Lab Resource: Stem Cell Line

Generation of iPSC line iPSC-FH2.1 in hypoxic conditions from human foreskin fibroblasts

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ABSTRACT

Human foreskin fibroblasts were used to generate the iPSC line iPSC-FH2.1 using the EF1a-hSTEMCCA-loxP vector expressing OCT4, SOX2, c-MYC and KLF4, in 5% O_2 culture conditions. Stemness was confirmed, as was pluripotency both *in vivo* and *in vitro*, in normoxia and hypoxia. Human Embryonic Stem Cell (hESC) line WA-09 and reprogrammed fibroblast primary culture HFF-FM were used as controls.

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Resource table

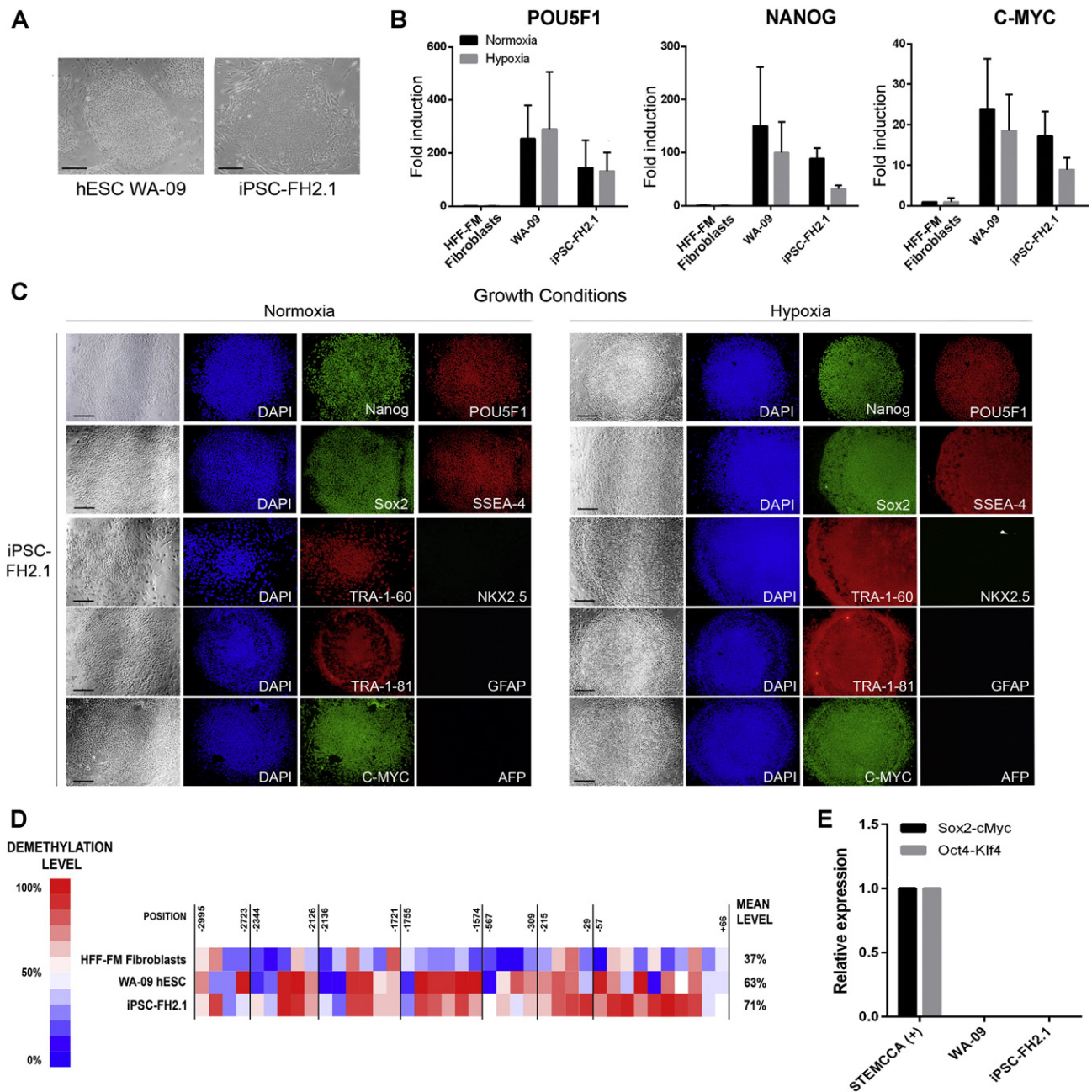|	
Name of stem cell line	iPSC-FH2.1
Institution	LIAN-CONICET, FLENI
	Laboratorio de Regulación de la Expresión Génica en Células Madre, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires
Person who created resource	María Questa
Contact person and email	mariaquesta@gmail.com
Date archived/stock date	2010
Origin	Human foreskin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); derived from human healthy fibroblasts, in 5% O_2
Sub-type	Cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed (Figs. 1 and 2)
Link to related literature	None
Information in public databases	No
Ethics	Patient informed consent obtained Ethics Review Board approval obtained

Resource details

We obtained fibroblasts from a healthy donor (primary culture HFF-FM) and reprogrammed them using the STEMCCA vector to generate the iPSC-FH2.1 line, in hypoxic conditions. Control lines iPSC-FN2.1 and iPS6.1 were generated in parallel, in normoxia, showing the same results as iPSC-FH2.1, regarding their identity and purity. The iPSC-FH2.1 line exhibited classic pluripotent stem cell morphology: small cells with low nucleus:cytoplasm ratio, organized in colonies with defined borders that grew as three-dimensional structures (Fig. 1A). As assessed by RT-qPCR, the expression of stemness markers POU5F1, NANOG and C-MYC was induced when cells were reprogrammed, compared to donor fibroblasts (Fig. 1B). The expression of several human stemness markers, including NANOG, POU5F1, SOX2, SSEA-4, TRA1–60, TRA-1–81 and C-MYC, was also studied by immunostaining (Fig. 1C), exhibiting clear expression of these markers when cultured in normoxic and hypoxic conditions.

To assess if reprogramming had any effect on promoter DNA modifications, we studied the methylation of the POU5F1 promoter (Fig. 1D). We found that on a global level the POU5F1 promoter DNA region is demethylated on several CpG islands in pluripotent cells; whereas in fibroblasts that originated them the promoter is widely methylated. This supports the occurrence of a profound remodeling of gene expression.

The transgene inserted by the STEMCCA lentiviral vector was reported to become silenced after the reprogramming event (Sommer et al., 2009). In order to study the endogenous expression of the endogenour genes (as in Fig. 1A), we needed to be sure that the expression of the exogenous genes carried in the vector had been silenced and we could,

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Fig. 1. iPSC-FH2.1 iPSC line's stemness. Description of data: (A) Phase contrast image of representative colonies cultured in normoxia. Scale bars: 200 μm. (B) RT-qPCR analysis of expression of stemness markers *POU5F1*, *NANOG* and *C-MYC*, in cells cultured either in normoxia or hypoxia for 72 h. Fold-induction *vs.* control is shown in bars representing the mean ± SEM of three independent experiments. Inductions marked with * are significant with respect to HFF-FM Fibroblasts in the same O_2 condition, with $p < 0.05$. (C) Representative phase contrast and immunostaining images for stemness markers, in normoxia and chronic hypoxia. Nuclei were stained with DAPI. Scale bars: 200 μm. (D) Demethylation levels of the *POU5F1* promoter. Each square represents a CpG and each section delimited by a black line represents an amplicon of PCR. The numbers indicate the base positions relative to the transcription start site. (E) RT-qPCR analysis of the expression of the transgenes carried by the lentiviral reprogramming vector. STEMCCA is the vector itself used as a positive control. Fold-induction *vs.* control is shown in bars representing the mean ± SEM of three independent experiments. Repression for hESC and iPSC line is significant with $p < 0.05$.

therefore, measure only endogenous expression from cDNA (Fig. 1E). There is no detectable expression of the transgenes, as studied by RT-qPCR on established iPSC lines, using primers specific for the exogenous expression of the lentiviral genes.

Stemness markers that are induced during reprogramming are down-regulated during differentiation. The stem cell line was induced to undergo differentiation in normoxia and *POU5F1*, *SOX2* and *NANOG* expression decreased between day 0 (undifferentiated state) and day 21 of the differentiation process (Fig. 2A). Moreover, germ layer lineage marker expression was induced during the differentiation when cells were cultured both in normoxia or hypoxia for a long-term period (Fig. 2B). Germ layer markers were all induced from a basal expression at day 0 to an increased expression at day 7 or day 21, depending on the kinetics of each gene. All the cell lines were able to differentiate *in vitro* to the three germ layers. The presence of differentiation marker proteins was also observed by immunostaining (Fig. 2C), both when grown in normoxia or hypoxia. The iPSC lines established also showed pluripotency *in vivo*, being able to generate teratomas in immunosuppressed mice (Fig. 2D). The teratomas generated had cells of the three germ layers, which could be assigned to different tissues and structures. Finally, we found that all the cell lines generated and studied possessed a normal human karyotype, and there were no significant genetic anomalies introduced by the reprogramming process (Fig. 2E).
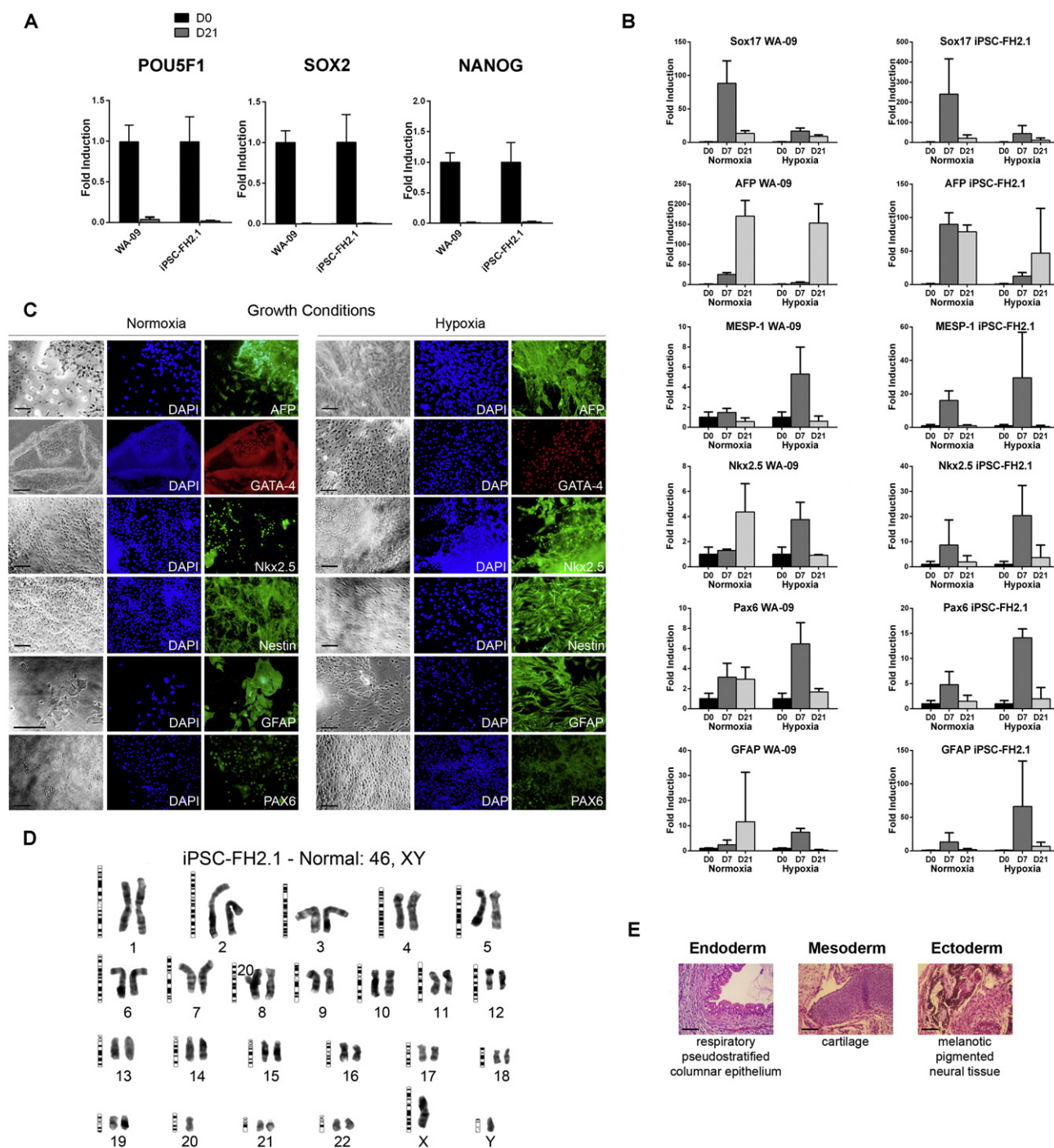
Fig. 2. iPSC-FH2.1 iPSC line's pluripotency and chromosomal integrity. (A) RT-qPCR analysis of stemness markers *POU5F1*, *SOX2* and *NANOG* expression during differentiation, in normoxia. D0: day 0 of differentiation (control), D21: D21 post-differentiation induction. Fold-induction *vs.* control is shown in bars representing the mean ± SEM of three independent experiments. Repression is significant with p < 0.05. (B) RT-qPCR analysis of the expression of endoderm markers *αFP* and *SOX17*, mesoderm markers *MESP-1* and *MYOD*, and ectoderm markers *NES* and *TUBB3*, in iPSC-FH2.1 cultured in normoxia or hypoxia. Representative images of 3 independent experiments are shown. D7: day 7. (C). Representative phase contrast and immunostaining images for endoderm markers AFP and GATA-4, mesoderm marker NKX2.5 and ectoderm marker NESTIN, GFAP and PAX6 in iPSC-FH2.1, cultured in normoxia or hypoxia. Nuclei were stained with DAPI. Scale bars: 200 μm. (D) Karyogram of iPSC-FH2.1 line in normoxia. Karyotype is a normal 46 chromosome human karyotype. (E) Representative brightfield microscopy images of hematoxylin–eosin stained teratoma sections generated in nude mice, showing the presence of tissues from the three germ layers. Scale bars: 200 μm.

Materials and methods

Cell culture

hESC line WA-09 was purchased from WiCell Research Institute. Cell lines were maintained and differentiated, according to (Romorini et al., 2012; Scassa et al., 2011; Sommer et al., 2009).

The HFF-FM fibroblast primary culture was established according to (Meng et al., 2008) from a sample of human foreskin, obtained after informed consent. The protocol was approved by the Ethics Committee of our institution.

Incubation was performed at 5% CO_2 levels, in humidified atmosphere at 37 °C. Normoxic cultures were performed at atmospheric O_2 levels. For hypoxia, cells were cultured at 5% O_2.

Lentiviral vector production and iPSC generation

The EF1a-hSTEMCCA-loxP (STEMCCA) lentiviral cassette and helper plasmids were generously provided by Dr. Gustavo Mostoslavsky and lentiviral vectors were produced as previously described (Sommer et al., 2009), with minor modifications. Briefly, the five-plasmid transfection system was introduced into HEK-293 T cells using the FuGENE6 transfection reagent (Roche, 1814443), according to manufacturer's recommendations. Optimal transfection efficiency was obtained using a FuGENE6:DNA ratio of 6:1. Lentiviral vector-containing supernatants were collected 48, 64, and 80 h post-transfection, filtered through a 0.45 μm low-binding filter and concentrated in an Amicon Ultra 15 100.000 NMWL column (EMD Millipore, UFC910024), according to manufacturer's instructions. Concentrate was aliquoted, stored in liquid nitrogen, and used within 12 months. Transduction efficiency of the lentiviral concentrate was assessed on the fibroblast line HFF-FM using concentrate defined as: Lentiviral Particles/μl = Number of Oct4 + DAPI + nuclei/μl of concentrate used for assay.

RT-Quantitative real time PCR (RT-qPCR) and endpoint PCR

RNA isolation and cDNA synthesis were performed as previously described (Romorini et al., 2012). cDNA samples were diluted ten-fold. RT-qPCR was conducted using Sybr Green ER qPCR Super Mix (Invitrogen), following manufacturer's instructions, at an annealing temperature of 60 °C in a StepOne Plus Real Time PCR System (Applied Biosystems) with StepOne Software v2.1. A list of primers is shown in Supplementary Table S1. In all hypoxia experiments, housekeeping gene *HPRT1* was used. In all other cases, housekeeping gene *GAPDH* was used.

Endpoint PCR was performed as previously described (Romorini et al., 2012).

To assess expression of transgenes, RT-qPCR was performed on cDNA from iPSC mRNA or the STEMCCA DNA as a positive control, using primers that bridge over two of the four transgenes on the STEMCCA vector sequence. This design allows amplification of only cDNA generated from the exogenous mRNA expression of these genes (Sommer et al., 2009). A list of primers is shown in Supplementary Table S1.

Promoter methylation analysis was conducted as previously described (Luzzani et al., 2015).

Immunostaining

Immunostaining was conducted as previously described (Scassa et al., 2011) using primary antibodies: anti-OCT-3/4 (SC-5279), SSEA4 (SC-21704), AFP (SC-166325), TRA1–60 (SC-21705), TRA-1–81 (SC-21706), GATA-4 (SC-25310) (all from Santa Cruz Biotechnology–SCB–), NANOG (Cell Signaling, #4903), SOX2 (Cell Signaling, #3579), NESTIN (Millipore, AB5922), NKX2.5 (SCB, SC-14033), C-MYC (Cell Signaling, #5605), GFAP (Millipore, AB5804), PAX6 (Abcam, AB5790), according to manufacturer's instructions. Fluorescent secondary antibodies, Alexa Fluor (AF)-488-conjugated anti-mouse IgG (A11029), AF-488-conjugated anti-rabbit IgG (A11034), AF-568-conjugated anti-mouse IgG (A11031), and AF-568-conjugated anti-rabbit IgG (A11036) (all Molecular Probes/Invitrogen) were used according to manufacturer's instructions.

Teratoma formation and hematoxylin–eosin staining

Cells were grown to confluence and dissociated. 4×10^6 cells in 1/4 Matrigel dilution were injected subcutaneously in the dorsal flanks of 6–8 week-old male N:NIH(S)-*Fox1*[nu] nude mice. After tumor formation, animals were anesthetized in a CO_2 atmosphere chamber and sacrificed by cervical dislocation. Tumors were dissected, fixed, embedded in paraffin, hematoxylin–eosin (Biopur) stained, according to manufacturer's instructions, mounted on microscope slides and photographed under a Nikon Eclipse E400 microscope with a Nikon Coolpix-S4 digital camera.

Karyotyping

Cells were cultured to exponential growth rate, colchicine (Sigma-Aldrich) was added and cells incubated for 1.5–3 h. Cells were dissociated, KCl hypotonic solution was added, and the sample was incubated for 20 min. Cells were resuspended in glacial acetic acid/methanol 3:1 (Van Rossum, JT Baker), with a 24-hour incubation at 4 °C. Microscope slides were prepared and stained with Giemsa (Biopur) in Phosphate Buffer Solution pH = 7, rinsed, dried and mounted, observed under an Olympus BX60 microscope at 1000× magnification and photographed using an Olympus NO-C-5060 camera. 50 metaphases were analyzed for each cell line.

Statistical analysis

Results are shown as mean ± SEM. p Values below 0.05 were considered statistically significant. Student's t test was used to determine significant differences between means, except for the transgene expression study, where a One-Way ANOVA followed by Dunnett's multiple comparisons was used.

Ethics, consents and permissions

The FLENI Ethics Committee approved all animal and human procedures. All human samples were obtained after written informed consent. The work described was carried out in accordance with The Code of Ethics of the World Medical Association.

Animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.12.026.

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