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Lab Resource: Stem Cell Line

Establishment of induced pluripotent stem cell (iPSC) line from 55-year old male patient with hemorrhagic Moyamoya disease

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ABSTRACT

Peripheral blood mononuclear cells (PBMCs) were collected from 55-year old male patient with a confirmed diagnosis of hemorrhagic Moyamoya disease (MMD). PBMCs were reprogrammed using Sendai virus particles delivering the four Yamanaka factors. A footprint-free hiPSC line was characterized by the expression of pluripotency markers and a normal karyotype. These cells were able to give rise to Embryoid Bodies and to a progeny of differentiated cells belonging to the 3 germ layers. This hiPSC line represents a suitable tool for modelling *in vitro* MMD disease to investigate the cellular mechanisms underlying the occurrence of this pathology.

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Resource Table:

Name of Stem Cell line	MMD HEM#30 iPSC line
Institution	Department of Neuroscience and Surgery of the Nervous System, Papa Giovanni XXIII Hospital, Bergamo, Italy; Centre for Integrative Biology, Università degli Studi di Trento, Trento, Italy
Person who created resource	Luigi Andrea Lanterna, Luciano Conti
Contact person and email	Luigi Andrea Lanterna, l.lanterna@gmail.com; Luciano Conti, Luciano.Conti@unitn.it.
Date archived/stock date	October 2015
Origin	Peripheral blood mononuclear cells (PBMCs)
Type of resource	Biological reagent: induced pluripotent stem cell (iPSC); derived from a sporadic hemorrhagic Moyamoya disease (MMD) patient
Sub-type	Induced pluripotent stem cells (iPSCs)
Key transcription factors	hOCT4, hSOX2, hc-MYC, hKLF4 (CytoTune™-iPS 2.0 Sendai Reprogramming Kit - Thermo Fisher Scientific)
Authentication	Cell line was characterized for its homogeneity and identity by these assays: RT-PCR, WB and immunofluorescence to test the expression of pluripotency genes, RT-PCR to identify the expression of SeV specific genome, karyotype to confirm chromosome stability, embryoid body assay to evaluate the *in vitro* differentiation in the 3 germ-layer derivatives
Link to related literature	/
Information in public databases	/
Ethics	Patient informed consent obtained; Ethics Review Board-competent authority approval was obtained by the Papa Giovanni XXIII Hospital Ethical Board.

Resource details

Blood sample was donated by a 55-year old male patient affected by Moyamoya disease (MMD; Papa Giovanni XXIII Hospital, Bergamo, Italy). Patient was admitted to the emergency room for a large intracerebral hematoma of the left frontal lobe. The computed tomography angiography did not show any vascular malformation but, on the other side, disclosed a significant stenosis of the distal intracranial internal carotid artery on both sides suggestive of MMD. Postoperative digital subtraction angiography confirmed the diagnosis of MMD.

Patient's PBMCs were purified and infected with Sendai virus particles (Yang et al. 2008), delivering 3 different vectors encoding for the Yamanaka reprogramming genes (OCT4, SOX2, KLF4, and c-Myc; Takahashi et al., 2007). After two weeks, several colonies with the typical iPSC-like phenotype appeared in the cultures. These were then

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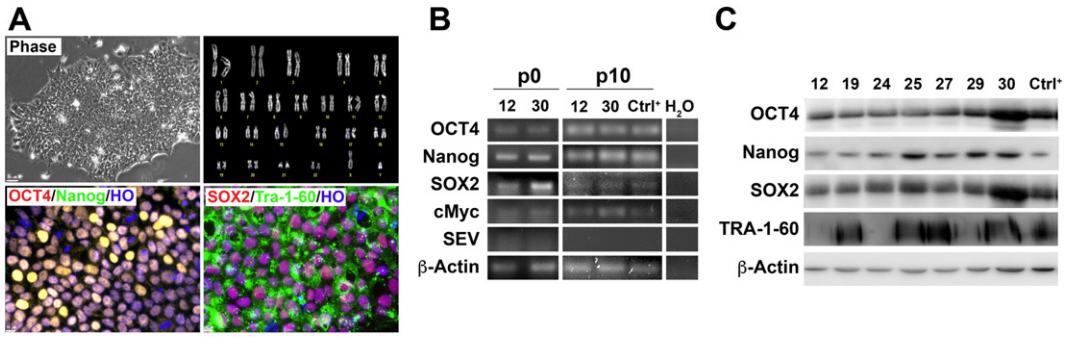

Fig. 1. Characterization of MMD HEM#30 iPSC line. A: Typical phenotype of a MMD HEM#30 iPSC colony and karyogram showing normal 46 chromosomes (XY). Immunophenotypical analysis revealing the homogeneous expression of the pluripotency markers OCT4, Nanog, TRA-1-60, SOX2 (40×) performed on passage 10 cultures. B: RT-PCR displaying the expression of the pluripotency-associated genes at passages 0 and 10 MMD HEM#30 iPSCs cultures and another MMD HEM hiPSC clone (#6). Loss of Sendai virus genome is shown in passage 10 cultures. Commercial hiPSCs were used as positive control for pluripotency-associated genes. C: Western blot analysis showing protein expression levels of pluripotency markers in MMD HEM#30 iPSCs and in several clones isolated from reprogrammed PBMCs by the same patient. Commercial hiPSCs were used as positive control.

picked about 20 days post-transduction. Different clones were isolated, expanded and their phenotype was monitored along the time. MMD HEM#30 iPSC line and other clones were further characterized by different assays. MMD HEM#30 iPSC was shown to be regularly expandable at purity, exhibited a normal diploid 46, XY karyotype, without detectable abnormalities, and the expression of OCT4, Nanog, SOX2 and TRA-1-60 pluripotency markers (Fig. 1A) (Itskovitz-Eldor et al., 2000; Carpenter et al., 2003). The lack of residual Sendai virus particles was confirmed at passage 10 (Fusaki et al., 2009), suggesting that the expression of OCT4, SOX2, KLF4, and c-Myc was indicative of an effective complete reprogramming process (Fig. 1B). The *in vitro* differentiation competence was assessed by Embryoid Body assay, giving rise to mature cells belonging to the three different germ layers, immunoreactive for ectoderm (βIII-Tubulin), mesoderm (α-SMA) and endoderm (TROMA-1) markers (Fig. 2A) (Itskovitz-Eldor et al., 2000; Carpenter et al., 2003). Moreover, differentiated MMD HEM#30 iPSCs expressed transcript levels of FGF5 (ectoderm), Nestin (neuro-ectoderm), T-Brachyury (mesoderm), SOX-17 (endoderm) comparable to those assessed in a differentiated reference commercial hiPSC line (Fig. 2B).

Materials and methods

PBMCs collection and freezing

Patient's PBMCs were isolated in BD Vacutainer CPT Cell Preparation tubes and separated by centrifugation (30 min, 1800*g* at room temperature). Then they were resuspended in 35 ml of PBS, centrifuged at 300 g for 15 min at room temperature and aliquots of $2 * 10^6$ cells were cryopreserved in fetal bovine serum (FBS) supplemented with 10% DMSO.

PBMCs thawing and infection with Sendai virus

Thawed cells were cultured in 24 well-plates in PBMC expansion medium, composed by StemPro-34 Serum Free Medium Basal Medium (Thermo Fisher Scientific), StemPro-34 Nutrient Supplement, 200 mM GlutaMAX (Thermo Fisher Scientific), 1% penicillin/streptomycin (Life Technologies), 100 ng/ml stem cell factor (SCF, Prepotech), 100 ng/ml FLT-3 (Thermo Fisher Scientific), 20 ng/ml Interleukin-6 (IL-6) (Thermo Fisher Scientific), 20 ng/ml Interleukin-3 (IL-3) (Thermo Fisher Scientific). The medium was replaced daily.

After 4 days, PBMCs were transduced in feeder-free conditions with the components provided with the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific), according the manufacturer's instructions. 20 days post-infection, optimal iPSC-like colonies were marked, picked and transferred in Essential 8 medium (E8 medium; Thermo Fisher Scientific) on Geltrex-coated wells. Both MMD HEM#30 iPSC line and commercial hiPSC line (GIBCO, Thermo Fisher Scientific) were maintained in E8 medium, performing daily medium change and regular splitting every 3–4 days with an EDTA-based dissociation solution.

In vitro differentiation

hiPSCs were collected and dissociated in cell clumps, plated in low attachment wells in 4 ml of Essential 8 medium supplemented with 2 mg/ml of PVA (polyvinyl alcohol, Santa Cruz Biotechnology) and

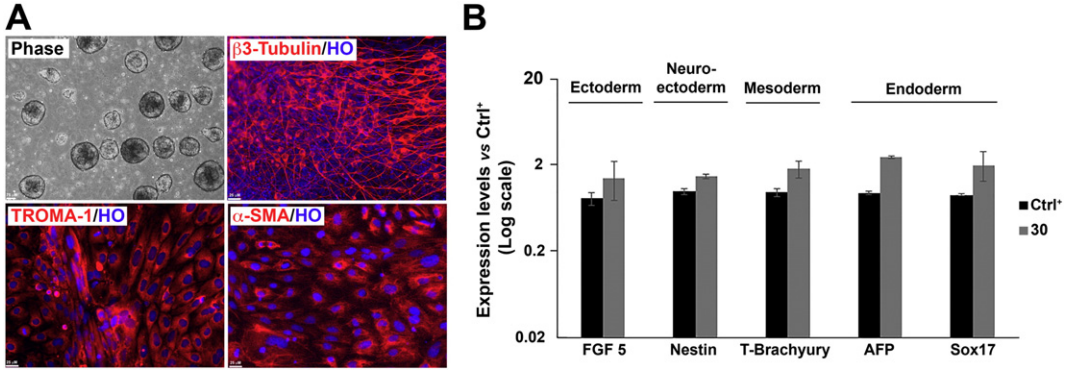

Fig. 2. In vitro differentiation competence of MMD HEM#30 iPSC line. A: Day 4 Embryoid bodies culture (5×). After 14 days, differentiated cultures exhibited the presence of cells immunopositive for ectodermal (β3-Tubulin), mesodermal (α-SMA) and endodermal (TROMA-1) (20×) germ-layers markers. B: FGF5 (ectoderm), Nestin (neuro-ectoderm), T-Brachyury (mesoderm) and SOX-17 (endoderm) expression levels assessed by qRT-PCR were comparable between 14 days differentiated EBs cultures obtained from MMD HEM#30 iPSCs and commercial hiPSCs used as reference.

Table 1
List of primer sequences, amplicon sizes and number of PCR cycles.

Gene	Primer sequence	Amplicon size	Cycles (PCR)
β actin	F:GACAGGATGCAGAAGGAGATTACTG R: CTCAGGAGGAGCAATGATCTTGAT	72 bp	25
OCT4	F: GGAAGGAATTGGGAACACAAAGG R: AACTTCACCTTCCCTCCAACCA	71 bp	30
SOX2	F: GCTACAGCATGATGCAGGACCA R: TCTGCGAGCTGGTCATGGAGTT	135 bp	30
c-Myc	F: CCTGGTGCTCCATGAGGAGAC R: CAGACTCTGACCTTTTGCCAGG	128 bp	30
KLF4	F: CATCTCAAGGCACACCTGCGAA R: TCGGTCGCATTTTTGGCACTGG	156 bp	30
Nanog	F: CCTGTGATTTGTGGGCCTG R: GACAGTCTCCGTGTGAGGCAT	78 bp	30
SeV	F:GGATCACTAGGTGATATCGAGC R: ACCAGACAAGAGTTTAAGAGATATGTATC	181 bp	35
Nestin	F: GGAGAAGGACCAAGAACTG R: ACCTCCTCTGTGGCATTC	153 bp	qRT-PCR
T-Brachyury	F: CCTTCAGCAAAGTCAAGCTCACC R: TGAACTGGGTCTCAGGGAAGCA	153 bp	qRT-PCR
FGF5	F: GGAATACGAGGAGTTTTCAGCAAC R: CTCCCTGAACTTGCAGTCATCTG	99 bp	qRT-PCR
AFP	F: GCAGAGGAGATGTGCTGGATTG R: CGTGGTCAGTTTGCAGCATTCTG	113 bp	qRT-PCR
β3-Tubulin	F: TCAGCGTCTACTACAACGAGGC R: GCCTGAAGAGATGTCCAAAGGC	120 bp	qRT-PCR
GAPDH	F: CCACTCCTCCACCTTTGAC R: ACCCTGTTGCTGTAGCCA	102 bp	qRT-PCR

10 μg/ml of y-27632 ROCK inhibitor (TebuBIO). After two days, cell aggregates were shifted to a 1:1 mix of Essential 6 medium (E6 medium; Thermo Fisher Scientific) and E8 medium supplemented with 2 mg/ml of PVA. From the following day until the end of the experiment, cells were maintained in E6 medium. At day 7, Embryoid Bodies (EBs) were collected and plated on Geltrex-coated wells, and let to differentiate for further 7 days. Medium was changed every other day.

RNA isolation, polymerase chain reaction (PCR) and quantitative-PCR (qPCR)

RNA was isolated with the TRIzol Reagent (Thermo Fisher Scientific) following the manufacture's protocol, then retro-transcribed with iScript cDNA Synthesis Kit (BioRad). cDNA was used to verify the expression of specific target genes by RT-PCR (Reverse Transcription-PCR, primers reported in Table 1) or qRT-PCR (quantitative RT-PCR), using the SsoAdvanced Universal SYBR Green Supermix Kit. qRT-PCR data were analyzed according to the comparative ΔΔCt method and normalized by using GAPDH housekeeping gene.

Immunofluorescence analysis

Cells were fixed in PFA 4% for 15 min at room temperature, permeabilized with Triton 0.3% for 10 min at room temperature, and incubated in blocking solution (FBS 5%, Triton 0.3% in PBS) for 1 h. Hybridization with the primary antibodies reported in the Table 2 was performed overnight at 4 °C in antibody solution (FBS 2% and Triton 0.2% in PBS). The signal was revealed by the appropriate secondary antibodies (Table 2), while nuclei were counterstained with Hoechst 33258 1 μg/ml (Thermo Fisher Scientific). Images were acquired with the microscope Leica DM IL Led Fluo and the camera Leica DFC450 C (Leica Microsystem).

Western blot assay

Cells were lysed in RIPA buffer and 20 μg of total lysate were fractionated on 8% SDS-polyacrylamide gel, blotted onto PVDF membranes with the TransBlot Turbo apparatus (BioRad) and hybridized overnight at 4 °C with the primary antibodies (Table 2). The signal was detected with appropriate HRP-conjugated secondary antibodies (BioRad) and ECL Clarity system (BioRad), using the chamber UVITECH Cambridge (Uvitech).

Karyotyping

Cell cultures were treated with 10 ng/ml colcemid (Gibco KaryoMAX Colcemid solution in PBS, Thermo Fisher Scientific) for 16 h (overnight) at 37 °C. Metaphases were collected according to standard protocols. Briefly, cells were incubated with hypotonic solution (0.075 M KCl for 15 min at room temperature) and fixed in acetic acid/methanol (1:3 *v*/*v*). Air-dried metaphase spreads slides were then analyzed by QFQ banding. Microscope observation was performed using a Nikon Eclipse 90i (Nikon Instruments) equipped with the acquisition and analysis Genikon software (Nikon Instruments).

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Table 2
List of the antibodies used in for immunocytochemistry (IC) and Western Blot (WB) assays, working dilutions and species in which they have been produced.

Antibody	Company	Dilution	Species
TRA 1–60	Santa Cruz Biotech	1:1000 (WB) 1:200 (IC)	Mouse IgM
Nanog	Santa Cruz Biotech	1:1000 (WB) 1:100 (IC)	Mouse
SOX2	Millipore	1:2000 (WB) 1:300 (IC)	Rabbit
OCT4	Santa Cruz Biotech	1:1000 (WB) 1:100 (IC)	Rabbit
α-SMA	Sigma	1:1000 (IC)	Mouse
TROMA-1	Iowa DHB	1:100 (IC)	Mouse
β3-Tubulin	Promega	1:1000 (IC)	Mouse
Anti-rabbit HRP	BioRad	1:3000	Goat
Anti-mouse HRP	BioRad	1:3000	Goat
Anti-mouse IgM FITC	Santa Cruz Biotech	1:200	Goat
Alexa Fluor IgG anti-rabbit 568	Life Technologies	1:300	Goat
Alexa Fluor IgG anti-rabbit 488	Life Technologies	1:300	Goat
Alexa Fluor IgG anti-mouse 568	Life Technologies	1:300	Goat
Alexa Fluor IgG anti-mouse 488	Life Technologies	1:300	Goat

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