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Lab Resource: Multiple Cell Lines

# A collection of three integration-free iPSCs derived from old male and female healthy subjects

Eva Rodríguez-Traver[a,b,1], Eva Díaz-Guerra[a,b,1], César Rodríguez[c], Fabián Arenas[b,d], María Orera[c], Jaime Kulisevsky[b,d], Rosario Moratalla[a,b], Carlos Vicario[a,b,*]

[a] Instituto Cajal-Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain
[b] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain
[c] Servicio de Bioquímica Clínica, Hospital General Universitario Gregorio Marañón, Madrid, Spain
[d] Movement Disorders Unit, Neurology Dept., Hospital Sant Pau, Barcelona, Spain

ABSTRACT

Here, we present the characterization of three iPSC lines derived from dermal fibroblasts of old healthy subjects. Fibroblasts were reprogrammed using Sendai viral vectors encoding OCT4, SOX2, KLF4 and c-MYC. The iPSCs expressed endogenous pluripotency markers, could generate the three germ layers (ectoderm, mesoderm and endoderm), maintained a stable karyotype, and were free from Sendai vectors and reprogramming factors. These integration-free iPSCs can serve for establishing control cell cultures in studies searching for phenotypes and mechanisms that could potentially be dysregulated in degenerative diseases.

## 1. Resource table

| | |
|---|---|
| Unique stem cell lines identifier | ICCSICi012-A<br>ICCSICi013-A<br>ICCSICi014-A |
| Alternative names of stem cell lines | ICCSICi012-A: IC-Ctrl1-F-iPS-4F-1<br>ICCSICi013-A: IC-Ctrl2-F-iPS-4F-1<br>ICCSICi014-A: IC-Ctrl3-F-iPS-4F-1 |
| Institution | Instituto Cajal-CSIC and CIBERNED (Madrid, Spain) |
| Contact information of distributor | Carlos Vicario, cvicario@cajal.csic.es |
| Type of cell lines | iPSC |
| Origin | Human |
| Cell Source | Dermal fibroblasts |
| Clonality | Clonal |
| Method of reprogramming | Non-integrative (Sendai viral vectors expressing OCT4, KLF4, SOX2 and c-MYC) |
| Multiline rationale | Healthy subjects, non-isogenic cell lines |
| Gene 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 | ICCSICi012-A: 23/08/2018<br>ICCSICi013-A: 20/03/2018<br>ICCSICi014-A: 20/03/2018 |
| Cell line repository/bank | IC-Ctrl1-F-iPS-4F-1<br>IC-Ctrl2-F-iPS-4F-1<br>IC-Ctrl3-F-iPS-4F-1<br>Banco Nacional de Líneas Celulares (BNLC, Spanish Stem Cell 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 | The study was approved by the Commission of Guarantees for the Donation and Utilization of Human Cells and Tissues, Instituto de Salud Carlos III (ISCIII; approval numbers 3,212,701 and 4,103,331). Informed consents were obtained from the subjects. |

## 2. Resource utility

Studying neurodegeneration has been difficult due to the scarcity of human cellular models. Our three iPSC lines are valuable tools for obtaining differentiated cells from healthy subjects. They can be used as controls for other disease lines derived from geriatric patients.

* Corresponding author at: Instituto Cajal-Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.
*E-mail address:* cvicario@cajal.csic.es (C. Vicario).

[1] E.R-T and E.D-G contributed equally to this study.

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**Table 1**
Summary of lines.

| iPSC line names | Abbreviation in figures | Gender | Age | Ethnicity | Genotype of locus | Disease |
|---|---|---|---|---|---|---|
| ICCSICi012-A (IC-Ctrl1-F-iPS-4F-1) | IC12-HS | Male | 85 | Caucasian | N/A | N/A |
| ICCSICi013-A (IC-Ctrl2-F-iPS-4F-1) | IC13-HS | Female | 66 | Caucasian | N/A | N/A |
| ICCSICi014-A (IC-Ctrl3-F-iPS-4F-1) | IC14-HS | Male | 72 | Caucasian | N/A | N/A |

## 3. Resource details

Human iPSC technology has brought new approaches for disease modeling, drug discovery and cell therapy development. Advantages of using human iPSCs in disease modeling include their easy accessibility and their ability to give rise to almost any desired cell type such as neurons and glia cells for neurodegeneration studies (Brennand et al., 2015; Shi et al., 2017). In this work, we obtained and characterized three iPSC lines derived from dermal fibroblasts of old healthy subjects using a non-integrative reprogramming method (Sendai viral vectors encoding OCT4, SOX2, KLF4 and c-MYC transcription factors). Three weeks after reprogramming, primary colonies were picked and expanded, and the iPSC clones ICCSICi012-A, ICCSICi013-A and ICCSICi014-A were chosen for complete characterization (Tables 1 and 2). The normal iPSC morphology (flat, round or polygonal with defined borders) in these iPSCs was confirmed by light microscopy observations (Fig. 1A). These clonal lines expressed NANOG, TRA-1-60, TRA-1-81 and SSEA-3 human pluripotency markers (Table 3), as demonstrated by immunofluorescence (Fig. 1B–E) and OCT4, SOX2 and NANOG by RT-qPCR (Fig. 1F), whereas fibroblasts showed no expression of these transcripts (Supplementary Fig. 1A). The iPSCs presented normal karyotypes (46, XY and 46, XX) at passages 14–19 (Fig. 1G). To confirm the pluripotency of these iPSCs, the clone lines were differentiated into the three germ layers (ectoderm, mesoderm or endoderm) using three distinct protocols. Immunocytochemistry with specific antibodies (Table 3), revealed the expression of PAX6 and ß-III-TUBULIN (ectoderm), DESMIN (mesoderm) as well as AFP (endoderm; Fig. 1H). In addition, the iPSC lines were integration-free as confirmed by the absence of the SeV genome and transgenes (tested at passages 5–10 by RT-PCR; Fig. 1I and Supplementary Fig. 1B). Furthermore, the iPSC lines were free from mycoplasma (Supplementary Fig. 1C). The analysis of nine short tandem repeat (STR) loci plus the Amelogenin (AMEL) locus for gender determination, showed that the parental fibroblasts and the iPSC lines shared alleles with a 100% match (available with the authors).

## 4. Materials and methods

### 4.1. Reprogramming of dermal fibroblasts

Fibroblasts were isolated from skin biopsies which were taken from the ventral side of the forearm of healthy subjects. Firstly, fat was removed from the biopsy and the tissue was incubated with 0.25% Typsin (Labclinics) and 2.4 mM EDTA (Sigma-Aldrich) in Hanks' Balanced Salt Solution (Thermo Fisher) at 37 °C for 1–2 h. The dermis was isolated and cut into small pieces that were incubated with 2.75 mg/mL Collagenase, 1.25 mg/mL Hyaluronidase (all Sigma-Aldrich), 0.1 mg/mL DNAse I (Labclinics) in RPMI supplemented with 0.25 µg/mL Amphotericin, 50 U/mL Penicillin and 50 µl/mL Streptomycin B (all Thermo Fisher) at 37 °C for 2–3 h. Next, the tissue was mechanically dissociated, centrifuged at 400x g, 10 min and the cells were counted and plated onto 0.1% (w/v) gelatin-coated (Sigma-Aldrich) dishes at 10,000 cells/cm$^2$ in fibroblast medium consisting of DMEM with 110 mg/L pyruvate and high glucose (4,5 g/L) supplemented with 100 µM non-essential amino acids (NEAA), 100 U/mL Penicillin and 100 µl/mL Streptomycin B and 10% (v/v) fetal bovine serum (all Thermo Fisher). Finally, fibroblasts were expanded and two days before reprogramming, they were plated at 10,000 cells/cm$^2$, transduced on day 2 using the CytoTune™-iPS Sendai Reprogramming Kit (Thermo Fisher) and incubated (37 °C, 5% $CO_2$). Seven days later, transduced human fibroblasts were seeded on mitotically inactivated mouse embryonic fibroblasts (MEFs) in hiPSC medium (KnockOut™ DMEM/F12, 100 µM NEAA, 2 mM glutaMAX, 55 µM ß-mercaptoethanol, 100 U/mL Penicillin, 100 µL/mL Streptomycin B, 20% KnockOut serum replacement; Thermo Fisher) supplemented with 20 ng/mL FGF-2 (Peprotech), and incubated (37 °C, 5% $CO_2$). Three-four weeks after, iPSC colonies were mechanically sliced into several fragments of cell clumps, seeded onto mitotically inactivated MEFs and expanded every week in hiPSC medium supplemented with 20 ng/mL FGF-2 and incubated as above. Ten colonies per well of multiple well plate, size 6 wells, were passaged.

**Table 2**
Characterization and validation.

| Classification | Test | Result | Data |
|---|---|---|---|
| Morphology | Photography | Normal morphology | Fig. 1A |
| Phenotype | Qualitative analysis Immunocytochemistry | Positive for NANOG, TRA-1-60, TRA-1-81 and SSEA-3 pluripotency markers | Fig. 1B–E |
| | Quantitative analysis RT-qPCR | Positive for OCT4, SOX2 and NANOG transcription factors | Fig. 1F and Supplementary Fig. 1A |
| Genotype | Karyotype (G-banding) and resolution | 46, XY and 46, XX Resolution 450–500 bands | Fig. 1G |
| Identity | Microsatellite PCR (mPCR) OR STR analysis | N/A | N/A |
| | | 10 sites tested, identity verified (100% match) | Available with the authors |
| Mutation analysis (IF APPLICABLE) | Sequencing | N/A | N/A |
| | Southern Blot OR WGS | N/A | N/A |
| Microbiology and virology | Mycoplasma | Mycoplasma testing by PCR: Negative | Supplementary Fig. 1C |
| Differentiation potential | Embryoid body formation followed by directed differentiation | Proof of three germ-layers formation by positive immunostaining for PAX6 and ß-III-TUBULIN (ectoderm), DESMIN (mesoderm) and AFP (endoderm) | Fig. 1H |
| 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 |

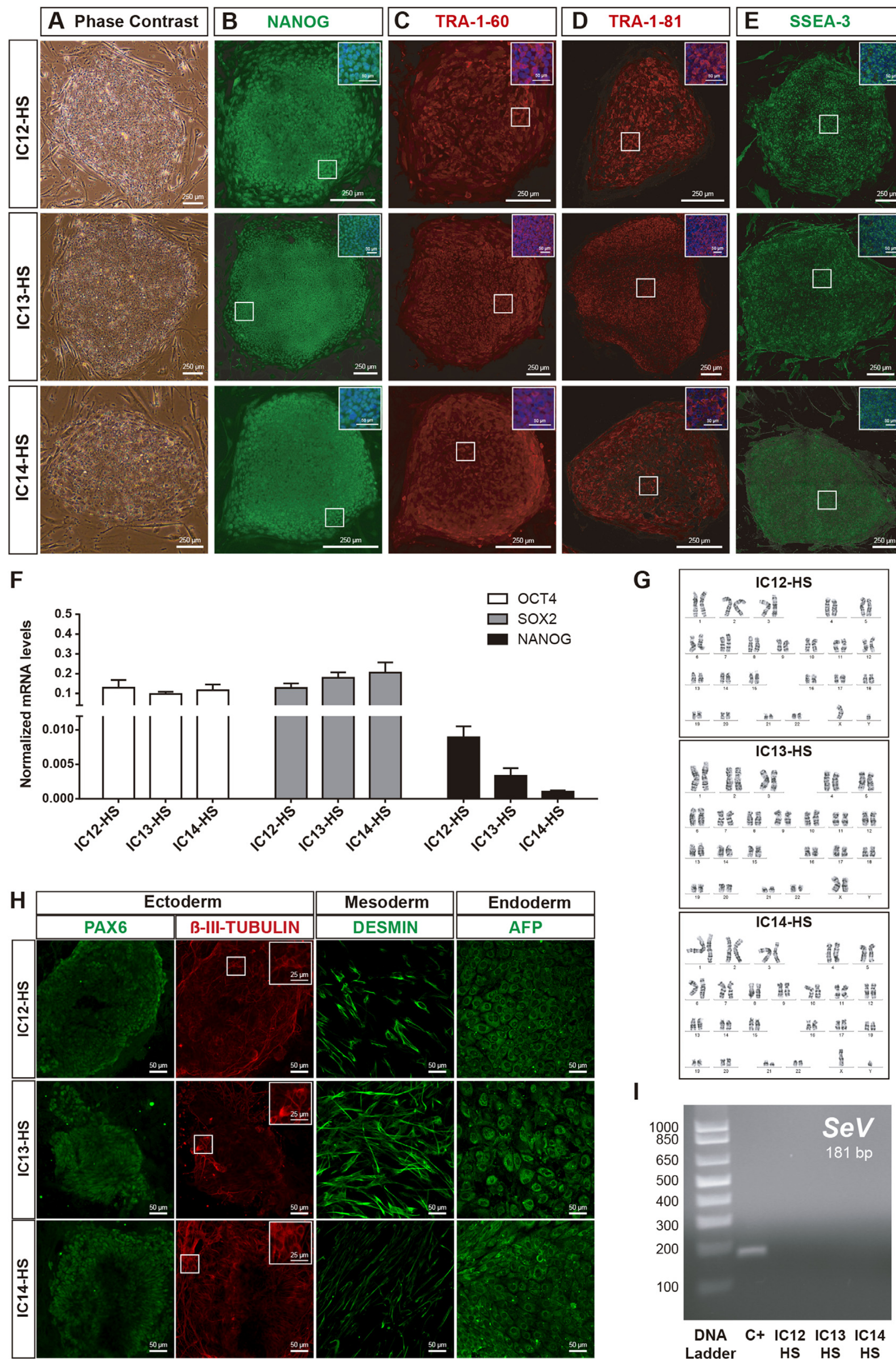


**Fig. 1.** Characterization of iPSCs derived from old male and female healthy subjects.

*4.2. Embryoid body (EB) formation and three-lineage differentiation*

iPSC colonies were mechanically divided into cell clumps and plated onto low attachment dishes in hiPSCs medium supplemented with 10 µM Rock inhibitor (Tocris) and without FGF-2. After 5 days, embryoid bodies were formed and plated onto 0.2% (w/v) gelatin-coated thermanox coverslips (Thermo Fisher) in the same medium without Rock inhibitor. Two-three days later, trilineage differentiation was

**Table 3**
Reagents details.

| Antibodies used for immunocytochemistry/flow-cytometry | | | |
|---|---|---|---|
| | Antibody | Dilution | Company Cat # and RRID |
| Pluripotency Markers | Rabbit anti-NANOG | 1:250 | Stemgent Cat#09-0020 RRID:AB_2298294 |
| | Rat anti-SSEA-3 | 1:100 | Stemgent Cat#09–0014 RRID:AB_1512168 |
| | Mouse anti-TRA-1-60 | 1:200 | Stemgent Cat#09-0010 RRID:AB_1512170 |
| | Mouse anti-TRA-1-81 | 1:200 | Stemgent Cat#09-0011 RRID:AB_1512171 |
| Differentiation Markers | Mouse anti-AFP | 1:100 | R&D Systems Cat#MAB1368 RRID:AB_357658 |
| | Rabbit anti-DESMIN | 1:250 | Lab Vision Cat#RB-9014-P0 RRID:AB_149768 |
| | Rabbit anti-PAX6 | 1:300 | Covance Cat#PRB-278P RRID:AB_291612 |
| | Mouse anti-β-III-TUBULIN | 1:1000 | Covance Cat#MMS-435P RRID:AB_2313773 |
| Secondary antibodies | Alexa Fluor 488 Goat Anti-Rabbit IgG | 1:750 | Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165 |
| | Alexa Fluor 488 Donkey Anti-Mouse IgG | 1:750 | Thermo Fisher Scientific Cat# A-21202, RRID:AB_141607 |
| | Alexa Fluor488 Goat Anti-Rat IgM | 1:500 | Thermo Fisher Scientific Cat# A-21212, RRID:AB_2535798 |
| | Alexa Fluor 594 Donkey Anti-Mouse IgG | 1:750 | Thermo Fisher Scientific Cat# A-21203, RRID:AB_2535789 |
| | Texas Red Goat Anti-Mouse IgM | 1:400 | Jackson ImmunoResearch Labs Cat# 115-076-075, RRID:AB_2338588 |
| Primers | | | |
| | Target | Forward/Reverse primer (5′ – 3′) | |
| Sendai Viral Vectors (PCR) | SeV, 181 bp | GGATCACTAGGTGATATCGAGC/ ACCAGACAAGAGTTTAAGAGATATGTATC | |
| | OCT4, 483 bp | CCCGAAAGAGAAAGCGAACCAG/ AATGTATCGAAGGTGCTCAA | |
| | KLF4, 410 bp | TTCCTGCATGCCAGAGGAGCCC/ AATGTATCGAAGGTGCTCAA | |
| | SOX2, 451 bp | ATGCACCGCTACGACGTGAGCGC/ AATGTATCGAAGGTGCTCAA | |
| | c-MYC, 532 bp | TAACTGACTAGCAGGCTTGTCG/ TCCACATACAGTCCTGGATGATGATG | |
| Pluripotency Markers (qPCR) | OCT4 | CCCCTGTCTCTGTCACCACT/ TGAATGAAGAACTTAATCCCAAA | |
| | SOX2 | ACACTGCCCCTCTCACACAT/ TCCCATTTCCCTCGTTTTT | |
| | NANOG | TCTGCTGAGATGCCTCACACGG/ CACACAGCTGGGTGGAAGAGAAC | |
| House-Keeping Genes (qPCR) | GADPH | AACCATGAGAAGTATGACAACAGCC/ TGAGTCCTTCCACGATACCAAAGT | |

started. Noggin (250 ng/mL; Peprotech) and A83 (5 µM; Miltenyi-Biotec) were used to induce ectodermal differentiation. On day 11, the hiPSC medium was changed to Neurobasal medium (Thermo Fisher) with 2% B27 (Thermo Fisher), 2 mM Glutamax, 0.02 mM ascorbic acid (Sigma-Aldrich), 0.05 mM cAMP (Sigma-Aldrich), 20 ng/mL BDNF (Peprotech), 20 ng/mL GDNF (Peprotech) and 1 ng/mL TGFß-3 (Peprotech). For mesodermal differentiation, the hiPSC was supplemented with 0.01 mM ascorbic acid. For endodermal differentiation, no growth factors or small molecules were added. All cultures were fixed on days 16–17.

### 4.3. Immunocytochemistry

Immunostaining was carried out following our standard procedures (Nieto-Estevez et al., 2016). The primary and secondary antibodies used are listed in Table 3. Images of the cells were taken on a Leica SP-5 confocal microscope.

### 4.4. RT-qPCR

Total RNA was extracted from the fibroblasts and iPSCs using RNeasy Mini Kit (Qiagen) to perform RT-qPCR following our standard protocols (Nieto-Estevez et al., 2016) using specific primers (Table 3). The gene expression level analysis was conducted in triplicate and normalized *versus GAPDH*.

### 4.5. Sendai virus and transgene detection

To detect the SeV genome and transgenes, 500 ng of total RNA were converted to cDNA using SuperScript III (Thermo Fisher) and oligo(dT) primers according to the manufacturer's information. The PCR reactions were performed using Taq DNA polymerase (Applied Biosystem) following the manufacturer's protocol in a thermocycler (Techne, TC-312). Different amplification conditions were applied for different primers: 1 min 45 s at 95 °C, 45 s at 95 °C, 45 s at 55–60 °C, 1 min at 72 °C (35 cycles), 5 min at 72 °C (primers listed in Table 3).

### 4.6. Karyotyping

Colcemid (10 µg/mL; Irvine-Scientific; 2 h at 37 °C) was used to stop iPSC mitosis at metaphase. Then, iPSCs were incubated with Trypsin-EDTA (Euroclone) 10 min at 37 °C. Hypotonic treatment was achieved with 0.075 M KCl (30 min at 37 °C) and cells were fixed with methanol / acetic acid (3:1). Cells were dropped on a microscope slide and metaphases were G-banded with Wright stain. At least 10–20 metaphases were counted and structurally evaluated (sensitivity of 450–500 bands per haploid number).

### 4.7. Mycoplasma detection

The absence of mycoplasma in the culture medium was analyzed by PCR using Mycoplasma Detection Kit (SoutherBiotech).

### 4.8. Short tandem repeat (STR) analysis

DNAs of the parental fibroblasts and iPSC lines were extracted using Puregene Core kit B (Qiagen) and analyzed at the Genomics Unit of CNIO-ISCIII (Spain). Nine STR loci plus the AMEL locus were PCR

amplified (GenePrint 10 kit; Promega). Each DNA was subsequently analyzed using an ABI Prism 3730xl Genetic Analyzer and the Osiris v2.6 software (NIH).

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Acknowledgments

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101663.

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