Stem Cell Research 51 (2021) 102140

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Multiple Cell Lines

Generation of multiple iPSC clones from a male schizophrenia patient carrying de novo mutations in genes KHSRP, LRRC7, and KIR2DL1, and his parents

Edit Hathy [a,b], Eszter Szabó [c], Katalin Vincze [a,b], Irén Haltrich [d], Eszter Kiss [d], Nóra Varga [c], Zsuzsa Erdei [c], György Várady [c], László Homolya [c], Ágota Apáti [c], János M. Réthelyi [a,b,*]

[a] Molecular Psychiatry Research Group, National Brain Research Program (NAP), Hungarian Academy of Sciences and Semmelweis University, Hungary
[b] Department of Psychiatry and Psychotherapy, Semmelweis University, Budapest, Hungary
[c] Institute of Enzymology, Research Center for Natural Sciences, Eötvös Loránd Research Network, Hungary
[d] 2nd Department of Pediatrics, Semmelweis University, Budapest, Hungary

A B S T R A C T

Here we describe the generation of induced pluripotent stem cell lines from each member - male proband, mother, father - of a schizophrenia case-parent trio that participated in an exome sequencing study, and 3 de novo mutations were identified in the proband. Peripheral blood mononuclear cells were obtained from all three individuals and reprogrammed using Sendai virus particles carrying the Yamanaka transgenes. These 3 iPSC lines (iPSC-SZ-HU-MO 1, iPSC-SZ-HU-FA 1, and iPSC-SZ-HU-PROB 1) represent a resource for examining the functional significance of the identified de novo mutations in the molecular pathophysiology of schizophrenia.

1. Resource Table

Unique stem cell lines identifier	SUi001-A SUi002-A SUi003-A
Alternative names of stem cell lines	iPSC-SZ-HU-PROB 1 iPSC- SZ-HU-MO 1 iPSC-SZ-HU-FA 1
Institution	1. Molecular Psychiatry Research Group, National Brain Research Program (NAP), Hungarian Academy of Sciences and Semmelweis University 2. Institute of Enzymology, Research Center for Natural Sciences, Eötvös Loránd Research Network
Contact information of distributor	Ágota Apáti, apati.agota@ttk.mta.hu János M. Réthelyi, rethelyi.janos@med.semmelweis-univ.hu
Type of cell lines	iPSC lines
Origin	human
Cell Source	PBMCs
Clonality	Clonal
Method of reprogramming	Sendai virus reprogramming
Multiline rationale	iPSCs derived from a case-parent trio
Gene modification	Yes
Type of modification	de novo mutations in proband
Associated disease	Schizophrenia
Unique stem cell lines identifier	SUi001-A SUi002-A SUi003-A
Gene/locus	Proband carrier of 3 missense DNMs in genes KHSRP (19:6416869C>A) LRRC7 (1:70505093G>A) KIR2DL1 (19:55286658A>T)
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	June 15, 2015
Cell line repository/bank	NA
Ethical approval	Health Care Research Council, Human Reproduction Committee in Hungary (in Hungarian: Egészségügyi Tudományos Tanács, Humán Reprodukciós Bizottság, ETT HRB) Approval number: 33873-3/2014-EHR

2. Resource utility

De novo mutations (DNMs) have been implicated in the etiology of schizophrenia (SZ), a chronic psychiatric disorder characterized by

* Corresponding author.
E-mail address: rethelyi.janos@med.semmelweis-univ.hu (J.M. Réthelyi).

https://doi.org/10.1016/j.scr.2020.102140
Received 24 June 2020; Received in revised form 1 December 2020; Accepted 21 December 2020
Available online 25 December 2020

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
iPSC- SZ-HU-MO 1	iPSC- SZ-HU-MO 1	Female	55	Caucasian	19:6416869CC	healthy control
iPSC-SZ-HU-FA 1	iPSC-SZ-HU-FA 1	Male	59	Caucasian	(19:6416869CC	healthy control
iPSC-SZ-HU-PROB 1	iPSC-SZ-HU-PROB 1	Male	24	Caucasian	19:6416869CA	schizophrenia patient

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Olympus CKX 41	Normal iPSCs formation	Fig. 1 panel A and Fig. 2 panel A
Phenotype	Qualitative analysis	Positive for pluripotency markers: Oct4, Nanog	Fig. 1 panel A
	AP staining	Positive for AP staining	
	Immunocytochemistry		
	Quantitative analysis Flow cytometry, RT-qPCR	Cell surface markers: SSEA-4, TRA1-60, and TRA1-81: >90% all of cell lines	Fig. 1 panels A, B and Fig. 2 panel A.
		RT-qPCR: Nanog, Brachyury, AFP	
		Sendai clearance qPCR: SevFam, cMyc, Klf4, Oct3/4, Sox2	
Genotype	Karyotype (G-banding) and resolution	iPSC- SZ-HU-MO 1:46 XX, iPSC-SZ-HU-FA 1: 46 XY, iPSC-SZ-HU-PROB 1: 46XY	Fig. 1 panel C
		Resolution: 450–500 bands per haploid chromosome set.	
Identity	STR analysis	DNA Profiling	Submitted in archive with journal
		Performed	
		17 sites tested, all matching between PBMC and iPSC lines.	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	IPSC-SZ-HU-PROB 1: heterozygous missense mutation in LRRC7 and KHSRP	Fig. 1 panel D
		iPSC- SZ-HU-MO 1	
		iPSC-SZ-HU-FA 1: homozygous in LRRC7 and KHSRP	
	Southern Blot OR WGS	NA	
Microbiology and virology	Mycoplasma test by MycoAlert	Mycoplasma testing by luminescence	not shown but available with author
		Negative	
Differentiation potential	Spontaneous differentiation	Immunocytochemistry: iPSCs state: Nanog, Oct4,	Fig. 1 panels A, E and Fig. 2 panel B.
		EBs state: SMA, AFP, β-tubulin, NESTIN, BMP4, SOX17	
Donor screening	HIV 1 + 2, Hepatitis B, Hepatitis C	Negative	not shown but available with author
Genotype additional info	Blood group genotyping	NA	
	HLA tissue typing	NA	

severe symptoms and impaired community functioning. By generating iPSC lines from patients carrying DNMs and using neuronal differentiation we can investigate directly the biological significance of DNMs (Tables 1 and 2).

3. Resource details

Schizophrenia is a chronic debilitating psychiatric disorder characterized by diverse clinical symptoms, i.e. hallucinations, delusions, social withdrawal, neurocognitive dysfunction, and as a consequence of these, decreased educational and workplace functioning (van Os and Kapur, 2009). Strong genetic effects and corresponding levels of heritability have been demonstrated in genetic studies of schizophrenia, however the emerging genetic architecture is complex, implicating a role for both common and rare genetic variants. The polygenic effect of common variants, i.e. single nucleotide polymorphisms, have been shown in genome-wide association studies. Rare variants such as copy number variants (CNVs) and DNMs are present in a small proportion of schizophrenia patients, however the associated risk is higher for these variants. The large scale identification of disruptive DNMs in schizophrenia has become feasible with the advent of next generation sequencing, whole exome and genome sequencing (Takata et al., 2016). While several DNMs have been demonstrated by examining schizophrenia cases and their unaffected parents, and DNMs can be evaluated using the predictions of bioinformatics tools with regard to their disease-causing effects, in most cases their biological significance remains inconclusive. iPSC-based in vitro disease modelling has been exploited successfully to elucidate the molecular disease pathways giving rise to schizophrenia (Brennand et al., 2011). Therefore it is plausible to use this model system for the investigation of disruptive DNMs in schizophrenia.

Here we describe the generation of iPSCs from a schizophrenia case-patient trio, in which the male proband is a carrier of 3 nonsynonymous DNMs in genes leucine rich repeat containing 7 (LRRC7), KH-Type Splicing Regulatory Protein (KHSRP), and Killer Cell Immunoglobulin-Like Receptor, Two Domains, Long Cytoplasmic Tail, 1 (KIR2DL1). iPSC lines were generated from the patient's and parents' peripheral blood mononuclear cells using Sendai virus-based reprogramming. iPSCs were investigated and characterized using brightfield microscopy (Fig. 1A upper panel) and alkaline phosphatase staining (Fig. 2A upper panel), immunocytochemistry (Fig. 1A middle panel), flow cytometry (Fig. 1A lower panel and Fig. 2A), qPCR (Fig. 1B), and karyotyping (Fig. 1C). These assays confirmed the pluripotency and genomic integrity of the generated iPSCs. Virus clearance was monitored using qPCR (Fig. 1B). Spontaneous differentiation experiments demonstrated the expression of multiple germline markers, thus confirming that the iPSC lines are able to differentiate into all three germ layers (Fig. 1D and Fig. 2B). The DNMs were validated in the iPSC lines by resequencing the genomic loci of interest (Fig. 1E). Rigorous characterization and quality control has indicated the pluripotency and genomic integrity of the derived iPSC clones (Fig. 1 and Fig. 2). The significance of these cell lines lies in their utility for examining the functional role of the DNMs harboured in the proband, who is a schizophrenia patient. In subsequent experiments, we will seek to identify molecular and cellular phenotypes that differ between the progenies of the individual cell lines. As schizophrenia is the disorder of the central nervous system, more precisely of forebrain glutamatergic and GABAergic neurons and their network circuitry, we plan to use targeted neuronal differentiation protocols to study homogenous neuronal populations in terms of transcriptomics, synaptic connectivity, single-cell electrophysiology and calcium signalling. The process of reprogramming case-parent trios represents a means for investigating disease-causing mutations by

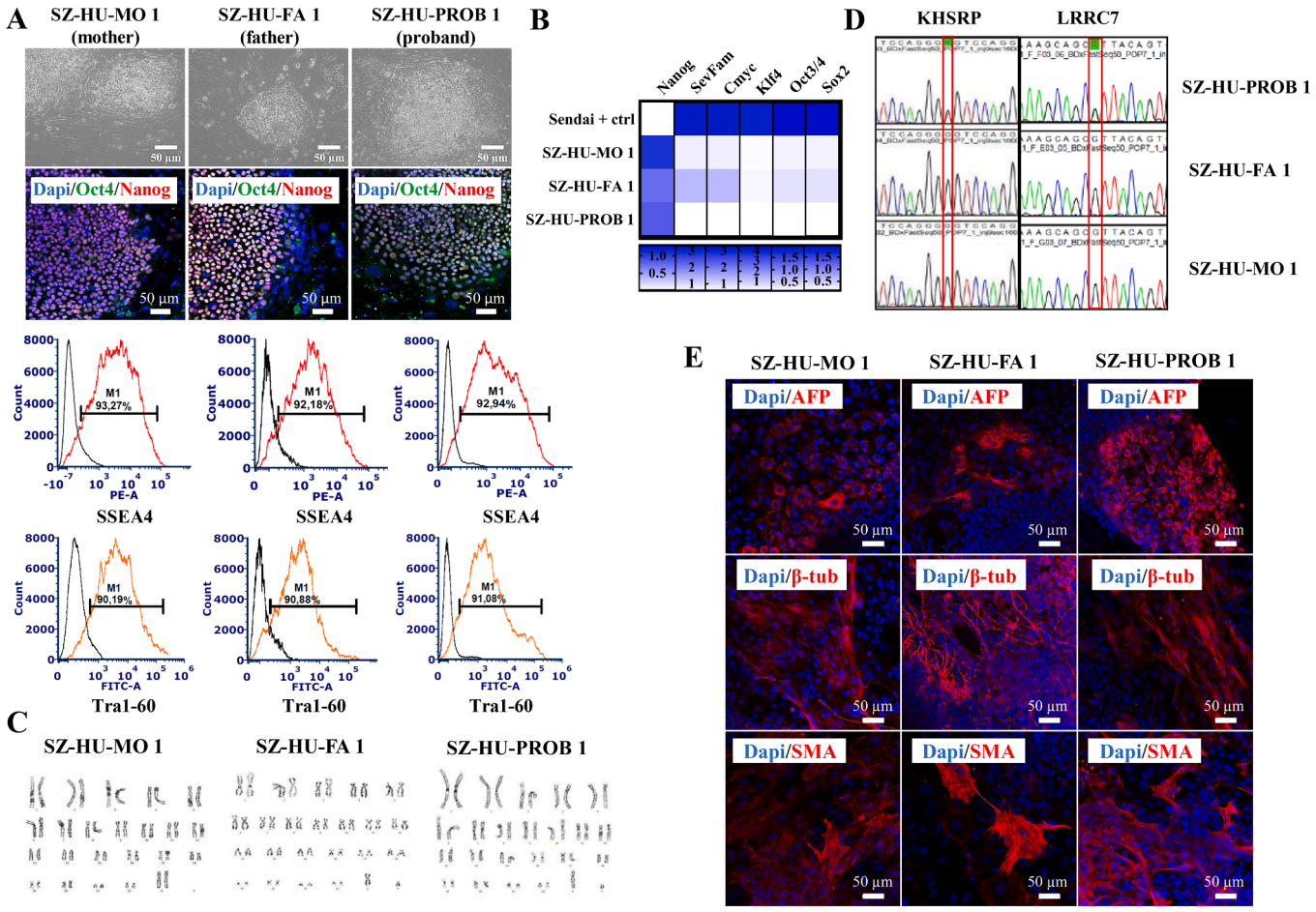

Fig. 1. Characterization and quality control of iPSC clones.

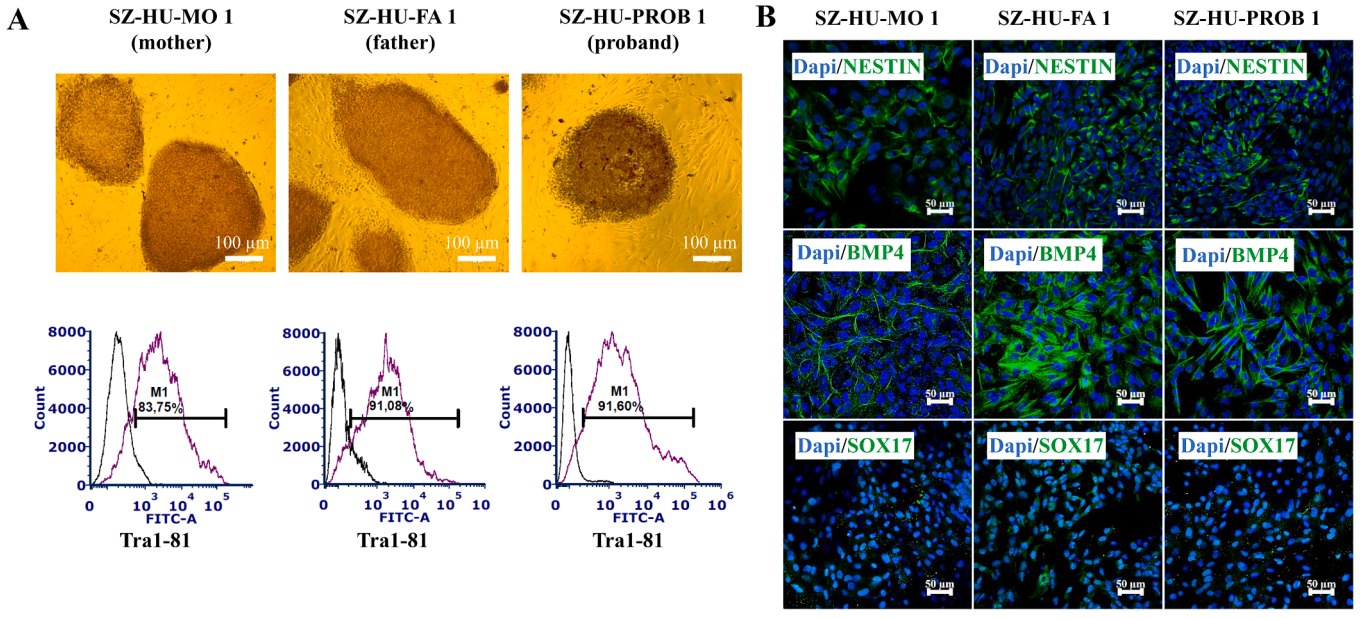

Fig. 2. Characterization of iPSC clones.

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT3/4	1:50 1:100	AB_628051, Cat.no.: Sc-5279, Santa Cruz Biotechnology, CA, USA
	Goat anti-Nanog	1:25	
	Mouse anti-SSEA4-PE	1:200	AB_355097, Cat.no.: AF1997, R&D System
	Mouse anti-TRA1-60	1:200	AB_357038, Cat.no.: FAB1435P, R&D Systems
	Mouse anti-TRA1-81		AB_891610, Cat. no.: 14–8863-82, eBioscience, MA, USA
			AB_891614, Cat. no.: 14–8883-82, eBioscience, MA, USA
Differentiation Markers	Mouse anti-AFP	1:500	AB_258392, Cat.no.: A8452, Sigma/Merck, Darmstadt, Germany
	Mouse anti-B III tubulin	1:2000 1:100	
	Mouse anti-SMA	1:500	AB_357520, Cat.no.: MAB1195, R&D Systems, Minneapolis, USA
	Mouse anti human Sox17	1:500	
	Mouse anti-Nestin		AB_262054, Cat.no.: ab7817, Abcam, Cambridge, UK
	Rabbit anti-BMP4		AB_1861437, Cat.no.: ab84990, Abcam, Cambridge, UK
			AB_2251134, Cat.no.: MAB5326, Sigma/Merck, Darmstadt, Germany
			AB_10974254, Cat.no.: ab124715, Abcam, Cambridge, UK
Secondary antibodies	Alexa Fluor 647-conjugated goat anti-mouse IgG	1:250 1:250	AB_2535804, Cat.no.: A21235, Thermo Fisher Scientific, Massachusetts, USA
	Alexa Fluor 568-conjugated goat anti-rabbit IgG	1:250	AB_143157, Cat.no.: A11011, Thermo Fisher Scientific, Massachusetts, USA
	Alexa Fluor 488-conjugated goat anti-mouse IgG		AB_138404, Cat.no.: A11029, Thermo Fisher Scientific, Massachusetts, USA
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Sendai TaqMan kit	Sendai-SevFam	AB_2760502, Cat.no.: A13640	
	Sendai-Oct3/4	TGCCCCAAGCAGACACCACCTGGCA	
	Sendai-Sox2	TCCCATGCATTCAAACTGACCGTAG	
	Sendai-Cmyc	CACATGTGACCGTAGTAAGAAAAAC	
	Sendai-Klf4	GGGTGAATGGGAAGCGGCCGCATGC CACATGAAGAGGCATTTTTAACCGT	
Pluripotency Markers (qPCR)	NANOG	Cat.no.: 4331182, Hs02387400_g1	
	Brachyury	Cat.no.: 4331182, Hs00610080_m1	
	AFP	Cat.no.: 4331182, Hs00173490_m1	
House-Keeping Genes (qPCR)	RPLP0	Cat.no.: 4331182, 1500979, HS99999902_m1	
Primers for Sanger sequencing	LRRC7	forward: CAATCCTCAAGGATCAGTGGA reverse: TTGTCACCATAGTTACCCAAGTTA	
	KHSRP	forward: GTGGTGTCTGCGCTGGAG reverse:CCGGATGATGAACAACTTGA	

comparing multiple cell lines, which share considerable levels of genetic background (Wright et al., 2014). This approach to decrease genetic heterogeneity could complement the application of genome editing technologies used to establish isogenic cell lines that are also emerging in the in vitro disease modelling field.

4. Materials and methods

4.1. Reprogramming of PBMCs, isolation of iPSC clones and in vitro spontaneous differentiation

Peripheral mononuclear cells (PBMCs) were prepared according to manufacturer's instruction (BD Vacutainer CPT). PBMCs were cultured for 4 days with daily medium changes at a density of 5×10^5 cells/ml in StemPro®-34 (Thermo Fisher Scientific) medium supplemented with 2 mM L-Glutamine and cytokines at the following final concentrations (SCF 100 ng/mL, FLT-3 100 ng/mL, IL-3 20 ng/mL, IL-6 20 ng/mL, all from Peprotech). On day 4, PBMCs were transduced with Sendai virus particles carrying KOS (hKlf4, hOct3/4, hSox2), hc-Myc, and hKlf4 at MOIs of 5,5, and 3, respectively (according to the Cytotune 2.0 Kit user guide (Thermo Fisher Scientific)). Two days after transduction the transduced cells were seeded onto mitomycin treated mouse embryonic fibroblasts (MEFs) and further propagated until pluripotent clumps appeared. Six days after transduction, the culturing medium over the cells was gradually changed to KO-DMEM, supplemented with 15% KO Serum Replacement (Thermo Fisher Scientific), 100 mM glutamine (Thermo Fisher Scientific), 1% nonessential amino acids (Thermo Fisher Scientific), 0.1 mM βMercaptoethanol (Thermo Fisher Scientific) and 4 ng/ml bFGF (Thermo Fisher Scientific). 14–18 days after transduction, individual iPSC colonies were mechanically transferred to new MEF to generate clones.

14–18 days after transduction, individual iPSC colonies were mechanically transferred to mouse embryonic fibroblasts to generate clones. To ensure virus clearance and monitor stability, the clones were repeatedly passaged and expanded up to p10 using trypsin. Heat treatment at 38.5 °C was used between passage p4-p8 to take advantage of the heat sensitivity of virus particles. *In vitro* spontaneous differentiation experiments were performed as described earlier (Erdei et al., 2014). Briefly, three confluent wells of iPS colonies maintained on MEF were removed using by Collagenase IV and transferred into ULA (Ultra low attachment) plates in EB medium (KO-DMEM supplemented with 20% FBS, 1 mM L-GLU, 1% non-essential amino acids, and 0,1 mM ß-mercaptoethanol (Thermofisher Scientific)) to ensure the formation of floating Embryoid Bodies (EBs). The medium was changed daily for 6 days. After 6 days of suspension culture, the EBs were further differentiated on gelatine-coated 8 well confocal chambers for immunofluorescence staining in DMEM supplemented with 10% FBS.

4.2. Alkaline phosphatase assay

Multiple iPSC clones were tested for alkaline phosphatase enzymatic activity using the Alkaline Phosphatase Detection kit (EMD Millipore).

4.3. Immunocytochemistry

iPSCs or iPSC-derived EBs were seeded on Matrigel-coated Lab-Tek chambers (Thermo Fisher Scientific). 1 days after plating of iPSCs or 6 days after plating EBs, the samples were fixed using 4% paraformaldehyde. After blocking and permeabilization with a buffer containing 0.2% BSA, 1% fish gelatine, 3% goat serum, and 0.1% TritonX-100, the cultures were incubated with primary antibodies (see Table 3) for 1 h. Next, the samples were incubated with secondary antibodies and counterstained with DAPI (Sigma). The pictures were taken by ZEISS LSM710 confocal laser scanning microscope.

4.4. Quantitative PCR and virus clearance assay

RNA was isolated with Trizol (Thermo Fisher Scientific). RNA samples were reverse transcribed to cDNA using GoScript™ Reverse Transcriptase (Promega). Taqman gene expression assays were used for quantification of mRNA expression level of Nanog, AFP, Brachyury, and Pax6 relative to that of RPLP0. For monitoring virus clearance, we used

the TaqMan IPSC Sendai detection Kit (Thermo Fisher Scientific).

The PCR reactions were performed following the thermal profile below: denaturation at 95° C for 10 min, followed by incubation at 95° C for 15 s, and annealing at 60° C for 1 min during 40 cycles, using Step One Plus PCR device (Applied Biosystems).

4.5. Flow cytometry analysis

Expression of pluripotency marker SSEA4 was assessed by flow cytometry (as described previously (Erdei et al., 2014)). Data was collected with an Attune Acoustic Focusing Cytometer (Applied Biosystems by Life Technologies) and analysed with the Attune Cytometric Software.

Flow cytometry was performed as described previously (Erdei et al., 2014). SSEA4-PE (R&D Systems, 1:25 dilution), TRA1-60 (eBioscience, 1:200 dilution) and TRA1-81 (eBioscience, 1:200 dilution) were used for investigation of pluripotency while anti-mouse Sca-1 (Ly-6A/E) -APC, (eBioscience, 1:25 dilution) antibody was employed for gating out the MEF. Isotype-matched control mAbs were used for SSEA4 (IgG3-PE (R&D Systems, 1:25 dilution) and for TRA1-60 and TRA1-81 (mouse IgM Isotype Control 11E10 (eBioscience, 1:200 dilution). Secondary antibody AlexaFluor-488 conjugated goat anti-mouse IgG, IgM (H + L) (Invitrogen, 1:250 dilution) was used in the case of TRA1-60 and TRA1-81. The cells were incubated with the antibodies for 30 min at 4 °C, without permeabilization. Before measurements Topro3-iodide (Invitrogen, 1:300 dilution) was used for gating the living cells. Data was collected with FACSAria III Cell Sorter (BD Biosciences) and analyzed with the FCS Express 6 Software.

4.6. Karyotyping and STR analysis

Karyotyping and STR analyses were performed by the Cytogenetic Laboratory of Semmelweis University and UD-GenoMed Medical Genomic Technologies Ltd (Hungary), respectively. Chromosomal abnormalities were screened by G-banding analysis of IPSC clones. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2016). iPSC clones were authenticated by STR analysis using GenePrint® 10 System (Promega).

4.7. Sanger sequencing

Genomic DNA (gDNA) was isolated from the iPSCs using Quick-DNA Miniprep Kit (Zymo Research). For PCR amplification the primers are listed in Table 3. Sanger sequencing reactions were carried out with the 3130 Genetic Analyzer (Applied Biosystems by Life Technologies).

4.8. Mycoplasma test

MycoAlert™ Mycoplasma Detection Kit was used according to the manufacturer's instructions (Lonza).

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.

Acknowledgements

The authors thank Beáta Haraszti, Gyöngyi Buchan and Gergő Vőfély for advice, training and technical assistance.

Funding

This study was funded by the National Brain Research Program (NAP) of Hungary (grant numbers: KTIA_NAP_13-1-2013-0001 to LH, KTIA_NAP_13-2014-0011 to JR, and 2017-1.2.1-NKP-2017-00002 to ÁA, LH, and JMR). The research was also supported by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the Neurology thematic programme of Semmelweis University.

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