

Lab resource: Stem cell line

Generation of a disease-specific iPS cell line derived from a patient with Charcot-Marie-Tooth type 2K lacking functional *GDAP1* gene



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ABSTRACT

Human CMT2-FiPS4F1 cell line was generated from fibroblasts of a patient with Charcot-Marie-Tooth disease harbouring the following mutations in the *GDAP1* gene in heterozygosis: *p.Q163X/p.T288NfsX3*. This patient did not present mutations in the *PM22*, *MPZ* or *GJB* genes. Human reprogramming factors OCT3/4, KLF4, SOX2 and C-MYC were delivered using a non-integrative methodology that involves the use of Sendai virus.

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Resource Table: *please write over examples provided, fill in all fields.*

Name of stem cell line	CMT2-FiPS4F1
Institution	Universidad de Valencia
Person who created resource	Salvador Martí
Contact person and email	Josema Torres, Josema.Torres@uv.es
Date archived/stock date	October 18, 2016
Origin	Human skin cells
Type of resource	Biological reagent: induced pluripotent stem cell (iPS cell) from a patient with Charcot-Marie-Tooth disease.
Sub-type	Cell line
Key transcription factors	OCT3/4, KLF4, SOX2 and C-MYC
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	If more than 5, include only links and place full bibliographic details in the reference list at the end of the manuscript
Information in public databases	http://www.orpha.net/consor/cgi-bin/Disease_Search.php?lng=EN&data_id=14808&Disease_Disease_Search_diseaseGroup=101097&Disease_Disease_Search_diseaseType=ORPHA&Enfermedade(s)/grupo%20de%20enfermedades=Enfermedad-de-Charcot-Marie-Tooth-autos-mica-recesiva-c http://www.ciberer-biobank.es http://treat-cmt.es/ http://www.dmd.nl/nmdb/home.php?select_db=GDAP1 http://www.molgen.ua.ac.be/CMTMutations/Home/Default.cfm
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

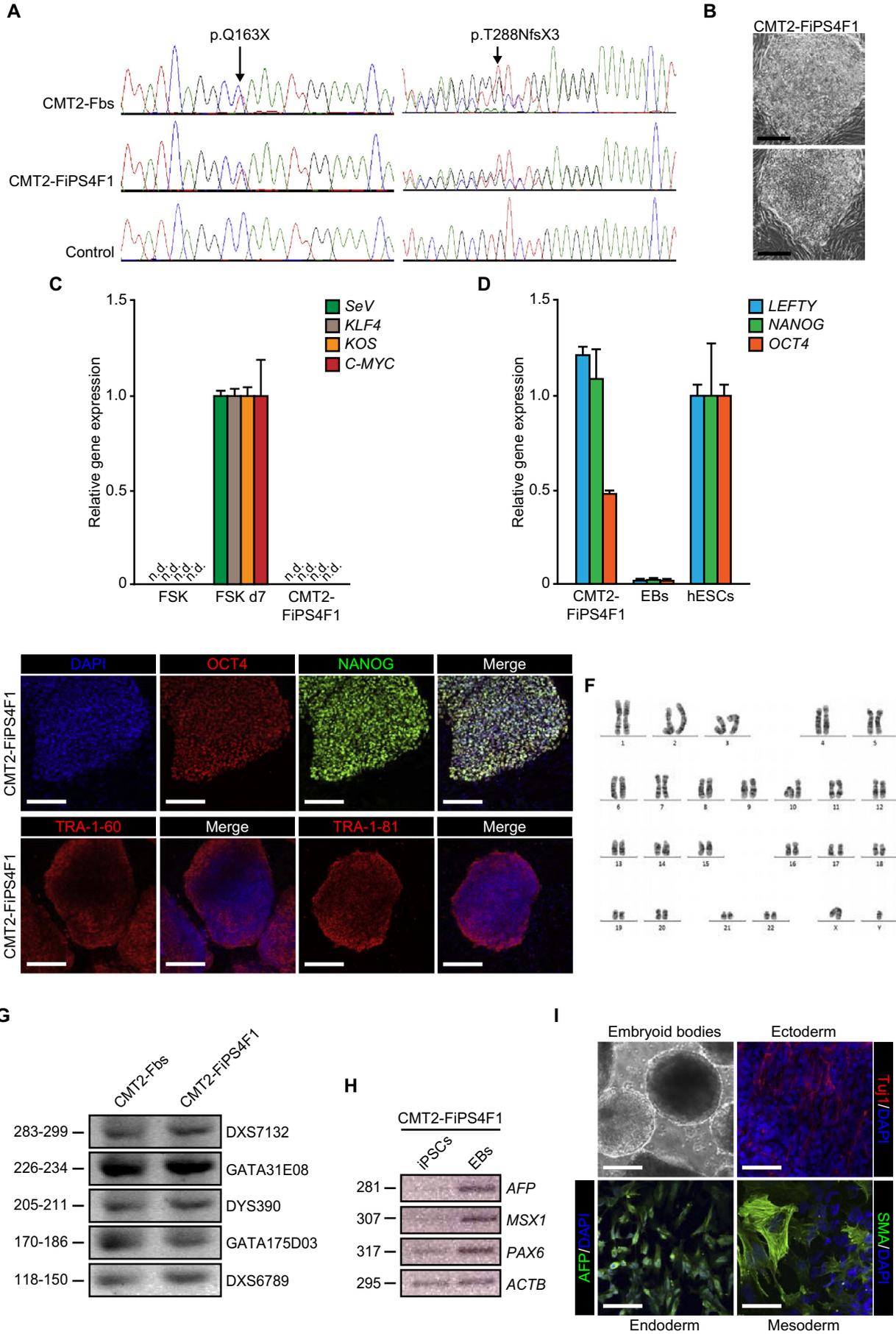
1. Resource details

The generation of the human iPS cell line, CMT2-FiPS4F1, was carried out using non-integrative Sendai viruses encoding the reprogramming factors OCT3/4, KLF4, SOX2 and cMYC, (Takahashi et al., 2007; Nishimura et al., 2011). For this purpose, fibroblasts from a described

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patient with recessive axonal Charcot-Marie-Tooth disease Type 2K ARCMT2K (Sevilla et al., 2008) were obtained from skin biopsies in the CIBERER Biobank. The patient's fibroblasts presented two mutations in *GDAP1* gene (*p.Q163X/p.T288NfsX3*) in heterozygosis. The presence of these mutations in CMT2-FiPS4F1 cells was confirmed by Sanger sequencing (Fig. 1A). The iPS cell colonies displayed a typical Embryonic Stem (ES) cell-like colony morphology and growth behaviour (Fig. 1B). We confirmed the clearance of the exogenous reprogramming factor genes by quantitative real-time polymerase chain reaction (qPCR) after eight cell culture passages (Fig. 1C). The endogenous expression of the pluripotency-associated transcription factors *LEFTY*, *NANOG* and *OCT3/4* was evaluated by qPCR (Fig. 1D). Immunofluorescence analysis revealed the expression of the *OCT3/4* and *NANOG* transcription factor proteins, and the surface antigens TRA-1-60 and TRA-1-81, features of pluripotent cells (Fig. 1E). The CMT2-FiPS4F1 cell line was adapted to feeder-free culture conditions without alterations in their karyotype (46, XY) after more than twenty passages (Fig. 1F). Using DNA fingerprinting analysis we confirmed that the CMT2-FiPS4F1 cell line was derived from the patient's fibroblasts (Fig. 1G). Finally, we examined the pluripotency of the CMT2-FiPS4F1 cell line by inducing their differentiation through their aggregation in embryoid bodies (EBs), and analysing the expression of markers from the three germ layers (endoderm, mesoderm and ectoderm) by PCR (Fig. 1H) and immunofluorescence (Fig. 1I).

2. Materials and methods

2.1. Non-integrative reprogramming of CMT2K fibroblasts into iPS cells

All the experimental protocols included in the present study were approved by the Institutional Ethical Committee of the University of Valencia and the Spanish Warranty Committee for the Donation and Use of Human Cells and Tissues, General Sub-Directorate for Research on Cellular Therapy and Regenerative Medicine, according to Spanish and European Union legislation. Human fibroblasts from a described patient with diagnosed CMT2K harbouring the mutations *p.Q163X/p.T288NfsX3* in *GDAP1* gene in heterozygosis were obtained from the CIBERER Biobank (<http://www.ciberer-biobank.es/>). These fibroblasts were reprogrammed using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo scientific) following the instructions of the manufacturer. After eight passages, the clearance of the exogenous reprogramming factors and Sendai virus genome was confirmed by RT-PCR following the manufacturer's instructions. The CMT2-FiPS4F1 iPS cell line was maintained and expanded both on feeder layers and feeder-free conditions. In the first case, CMT2-FiPS4F1 cells were plated on a layer of Mitomycin-C-inactivated human fibroblasts in hES medium containing: DMEM-F12 (Biowest), 20% Knockout serum replacement (Gibco), 1 × MEM non-essential amino acids, 2 mM Glutamine and 1 × penicillin/streptomycin (all from Biowest), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 8 ng/ml bFGF (Peprotech), in the presence of hLIF. Subsequently, CMT2-FiPS4F1 cell line was adapted and cultured in feeder-free conditions in PeptoGrow medium (Peprotech) on matrigel (BD Biosciences)-coated plates following the recommendations of the manufacturer. The CMT2-FiPS4F1 cell line was passaged by 5 min collagenase IV treatment followed by mechanical disaggregation of the colonies with a pipette.

2.2. Mutation analysis

Total DNA from the patient's fibroblasts and CMT2-FiPS4F1 cell line was extracted using a standard phenol-chloroform protocol. The amplification of the *GDAP1* gene region containing the *p.Q163X* mutation was carried out using the primers *GDAP1_E4_5*: 5'-GTCATGCTCTTGTTCAT TGAGTTT-3' and *GDAP1_E4_3*: 5'-CTGCTTGTTAAGCACTTGTCATCA-3', obtaining a fragment of 397 bp; and the region containing the mutation *p.T288NfsX3* using the primers *GDAP1_E6_5*: 5'-TCATCTTTTGTACT CACTCACC-3' and *GDAP1_E6_3*: 5'-TGCTAACTACTCAATAAGACAGACT CAGA-3', obtaining a fragment of 591 bp. Following PCR amplification, sequencing of amplicons was performed on both strands in an ABI3730 sequencer (Applied Biosystems, Foster City, CA) using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

2.3. qPCR analyses

Total RNA was isolated using TRI-Reagent (Sigma-Aldrich) and 0.5 µg was used to synthesize cDNA using PrimeScript RT III (Thermo Scientific). One µl of the reaction was used to analyse the expression of the endogenous pluripotency associated genes *LEFTY*, *NANOG* and *OCT3/4*, by qPCR. Primer sequences have been described elsewhere (Aasen et al., 2008). All the expression values were normalized to the *GAPDH* housekeeping gene expression. Plots are representative of three independent experiments carried out in triplicate. One µl of the PrimeScript™ RT III reaction was used to confirm the clearance of both the exogenous reprogramming factors and Sendai virus. Primer sequences are available in the information provided by the CytoTune-iPS 2.0 Sendai Reprogramming Kit. All the expression values were normalized to human foreskin fibroblast (FSK) expressing the reprogramming factors at day 7 post-transduction. Plots are representative of at least three independent experiments carried out in triplicate.

2.4. PCR analyses

One µl of cDNA (PrimeScript™ RT III reaction) was used to analyse by end-point PCR the expression of *AFP* (endoderm), *MSX1* (mesoderm), *PAX6* (ectoderm) or *ACTB* (endogenous control) genes. Primer sequences have been described elsewhere (Takahashi et al., 2007).

2.5. Karyotype analysis

Karyotype analysis of the CMT2-FiPS4F1 iPS cell line was carried out using cells with more than twenty culture passages. Exponentially growing cells were trypsinized, treated with hypotonic solution (KCl 0.075 M), fixed with Carnoy's fixative and stored at 4 °C until processed for the karyotype analysis. Cells were then dropped on a microscope glass slide and dried. Metaphase cells were G banded using Wright staining. At least 20 metaphases were karyotyped.

2.6. Immunofluorescence analysis

Cells were grown on matrigel-coated coverslips in hES cell medium for 5 days. Then, cells were fixed with 4% paraformaldehyde in PBS and processed for immunofluorescence using the following antibodies: TRA-1-60 (Millipore; MAB4360; 1:25); TRA-1-81 (Millipore; MAB4381; 1:25); *NANOG* (Cell Signaling Technology; #3580;

Fig. 1. Molecular and functional characterization of CMT2-FiPS4F1 cells. 1A. Electropherograms showing the *GDAP1* gene mutations *p.Q163X/p.T288NfsX3* in DNA samples extracted from patient's fibroblasts (CMT2-Fbs) or CMT2-FiPS4F1 iPS cells. Human dermal fibroblasts from a healthy donor were used as controls (control) 1B. Typical ES-like colony morphology of CMT2-FiPS4F1 cells; scale bars: 100 µm. 1C. Confirmation by qPCR of the exogenous reprogramming factors and Sendai virus clearance in CMT2-FiPS4F1 cells. 1D. qPCR showing the expression of the pluripotency markers *LEFTY*, *NANOG* and *OCT3/4* in CMT2-FiPS4F1 cells. 1E. Representative confocal images showing the expression of the pluripotency markers *OCT3/4*, *NANOG* (scale bars: 170 µm), TRA-1-60 and TRA-1-81 (scale bars: 340 µm). 1F. Karyotype analysis. CMT2-FiPS4F1 cells displayed a normal karyotype (46, XY). 1G. DNA fingerprinting analysis showing that CMT2-FiPS4F1 cells come from the patient's fibroblasts (CMT2-Fbs). 1H. Electrophoretic analysis showing the expression of the three germ layer markers *AFP*, *MSX1* and *PAX6* in undifferentiated (iPSCs) or differentiated (EBs) CMT2-FiPS4F1 cells; *ACTB* expression is shown as an endogenous control. 1I. Twenty-one days-old EBs from CMT2-FiPS4F1 cells; scale bars: 500 µm. Differentiated CMT2-FiPS4F1 cells were positive for the expression of representative markers from the three germ layers: *AFP* (endoderm), *TUJ1* (ectoderm) and *SMA* (mesoderm); scale bars: 40 µm.

1:100); OCT3/4 (Santa Cruz Biotechnology; Sc-5279; 1:50); neuron-specific Class III beta-Tubulin (TUJ1) (Covance; MMS-435p; 1:500); α -Fetoprotein (AFP) (R&D systems; MAB1368; 1:30); Smooth Muscle alpha-Actin (SMA) (Abcam; ab18147; 1:100). AlexaFluor-conjugated secondary antibodies were from Invitrogen (1:1000) (A31570; A21426; A21206). Images were taken using an Olympus FV1000 confocal microscope.

2.7. *In vitro* differentiation assay

CMT2-FiPS4F1 pluripotency was tested using an EB-based differentiation assay. For this, entire colonies of iPS cells from a p6w plate (at 80% confluence) were collected using Collagenase IV, resuspended in 10 ml of hES medium without hLIF and cultured in suspension in a poly-HEMA-coated p100 petri dish (10 ml/dish). After three days in suspension, culture medium was switched to EB-medium-1 (DMEM F12, 10% KSR, 10% FBS, 1 \times MEM, 1 \times P/S, 2 mM Glutamine and 0.1 mM β -Mercaptoethanol). Three days later, EB medium-1 was switched to EB medium-2 (EB medium-1 without KSR and with 20% FBS). Fifteen days after addition of EB medium-2, EBs were either seeded (2 EBs per well) on matrigel-coated coverslips in a p24 multiwell plate and either cultured 7 more days for IF analysis or collected by centrifugation for isolating total RNA.

2.8. DNA fingerprinting analysis

For DNA fingerprinting analysis, highly polymorphic regions containing short tandem repeated sequences of DNA were evaluated. For this purpose, the following markers: DXS7132, GATA31E08, DYS390, GATA175D03 and DXS6789, were amplified by end-point PCR and analysed by electrophoresis in 3% agarose (Pronadisa) gels for molecular screening of small DNA fragments and PCR products. Primer sequences and PCR conditions are available upon request.

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