Secondary antibody	Goat anti-rabbit IgG	1:500	ThermoFisher Cat# A11012 RRID:AB_2534079
Isotype control	Mouse IgG3 kappa-PE	1:800	STEMCELL Technologies Cat# 60073PE RRID:AB_2722595
Isotype control	Mouse IgM kappa-PE	1:40	BD Biosciences Cat# 555,584 RRID:AB_395960
Primers			
	Target	Forward primer (5′-3′) Reverse primer (5′-3′)	
Pluripotency Markers (qPCR)	OCT4	AAGCCCTCATTTCACCAGG CTTGGAAGCTTAGCCAGGTC	
Pluripotency Markers (qPCR)	NANOG	CCAAATTCTCCTGCCAGTGAC CACGTGGTTTCCAAACAAGAAA	
Pluripotency Markers (qPCR)	SOX2	TCACATGTCCCAGCACTACC CCCATTTCCCTCGTTTTTCT	
Pluripotency Markers (qPCR)	TERT	TGGCTGCGTGGTGAACTTG GCGGTTGAAGGTGAGACTGG	
Pluripotency Markers (qPCR)	REX1	CAGATCCTAAACAGCTCGCAGAAT GCGTACGCAAATTAAAGTCCAGA	
Pluripotency Markers (qPCR)	GDF3	CTTATGCTACGTAAAGGAGCTGGG GTGCCAACCCAGGTCCCGGAAGTT	
Pluripotency Markers (qPCR)	DPPA2	CCGTCCCCGCAATCTCCTTCCATC ATGATGCCAACATGGCTCCCGGTG	
House-keeping gene (qPCR)	GAPDH	ATCGTGGAAGGACTCATGACCACA CCCTGTTGCTGTAGCCAAATTCGT	
Sendai virus detection	SeV	GGATCACTAGGTGATATCGAGC ACCAGACAAGAGTTTAAGAGATATGTATC	

(*continued on next page*)

Table 3 (*continued*)

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Transgenes detection	KOS	ATGCACCGCTACGACGTGAGCGC ACCTTGACAATCCTGATGTGG	
Transgenes detection	c-Myc	TAACTGACTAGCAGGCTTGTCG TCCACATACAGTCCTGGATGATGATG	
Transgenes detection	Klf4	TTCCTGCATGCCAGAGGAGCCC AATGTATCGAAGGTGCTCAA	

5′-ATCCCAGTACCTGTAGGCCT-3′ and 5′-AGCAGCTGACGATAGGAGG-3′; to detect c.2356G>A and c.2370T>G, both on exon 16, the used primers were: 5′-GGCACTGCTGAGACCTCT-3′ and 5′-GCCTGGTCTA TGGACTCAGA-3′.

3.5. *In vitro differentiation assay*

For *in vitro* differentiation assay the colonies from a fully confluent 6-well plate were detached using Gibco® Versene Solution (GIBCO, #15040066) and cultured in suspension on 6-well Clear Flat Bottom Ultra-Low Attachment (Corning, #3471) to form embryoid bodies in hiPSCs media without bGFG and 10% KSR. After 7 days in suspension, embryoid bodies were transferred into 0.1% gelatin-coated plates and cultured for additional 15 days to allow spontaneous differentiation. Then, the cells were fixed and immunostained to detect cells from the three germ layers.

3.6. *Detection of pluripotency markers by RT-PCR*

Total RNA was isolated with the RNeasy Mini Kit (Qiagen), 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 manufactureŕs instructions. The PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH). The expression level of pluripotency markers was analyzed using the primers described in Table 3. Fibroblasts and hESC H9 (WiCell) were used as negative and positive control, respectively.

3.7. *Immunocytochemistry, flow cytometry, alkaline phosphatase staining and mycoplasma detection*

To characterize and validate the generated hiPSCs, the immunocytochemistry, the flow cytometry studies, the alkaline phosphatase staining and the detection of mycoplasma were performed as previously described (Arellano et al., 2018).

3.8. *hiPSC nomenclature*

The generated hiPSC line was named following Spanish National Stem Cell Bank recommendations. The lines are registered on https://hpscreg.eu/ as RCPFi004-A, RCPFi005-A, RCPFi006-A.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102338.

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