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Lab resource: Stem cell line

# Establishment of induced pluripotent stem cell (iPSC) line from an 8-year old female patient with ischemic Moyamoya disease

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ABSTRACT

Peripheral blood mononuclear cells (PBMCs) were collected from an 8-year old female patient affected by ischemic Moyamoya disease (MMD). Patient's PBMCs were reprogrammed using Sendai virus particles delivering the four Yamanaka factors. The footprint free hiPSC line expressed the major pluripotency markers and exhibited a normal karyotype. Cells were competent to give rise to progeny of differentiated cells belonging to the 3 germ layers. This hiPSC line represents a good tool to *in vitro* model MMD in order to shed light on the cellular and molecular mechanisms responsible for the occurrence of this syndrome.



Resource table.

| | |
|---|---|
| Name of stem cell line | MMD ISCH#6 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 ischemic 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 | Identity and purity of the cell line was confirmed by the following tests: expression of pluripotency genes by RT-PCR, western blot and immunofluorescence, expression of SeV specific genome by RT-PCR, chromosome stability by karyotyping, *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 from the Papa Giovanni XXIII Hospital Ethical Board |

## 1. Resource details

Blood sample was collected from an 8-year old female patient with Moyamoya disease (MMD; Papa Giovanni XXIII Hospital, Bergamo, Italy). Patient presented a minor stroke and multiple transient ischemic attacks (TIA) characterized by transient motor deficits of the right hand. The MR showed an infarct in the fronto-parietal region on the left side and multiple small ischemic lesions in the deep and superficial watershed areas of both hemispheres. A computed tomography angiography demonstrated the complete occlusion of the internal carotid artery distal to the origin of the anterior choroidal artery on both sides with the compensatory hypertrophy of the basal lenticulostriate arteries, indicative for MMD. A postoperative digital subtraction angiography was consistent with the diagnosis of MMD.

Purified patient's PBMCs were transduced with Sendai virus particles (Yang et al., 2008–2012), delivering the four Yamanaka factors

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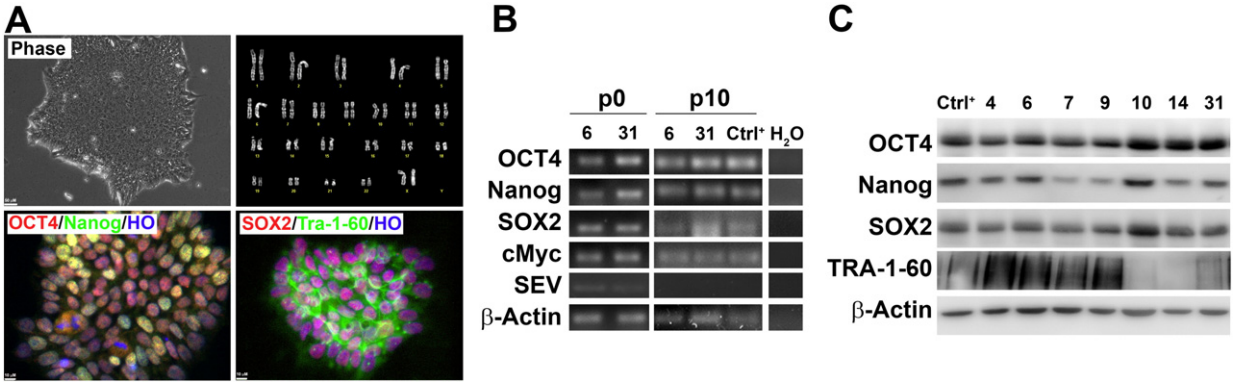


**Fig. 1.** Characterization of MMD ISCH#6 hiPSC line. A: Representative picture of a MMD ISCH-#6 hiPSC colony (5×) and its karyogram showing a normal diploid 46, XY karyotype with no evident cytogenetic aberrations. Immunophenotypical characterization showing the expression of the pluripotency markers OCT4, Nanog, TRA-1-60, SOX2 (40×). B: RT-PCR displaying the expression of the pluripotency-associated genes in MMD ISCH#6 iPSCs cultures and in another MMD ISCH hiPSC clone (#31) derived from the same patient at passages 0 and 10. Lack of the Sendai virus genome maintenance 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-associated markers in MMD ISCH#6 iPSCs and in other clones derived from the same patient.

(OCT4, SOX2, KLF4, and c-Myc; Takahashi et al., 2007). Colonies with typical iPSC-like morphology appeared 10–12 d after transduction and some of them were picked 16–18 d later. Clones were expanded and further characterized. One of the isolated clones gave rise to the MMD ISCH#6 iPSC line, characterized by homogeneous colonies, normal diploid 46, XX karyotype, without detectable abnormalities, and stable expression of the pluripotency markers OCT4, Nanog, SOX2 and TRA-1-60, verified by both immunofluorescence, RT-PCR and western blot (Fig. 1A–C) (Itskovitz-Eldor et al., 2000; Carpenter et al., 2003). Absence of residual episomal Sendai virus was confirmed at passage 10 (Fusaki et al., 2009), suggesting that the expression of OCT4, SOX2, KLF4, and c-Myc was indicative of a successful complete reprogramming process (Fig. 1B).

Differentiation potential of the MMD ISCH#6 iPSC line was assessed by Embryoid Body assay. Differentiated 14-day old cultures exhibited the presence of β3-Tubulin (ectoderm), α-SMA (mesoderm) and TROMA-1 (endoderm) immunopositive cells (Fig. 2A), thus confirming the pluripotent nature of the cells (Itskovitz-Eldor et al., 2000; Carpenter et al., 2003). The differentiation competency of this cell line was equivalent to that observed for a commercial hiPSC line, as shown by the comparable expression levels of the 3 germ layers markers FGF5 (ectoderm), Nestin (neuro-ectoderm), T-Brachyury (mesoderm), SOX-17 (endoderm) assessed by qRT-PCR (Fig. 2B).

## 2. Materials and methods

### 2.1. 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). They were collected in 35 mL of PBS, centrifuged at 300*g* for 15 min at room temperature. Stocks of $2 * 10^6$ cells were cryopreserved in fetal bovine serum (FBS) with 10% DMSO.

### 2.2. PBMCs thawing and reprogramming with Sendai virus particles

Cells were thawed in a well of a 24 well-plate and cultured in the 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 (Thermo Fisher Scientific), 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 for 3 d.

Four days later, viral particles provided with the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) were used to

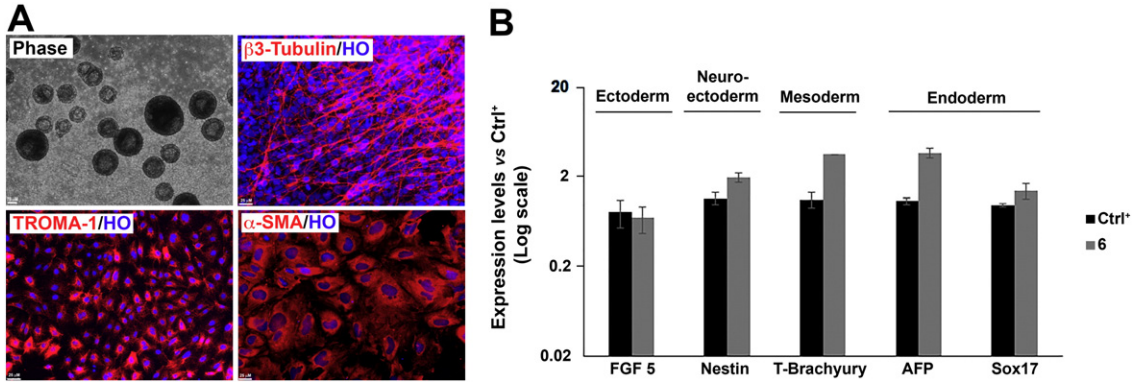


**Fig. 2.** *In vitro* differentiation of MMD ISCH#6 iPSC line. A: Picture of Embryoid Body after 4 d of suspension culture (5×). At D14 cultures expressed ectodermal (β3-Tubulin), mesodermal (α-SMA) and endodermal (TROMA-1) germ layer markers (20×). B: qRT-PCR showing a comparable expression levels of transcripts for the 3 germ-layers, FGF5 (ectoderm), Nestin (neuro-ectoderm), T-Brachyury (mesoderm) and SOX-17 (endoderm) between 14 d differentiated MMD ISCH#6 iPSCs and differentiated reference commercial hiPSCs.

**Table 1**
List of primers sequences, amplicons size and number of PCR cycles.

| Gene | Primers sequence | Amplicon size | Cycles (PCR) |
|---|---|---|---|
| β Actin | **Fw**: GACAGGATGCAGAAGGAGATTACTG; **Rev**: CTCAGGAGGAGCAATGATCTTGAT | 72 bp | 25 |
| OCT4 | **Fw**: GGAAGGAATTGGGAACACAAAGG; **Rev**: AACTTCACCTTCCCTCCAACCA | 71 bp | 30 |
| SOX2 | **Fw**: GCTACAGCATGATGCAGGACCA; **Rev**: TCTGCGAGCTGGTCATGGAGTT | 135 bp | 30 |
| c-Myc | **Fw**: CCTGGTGCTCCATGAGGAGAC; **Rev**: CAGACTCTGACCTTTTGCCAGG | 128 bp | 30 |
| KLF4 | **Fw**: CATCTCAAGGCACACCTGCGAA; **Rev**: TCGGTCGCATTTTTGGCACTGG | 156 bp | 30 |
| Nanog | **Fw**: CCTGTGATTTGTGGGCCTG; **Rev**: GACAGTCTCCGTGTGAGGCAT | 78 bp | 30 |
| SeV | **Fw**: GGATCACTAGGTGATATCGAGC; **Rev**: ACCAGACAAGAGTTTAAGAGATATGTATC | 181 bp | 40 |
| Nestin | **Fw**: GGAGAAGGACCAAGAACTG; **Rev**: ACCTCCTCTGTGGCATTC | 153 bp | qRT-PCR |
| T-Brachyury | **Fw**: CCTTCAGCAAAGTCAAGCTCACC; **Rev**: TGAACTGGGTCTCAGGGAAGCA | 153 bp | qRT-PCR |
| FGF5 | **Fw**: GGAATACGAGGAGTTTTCAGCAAC; **Rev**: CTCCCTGAACTTGCAGTCATCTG | 99 bp | qRT-PCR |
| AFP | **Fw**: GCAGAGGAGATGTGCTGGATTG; **Rev**: CGTGGTCAGTTTGCAGCATTCTG | 113 bp | qRT-PCR |
| β3-Tubulin | **Fw**: TCAGCGTCTACTACAACGAGGC; **Rev**: GCCTGAAGAGATGTCCAAAGGC | 120 bp | qRT-PCR |
| GAPDH | **Fw**: CCACTCCTCCACCTTTGAC; **Rev**: ACCCTGTTGCTGTAGCCA | 102 bp | qRT-PCR |

transduce Yamanaka's factors into the PBMCs in feeder-free conditions, following the manufacturer's instructions. 16–18 d post-infection, colonies with iPSC-like phenotype were identified, picked and transferred into Geltrex-coated wells and fed Essential 8 medium (E8 medium; Thermo Fisher Scientific), following the manufacture's instruction. Self-renewing MMD ISCH#6 iPSC line and commercial hiPSC line (GIBCO, Thermo Fisher Scientific) were routinely cultured in E8 medium, with daily medium change. Cells were passaged every 3–4 d with EDTA-based dissociation solution.

### 2.3. In vitro differentiation

hiPSCs were collected and dissociated in order to obtain cell clumps, then plated in low attachment wells in 2 mL of conditioned medium (E8 medium) and 2 mL of fresh E8 medium supplemented with 2 mg/mL of PVA (polyvinyl alcohol, Santa Cruz Biotechnology) and 10 μg/mL of Y-27632 ROCK inhibitor (Tebu-BIO). Two days after, cell clumps were resuspended in 4 mL of 1:1 mix composed of Essential 6 medium (E6 medium, Thermo Fisher Scientific) and E8 medium supplemented with 2 mg/mL of PVA. From the subsequent day until the end of the experiment cultures were maintained in E6 medium. At day 7, established embryoid bodies (EBs) were collected and transferred on Geltrex-coated wells, and let to differentiate for further 7 d.

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

RNA was purified with the TRIzol Reagent (Thermo Fisher Scientific) following the manufacture's protocol. cDNA was obtained by reverse-transcription with iScript cDNA Synthesis Kit (BioRad) and used to verify the expression of specific genes by RT-PCR (primers reported in Table 1). qRT-PCR (quantitative RT-PCR) was performed using SsoAdvanced Universal SYBR Green Supermix Kit (BioRad), following the recommended protocol. Data were analyzed according to the comparative ΔΔCt method and normalized by using GAPDH housekeeping gene.

### 2.5. 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. Incubation with the primary antibodies was performed overnight at 4 °C in FBS 2%, Triton 0.2% with the antibodies reported in the Table 2. The signal was revealed with the appropriate secondary antibodies (Table 2). Nuclei were counterstained with Hoechst 33,258 (1 μg/mL; Thermo Fisher Scientific). Pictures were detected with the microscope Leica DM IL Led Fluo and acquired with the camera Leica DFC450 C (Leica Microsystem).

### 2.6. 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 using the TransBlot Turbo apparatus (BioRad) and hybridized overnight at 4 °C with the primary antibodies reported in the Table 2. The signal was detected with appropriate HRP-conjugated secondary antibodies (BioRad) and ECL Clarity system (BioRad), using the chamber UVITECH Cambridge (Uvitech).

### 2.7. 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, PBS washed cells were treated with hypotonic solution (0.075 M KCl for 15 min at room temperature), fixed in acetic

**Table 2**
List of the antibodies used in for immunocytochemistry (IC) and Western blot (WB) assays, working dilution and species in which they are 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 |

acid/methanol (1:3 v/v) and air-dried metaphase spreads slides were 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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