Journal Pre-proofs

Lab Resource: Multiple Cell Lines

Generation of iPSC lines from two patients affected by febrile seizure due to inherited missense mutation in *SCN1A* gene

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Lab Resource: Multiple Stem Cell Lines template

Title: Generation of iPSC lines from two patients affected by febrile seizure due to inherited missense mutation in *SCN1A* gene.

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Abstract: Here, we described the generation of human induced pluripotent stem cell lines (hiPSCs) from fibroblasts isolated by punch biopsies of two siblings carrying inherited mutation (c.434T>C) in the *SCN1A* gene, encoding for the neuronal voltage gated sodium channel $Na_V1.1$. The mutation leads to the substitution of a highly conserved methionine with a threonine (M145T) in the protein sequence, leading to infant febrile seizures (FS). The older brother, affected by complex FS, also developed temporal lobe epilepsy (TLE) during adolescence.

Resource Table:

Unique stem cell lines identifier	UNIMGi001-A UNIMGi002-A
Alternative names of stem cell lines	SCN1A^{mut}-1 SCN1A^{mut}-2
Institution	University "Magna Graecia" of Catanzaro
Contact information of distributor	Stefania Scalise
Type of cell lines	iPSC
Origin	Human
Cell Source	Fibroblasts
Clonality	Mixed
Method of reprogramming	Non-integrating Sendai virus carrying OCT4, SOX2, c-MYC and KLF4
Multiline rationale	Siblings with the same mutation
Gene modification	Yes
Type of modification	Inherited mutation
Associated disease	SCN1A^{mut}-1: FS and TLE SCN1A^{mut}-2: FS
Gene/locus	*SCN1A* c.434T>C
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Stock date	27/07/2020
Cell line repository/bank	https://hpscreg.eu/user/cellline/edit/UNIMGi001-A https://hpscreg.eu/user/cellline/edit/UNIMGi002-A
Ethical approval	The human study was approved by the Ethics Committee of

	Ospedaliero - Universitaria "Mater Domini" (Approval number: AOM92_2020) with written informed consent obtained from each participant.

Resource utility

The generated iPSC lines represent a useful model to dissect the molecular mechanisms connecting prolonged FS of infancy with long-term alterations in the brain that can trigger epilepsy.

Resource Details

Febrile Seizure (FS) is a phenomenon that can occur in childhood during fever without evidence of any causative disease, such as central nervous system (CNS) infection or metabolic abnormality. FS are classified in simple and complex. Simple FS are not associated with permanent brain damage, do not occur more than once in 24 hours, present a maximum duration of 10 minutes, and resolve spontaneously. Complex FS, instead, can occur more than once within 24 hours, are longer lasting (>15 minutes), and are linked to development of hippocampal sclerosis and TLE during adolescence or adulthood. The patients enlisted in this study belong to a family in which fourteen members were affected by FS, disease trait that segregates as autosomal dominant with high penetrance. All affected individuals carry a c.434T>C missense mutation in *SCN1A* gene that alters a highly conserved methionine residue (Met145Thr) in the neuronal voltage gated sodium channel NaV1.1. The mutation is predicted to be a *loss of-function* mutation causing a 60% reduction of current density and a 10mV positive shift of the activation curve (Mantegazza et al., 2005). Interestingly, although the two patients carry the same mutation and both were affected by FS until the age of 6, only the older brother ($SCN1A^{mut}$-1), affected by complex FS, developed TLE in a later time (age 13). Sendai-based reprogramming vectors were used to generate iPSC cells from skin fibroblasts isolated from the two patients. Obtained iPSC clones showed human embryonic stem cell-like morphology with compact, flat and well-defined colonies edges (Fig.1-A) and loss of Sendai viral transgenes as confirmed by RT-PCR analysis (Suppl. Fig 1-A. SF = skin fibroblast; iSF = infected skin fibroblast). Karyotyping has been performed on both lines to assess chromosomal abnormalities by conventional cytogenetic analysis. Cells present a normal karyotype (46, XY), no numerical or structural aberrations have been detected (Suppl. Fig 1-B). The expression of pluripotency markers such as NANOG, OCT4 and TRA-1-60 was assessed by immunostaining (Fig. 1-B) and the reactivation of endogenous pluripotency-associated genes (*OCT4*, *SOX2*, *NANOG*, *REX1*, and *DMT3B*) was confirmed by quantitative RT-PCR (Fig. 1-C). Global pluripotency marker expression was confirmed by high-throughput PluriTest algorithm (Fig.1-D). Presence of the c.434T>C mutation was further confirmed by genomic DNA sequencing in patient-specific iPSCs (Fig.1-E). The differentiation capability of generated iPSCs was assessed via embryoid bodies (EBs) formation assay (Suppl. Fig 1-C), positively stained for NESTIN, BRACHYURY, and SOX17, specific markers of ectoderm, mesoderm, and endoderm lineages, respectively (Fig.1-F).

Materials and Methods

Reprogramming and cell culture. Skin biopsies were cultured in DMEM containing 10% FBS, 50U/ml penicillin and 50 µg/ml streptomycin allowing fibroblasts to outgrowth from the tissue. When 80% confluent, fibroblasts were trypsinized and 5 x 10^5 cells were seeded for reprogramming. Reprogramming process was carried out by CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) in feeder-free conditions. Emerged iPSC colonies were manually picked, transferred on Matrigel-coated dishes, and cultured in mTeSR1 medium (StemCell Technologies). **Immunofluorescence.** Cells were fixed in 4% (vol/vol) paraformaldehyde in PBS for 10 min at RT, washed in PBS, permeabilized and blocked for 2 h in PBS + 10% FBS + 0.1% Triton X-100. Cells were incubated overnight at 4°C with primary antibodies in PBS + 5% FBS + 0.1% Triton X-100. After washing with PBS, cells were incubated with secondary antibodies for 1 h at RT. Nuclei were stained with DAPI and the slides were mounted with Dako Fluorescent Mounting Medium. Images were acquired with a Leica imaging systems (DMi8), filter cubes and software from Leica microsystems. **RNA Extraction, RT-PCR and qRT-PCR**. Total RNA was extracted by TRIzol reagent (Thermo Fisher Scientific) and cDNA was made from 1 ug using the High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Sendai Virus detection was performed by standard PCR starting with 500 ng of cDNA. For real-time PCR, Power SYBR Green Master Mix (Applied

to normalize expression. Primers are detailed in Supplementary Table 3. **Sequencing**. Cells were lysed in 50mM Tris-HCl pH 8, 10mM EDTA and 1%SDS and gDNA was purified by UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Thermo Fisher Scientific). The region containing the mutation has been amplified and PCR product were send to Eurofins Genomics for sequencing. **EBs formation assay**. Cells were dissociated into single cells using StemPro Accutase (Thermo Fisher Scientific), seeded on low attachment dishes (poly-2-hydroxyethyl methacrylate-coated) and cultured in mTeSR1 medium + 10 uM Rho-kinase inhibitor, Y-27632 (Selleckchem). At day 7, floating EBs were transferred on 5µg/ml Biolaminin 521LN (Biolamina)-coated plates and cultured in adhesion for thirteen days in medium consisting of DMEM/F12 containing 20% knockout serum replacement (KSR, Thermo Fisher Scientific), 1% Glutamax (Thermo Fisher Scientific), 1% Non-Essential Amino Acids (Thermo Fisher Scientific), 100µM 2-mercaptoethanol, and 0.5% penicillin and streptomycin. **Karyotype**. Giemsa (G)-based chromosomal banding has been performed following standard cytogenetic procedures at the Medical Genetics Unit of Mater Domini University Hospital (Catanzaro, Italy). Briefly: after treatment with colcemid solution (10 ug/ml) (Thermo Fischer Scientific, USA) for 16h overnight at 37 °C, cultured cells have been detached, washed in PBS, treated with hypotonic solution (0,033 M KCl and 0,017 M $H_3CCOONa$) for 20 minutes at 37°C and fixed in methanol/acetic acid solution (3:1 v/v). G-banding has been performed according to conventional protocols on fixed metaphases. Images of banded chromosomes have been acquired on DMRA fluorescent microscope (Leica Microsystems, Germany) and karyotype analysis has been performed on 20 metaphases by using CytoVision® 7.3.1 software (Leica Biosystems, Germany).

References

Mantegazza M, Gambardella A, Rusconi R, et al. Identification of a Nav1.1 sodium channel (SCN1A) loss-of-function mutation associated with familial simple febrile seizures. Proc Natl Acad Sci U S A. 2005;102(50):18177-18182. doi:10.1073/pnas.0506818102.

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Table 1: Summary of lines

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
UNIMGi001-A	SCN1A^{mut}-1	Male	27	Caucasian	*SCN1A* c.434T>C	FS and TLE
UNIMGi002-A	SCN1A^{mut}-2	Male	19	Caucasian	*SCN1A* c.434T>C	FS

Table 2: Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	Human embryonic stem cell-like morphology	Figure 1 panel A
Phenotype	Qualitative analysis: ▪ Immunocytochemistry	Positive staining for pluripotency markers OCT4, NANOG and TRA-1-60	Figure 1 panel B
	Quantitative analysis: ▪ RT-qPCR ▪ PluriTest	All pluripotency genes tested presented a fold change (FC) expression of at least 364 relative to parental fibroblasts. SCORE PLURITEST: $SCN1A^{mut}$-1: novelty = 1.64 pluripotency = -4.60 $SCN1A^{mut}$-2: novelty = 1.60 pluripotency = 0.54	Figure 1 panels C and D
Genotype	Karyotype (G-banding) and resolution	46, XY for both lines 500-band resolution	Supplementary figure 1 panel B
Identity	STR analysis	Genotyping was performed according to ANSI/ATCC standard ASN-0002. 16 STR markers matched between iPSC lines and parental fibroblasts used for reprogramming	Submitted in archive to the journal
Mutation analysis	Sequencing	Confirmation of *SCN1A* c.434T>C mutation in both patients (in heterozygosity)	Figure 1 panel E
	Southern Blot OR WGS	N/A	
Microbiology and virology	Mycoplasma	Negative Micokit (Biowest)	Not shown but available from the authors
Differentiation potential	Embryoid body formation	Immunofluorescence assay for NESTIN (ectoderm), BRACHYURY (mesoderm) and SOX17 (endoderm)	Supplementary figure 1 panel C Figure 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

Table 3: Reagents details

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Mouse anti-OCT4	1:100	STEMCELL Technologies Cat# 60093, RRID:AB_2801346
Pluripotency Markers	Rabbit anti-NANOG	1:200	Thermo Fisher Scientific Cat# PA1-097, RRID:AB_2539867
Pluripotency Markers	Mouse anti-TRA-1-60	1:100	Thermo Fisher Scientific Cat# 41-1000, RRID:AB_2533494
Differentiation Markers	Mouse anti-NESTIN	1:1000	STEMCELL Technologies Cat# 60091AD, RRID:AB_2650581
Differentiation Markers	Goat anti-BRACHYURY	1:20	R&D Systems Cat# AF2085, RRID:AB_2200235
Differentiation Markers	Goat anti-SOX17	1:20	R&D Systems Cat# AF1924, RRID:AB_355060
Secondary antibodies	Alexa Fluor® 647 Goat anti-mouse IgG	1:500	Thermo Fisher Scientific Cat# A-21235, RRID:AB_2535804
Secondary antibodies	Alexa Fluor® 488 Goat anti-rabbit IgG	1:500	Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165
Secondary antibodies	Alexa Fluor® 594 Donkey anti-goat IgG	1:500	Thermo Fisher Scientific Cat# A-11058, RRID:AB_2534105
Primers			
	Target	**Forward/Reverse primer (5'-3')**	
Sendai virus (PCR)	SeV	GGATCACTAGGTGATATCGAGC/ ACCAGACAAGAGTTT AAGAGATATGTATC	
Transgene	c-MYC	TAACTGACTAGCAGGCTTGTCG/ TCCACATACAGTCCT GGATGATGATG	
Transgenes	Polycistronic KOS (KLF4, OCT3/4, SOX2)	ATGCACCGCTACGACGTGAGCGC/ ACCTTGACAATCCTG ATGTGG	
Pluripotency Markers (qPCR)	*OCT4*	GGAGGAAGCTGACAACAATGAA/ GGCCTGCACGAGGGTTT	
Pluripotency Markers (qPCR)	*SOX2*	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG	
Pluripotency Markers (qPCR)	*NANOG*	TGCAAGAACTCTCCAACATCCT/ ATTGCTATTCTTCGGCCAGTT	
Pluripotency Markers (qPCR)	*REX1*	GTGTGAACAGAACAGAAGAGGC/ CTGGTGTCTTGTCTTTGCCC	
Pluripotency Markers (qPCR)	*DNMT3B*	CCATGAAGGTTGGCGACAA/ TGGCATCAATCATCACTGGATT	
House-Keeping Genes (qPCR)	*GAPDH*	TCCTCTGACTTCAACAGCGA/ GGGTCTTACTCCTTGGAGGC	
Mutation analysis/sequencing	*SCN1A*	AGCTGCAGTTTGGGCTTTTC/ TGGTGGTCTTCAGGTGAACA	