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Lab Resource: Single Cell Line

Generation of an induced pluripotent stem cell line from a compound heterozygous patient in *TK2* gene

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A B S T R A C T

Autosomal recessive mutations in Thymidine kinase 2 (*TK2)* gene cause depletion and multiple deletions in mtDNA which normally lead to fatal and progressive neuromyopathy in infants and children. We have generated an induced pluripotent stem cell (iPSC) line by reprogramming fibroblasts derived from a patient carrying *TK2* mutations. New iPSC line pluripotency was evaluated by verifying the expression of pluripotency-related genes and the *in vitro* differentiation into the three germ layers. This human-derived model will be useful for studying the pathogenic mechanisms triggered by these mutations and for testing therapies in cell types normally affected in patients.

1. Resource table

Unique stem cell line identifier	UNIZARi001-A
Alternative name(s) of stem cell line	FiPSTK2-2
Institution	University of Zaragoza (Spain)
Contact information of distributor	seortiz@unizar.es
Type of cell line	iPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age: 15 Sex: Male Ethnicity: White Latino
Cell Source	Fibroblasts
Clonality	Clonal
Associated disease	TK2-deficient myopathy
Gene/locus	*TK2* gene c.388C > T (p.R130W) and c.604_606delAAG (p.K202del)
Date archived/stock date	2018
Cell line repository/bank	https://hpscreg.eu/cell-line/UNIZARi001-A
Ethical approval	All samples were collected with written informed consent and the Ethics Review Committees of the involved hospitals and the Government of Aragón approved the study (Comité Ético de Investigación Clínica de Aragón-CEICA-13/2017).

2. Resource utility

Despite several studies regarding TK2 deficiency have been previously described in mice models and patients, *in vitro* human-derived models are still limited to fibroblasts. Development of new cell models, such as this iPSC line, seems necessary to better understand pathogenic mechanisms triggered by these mutations and optimize new proposed therapies.

3. Resource details

Thymidine kinase 2 (TK2) is an enzyme implicated in salvage pathways of pyrimidine nucleosides in mitochondria. Autosomal recessive mutations cause TK2 deficiency leading to depletion and multiple deletions in mtDNA. Disorder is normally manifested as a fatal and progressive neuromyopathy in infants and children (Lopez-Gomez et al., 2021). Deoxynucleoside administration is under investigation as a promising therapy for this disorder (Cámara et al., 2014; Lopez-Gomez et al., 2017).

In this work, we have generated an iPSC from a 15-year-old compound heterozygous patient in *TK2* nuclear gene (c.388C > T; p.R130W and c.604_606delAAG; p.K202del). The patient suffered from progressive muscle weakness since the age of 3, ptosis, dysphagia and motor difficulties. Fibroblasts obtained from the patient skin explants were

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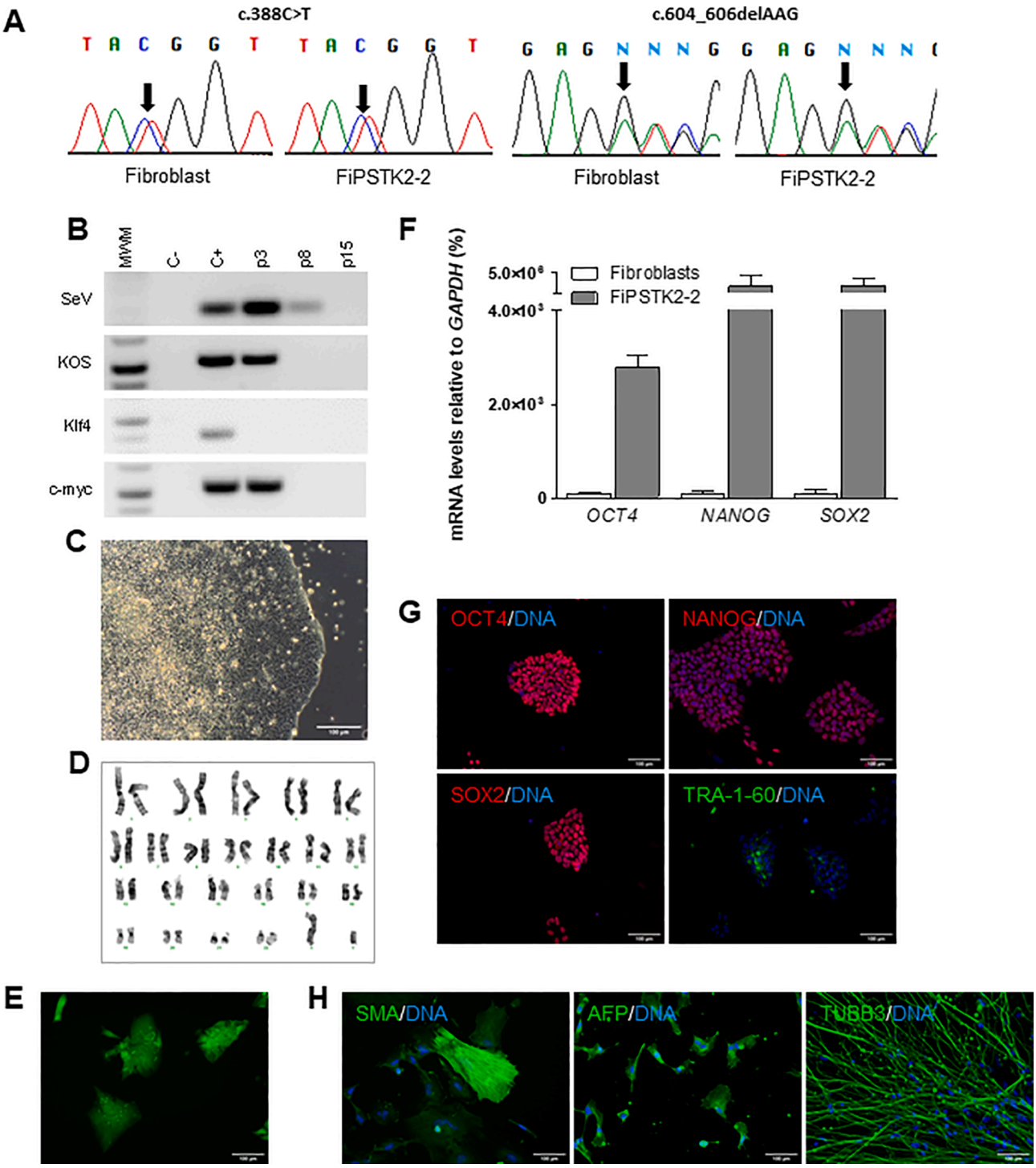

Fig. 1. Characterization of FiPSTK2-2 iPSC line.

reprogrammed using non-integrative Sendai-virus system to generate an iPSC line (FiPSTK2-2). We confirmed the presence of these mutations in fibroblasts and generated iPSC line by Sanger sequencing (Fig. 1A). We also checked by RT-PCR that vectors and exogenous reprogramming factor genes were completely removed after fifteen passages (Fig. 1B). New iPSC line showed human embryonic stem (hES)-like morphology (Fig. 1C, scale bar 100 μm), normal karyotype (46,XY) (Fig. 1D) and positive staining for alkaline phosphatase activity (Fig. 1E, scale bar 100 μm). DNA fingerprinting analysis confirmed the genetic identity between fibroblasts and iPSC. Furthermore, the expression of transcription factors OCT4, NANOG and SOX2, characteristic markers of pluripotent stem cells, were analysed by RT-qPCR (Fig. 1F) and immunofluorescence (Fig. 1G, scale bars 100 μm), as well as the surface marker TRA-1-60 (Fig. 1G, scale bar 100 μm). Finally, pluripotency capacity of iPSC line was evaluated by *in vitro* differentiation into three germ layers (endoderm, mesoderm and ectoderm) (Fig. 1H, scale bars 100 μm) (See Table 1).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1 panel C
Phenotype	Qualitative analysis	Staining/expression of pluripotency markers: OCT4, NANOG, SOX2 and TRA-1-60.	Fig. 1 panel G
	Immunofluorescence	Positive alkaline phosphatase activity	Fig. 1 panel E
	Quantitative analysis *(RT-qPCR)*	Expression of pluripotency markers *OCT4, NANOG, SOX2* in iPSC relative to fibroblasts	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution X	Normal: 46, XY 550 bands	Fig. 1 panel D
Identity	Microsatellite PCR (mPCR) OR STR analysis	NA All sites match	NA Available from author
Mutation analysis (IF APPLICABLE)	Sequencing	Two heterozygous mutations in *TK2* gene (c.388C > T; p.R130W and c.604_606delAAG; p.K202del)	Fig. 1 panel A
	Southern Blot OR WGS	NA	NA
Microbiology and virology	Mycoplasm	Mycoplasma was negative as tested by luminescence.	Table S1
Differentiation potential	Embryoid body formation and *in vitro* differentiation	Expression of specific markers of three germlayers evaluated by immunofluorescence with specific antibodies: Ectoderm: TUBB3/TUJ1 Endoderm: AFP Mesoderm: SMA	Fig. 1 panel H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	Not performed Not performed	

4. Materials and methods

4.1. iPSC generation and culture

Fibroblasts were reprogrammed using CytoTune-iPS 2.0 Sendai Reprogramming kit (Thermo Fisher Scientific). Nascent iPSC were cultured at 37 °C and 5% CO_2 for 3–4 weeks after transduction on feeder layers in iPSC medium containing *KnockOut*™ DMEM/F-12, 20% of *KnockOut*™ *Serum Replacement, MEM* Non-Essential Amino Acids Solution 1X, GlutaMAX™-I 1X, β-mercaptoethanol (100 μM), penicillin/streptomycin 1X and bFGF (4 ng/ml) (Thermo Fisher Scientific). Emerging iPSC colonies were mechanically picked and expanded on vitronectin coated

Table 2
Reagents details.

	Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-OCT4	1:200	Abcam Cat# ab19857	RRID: AB_445175
Pluripotency Markers	Rabbit anti-SOX2	1:200	Abcam Cat# ab92494	RRID: AB_10585428
Pluripotency Markers	Rabbit anti-NANOG	1:100	Abcam Cat# ab109250	RRID: AB_10863442
Pluripotency Markers	Mouse anti-TRA-1-60	1:150	Millipore Cat# MAB4360	RRID: AB_2119183
Differentiation Markers	Mouse anti-TUBB3 (Tubulin β-3)	1:500	Abcam Cat# ab18207	RRID: AB_444319
Differentiation Markers	Mouse anti-SMA (α-smooth muscle actin)	1:400	Sigma-Aldrich Cat# A2547	RRID: AB_476701
Differentiation Markers	Mouse anti-AFP (α-fetoprotein)	1:200	Abcam Cat# ab3980	RRID: AB_304203
Secondary antibodies	Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11001	RRID: AB_2534069
Secondary antibodies	Goat Anti-Rabbit IgG (H + L) Antibody, Alexa Fluor 488 Conjugated	1:1000	Molecular Probes Cat# A-11008	RRID: AB_143165
Secondary antibodies	Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1:1000	Thermo Fisher Scientific Cat# A-11012	RRID: AB_2534079
	Primers			
	Target	Size of band	Forward/Reverse primer (5′-3′)	
Sendai virus (RT-PCR)	SeV	181 bp	GGATCACTAGGTGATATCGAGC/ ACCAGACAAGAGTTTAAGAGATATGTATC	
Sendai virus (RT-PCR)	KOS	528 bp	ATGCACCGCTACGACGTGAGCGC/ ACCTTGACAATCCTGATGTGG	
Sendai virus (RT-PCR)	Klf4	410 bp	TTCCTGCATGCCAGAGGAGCCC/ AATGTATCGAAGGTGCTCAA	
Sendai virus (RT-PCR)	c-Myc	532 bp	TAACTGACTAGCAGGCTTGTCG/ TCCACATACAGTCCTGGATGATGATG	
Pluripotency Markers (qPCR)	NANOG Hs02387400_g1 (TaqMan® probe ID)			
Pluripotency Markers (qPCR)	POU5F1 (OCT4) Hs04260367_g1 (TaqMan® probe ID)			
Pluripotency Markers (qPCR)	SOX2 Hs01053048_s1 (TaqMan® probe ID)			
House-Keeping Genes (qPCR)	GAPDH Hs027866624_g1 (TaqMan® probe ID)			
Targeted mutation analysis/ sequencing	c.388C > T in *TK2*	465 pb	TCCTGCTTCACTCTCTCT/TCCCCAGACATCTCATTG	
Targeted mutation analysis/ sequencing	c.604_606delAAG in *TK2*	337 pb	GAGAATGAAAGTCGAGGC/AATGATCTCATGGGGGTG	

plates (0,5 μg/cm^2) with *Essential 8*™ culture media (Thermo Fisher Scientific), until no Sendai Virus genome was detected (Fig. 1B). Subsequently, iPSC were passaged every 3–4 days at a ratio of 1:3–1:6 using EDTA (0.5 mM) and media was replaced daily.

4.2. RT-PCR

Total RNA was isolated using NucleoSpin® RNA II kit (Macherey-Nagel). 1 μg of RNA was reversed-transcribed (RT) with the Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR was performed using specific primers for exogenous reprogramming vectors (Table 2) and analysed by agarose gel electrophoresis.

4.3. RT-qPCR

15 ng de RNA was amplified to detect the expression of endogenous pluripotency associated genes (*OCT4*, *NANOG* and *SOX2*) using TaqMan® RNA-to-CT™ 1-Step Kit (ThermoFisher Scientific) and TaqMan® probes (Table 2). Values were normalized to *GAPDH* mRNA levels. Plot represents biological triplicates.

4.4. Genetic mutation analysis

Total DNA was extracted using Cells and Tissue DNA Isolation Kit (Norgen Biotek Corp.). 100 ng of DNA was amplified using EmeraldAmp® Max PCR Master Mix (Takara) and specific primers (Table 2), and analyzed by sequencing (Fig. 1A).

4.5. Karyotype analysis

Cytogenic analysis were performed in Citogen, Center of Genetic Analysis in Zaragoza. At least 14 metaphase cells were analyzed.

4.6. STR analysis

16 markers were amplified by AmpFISTR® PCR Amplification Kit (ThermoFisher Scientific) and analysed by ABI Prism 3730xl DNA analyzer (Applied Biosystems) to evaluate the identity of fibroblasts and iPSC.

4.7. Alkaline phosphatase (AP) staining

Alkaline Phosphatase Live Stain (500X) (ThermoFisher Scientific) was used for determining the enzyme activity following manufacturer's instructions. Pictures were acquired using FLoid™ Cell imaging station (Life Technologies).

4.8. Immunofluorescence

Cells were washed once with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min. After blocking for 30 min with 5% bovine serum albumin, cells were incubated with a primary antibody against OCT4, SOX2, NANOG, TRA-1-60 and SMA for 1 h at room temperature, and against TUBB3 and AFP overnight at 4 °C. Subsequently, they were incubated with fluorescence-labeled secondary Alexa Fluor® 594 or 488 at room temperature for 1 h, protected from light. Cells were further incubated with DAPI for nuclear staining. Between incubations, samples were washed with 0.1% Triton X-100. Pictures were acquired using FLoid™ Cell imaging station (Life Technologies). Antibodies are listed in Table 2.

4.9. In vitro differentiation

In vitro differentiation of iPSC into the three germ layers was performed adapting a protocol previously described (Galera-Monge et al., 2016).

4.10. Mycoplasma

iPSC line was tested negative for mycoplasma contamination by MycoAlert™ Mycoplasma Detection kit (Lonza) and Synergy™ HT plate reader (BioTek).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102632.

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