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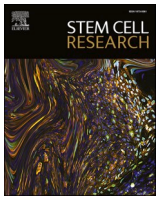

Generation and characterization of human induced pluripotent stem cell line METUi002-A from a patient with primary familial brain calcification (PFBC) carrying a heterozygous mutation (c.687dupT (p. Val230CysfsTer28)) in the *SLC20A2* gene

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A B S T R A C T

Primary familial brain calcification (PFBC) is a rare neurological condition characterized by abnormal calcification commonly in basal ganglia and multiple other brain regions, leading to neuropsychiatric, cognitive, and motor symptoms. *SLC20A2*, one of the known causative genes for PFBC, contains the highest number of variants directly associated with the disease. Here, we established an iPSC line (METUi002-A) from the peripheral blood mononuclear cells of a clinically diagnosed PFBC patient carrying a *SLC20A2* mutation (c.687dupT) using the integration-free Sendai reprogramming. METUi002-A can serve as a valuable tool to generate cellular models to investigate the mechanistic effects of this mutation in PFBC.

Resource Table:

Unique stem cell line identifier	METUi002-A
Alternative name(s) of stem cell line	N/A
Institution	Middle East Technical University, Ankara, Turkey
Contact information of distributor	Erkan Kiris; ekiris@metu.edu.tr
Type of cell line	iPSC
Origin	Human
Additional origin info required. for human ESC or iPSC	Age: 42 Sex: Male Ethnicity if known: N/A
Cell Source	PBMCs
Clonality	Clonal
Method of reprogramming	Sendai Virus-based transduction (CytoTune 2.0)
Genetic Modification	Yes
Type of Genetic Modification	Hereditary
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-PCR
Associated disease	Primary familial brain calcification (PFBC)
Gene/locus	SLC20A2 / 8p11.21c.687dupT

(*continued on next column*)

(*continued*)

Date archived/stock date	June 2023
Cell line repository/bank	https://hpscreg.eu/cell-line/METUi002-A
Ethical approval	Ethical approval was obtained from the Ethics Board of the Gazi University (Turkey). Approval No: 618.

1. Resource utility

There is no cure for PFBC, and mechanisms by which *SLC20A2* mutations lead to PFBC are poorly understood. Here, we report the first PFBC iPSC line with c.687dupT mutation in the *SLC20A2* gene (see Table 1). The iPSCs can be differentiated into disease-relevant cell types as valuable tools for research and drug discovery.

2. Resource details

PFBC is a rare neurological disorder characterized by abnormal accumulation of calcium deposits, bilaterally, in the basal ganglia and

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal hPSC Morphology	Fig. 1 Panel A
Phenotype	Qualitative analysis (Immunocytochemistry)	Colonies were positive for the following pluripotency markers: OCT4, SOX2, TRA1-60, SSEA-4	Fig. 1 Panel C
	Quantitative analysis (RT-qPCR)	Pluripotency markers *POU5F1*, *SOX2* and *NANOG* are endogenously expressed	Fig. 1 Panel D
Genotype	Karyotype (G-banding) and resolution	Normal Karyotype, 46 XY, Banding Resolution: 550–600	Fig. 1 Panel E
Identity	Microsatellite PCR (mPCR) OR	N/A	N/A
	STR analysis	16 loci were tested with a 100 % match	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	*SLC20A2* NM_001257180.2c.687dupT heterozygous mutation	Fig. 1 panel H
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma testing by RT-PCR	Negative	Fig. 1 Panel G
Differentiation potential	Embryoid body formation and *in vitro* spontaneous differentiation	Expression of markers specific for three germ layers detected by Immunocytochemistry (FOXA2& AFP for endoderm, TBX6 & SMA for mesoderm, and Pax6 & TUBB3 for ectoderm)	Fig. 1 Panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

various other brain regions, resulting in neuropsychiatric, cognitive, and motor symptoms (Carecchio et al., 2023). There are currently 7 known causative genes (*SLC20A2*, *PDGFRB*, *PDGFB*, *XPR1, MYORG, JAM2,* and *CMPK2*) for PFBC, and among these, the *SLC20A2* gene variants due to heterozygous mutations are responsible for the majority of PFBC cases (Chen et al., 2023). The *SLC20A2* gene encodes PiT2, a sodium-dependent phosphate co-transporter, which serves to import inorganic phosphate (Pi), and it has been shown that PiT2 defects or deficiencies affect Pi uptake and impaired Pi homeostasis may be critical for PFBC (Wang et al., 2012). Calcification is known to occur in all PFBC patients with gene mutations (Chen et al., 2023); however, the mechanism(s) by which specific *SLC20A2* mutations lead to PFBC is not well-understood. Patient-derived iPSCs carrying specific *SLC20A2* mutations can provide unique tools to study PFBC as these cells can be differentiated to obtain a limitless supply of cell types affected by the disease.

In this study, an iPSC line (METUi002-A) has been generated from a 42-year-old male, carrying a specific *SLC20A2* mutation in the exon 6 (c.687dupT, p.V230Cfs*28), who is diagnosed with PFBC following a thorough neurological examination. To the best of our knowledge, this mutation has been previously reported on another patient (Magistrelli et al., 2021), but its physiological or molecular effects remain poorly understood. The blood sample was obtained from the patient, with informed consent, and PBMCs were isolated and expanded as described previously (Begentas et al., 2022). PBMCs were reprogrammed using CytoTune-iPS Sendai Reprogramming Kit (CytoTune 2.0) and cultured on mitotically inactivated mouse embryonic fibroblast (MEF) feeder layer. Established colonies were comparable to the typical human pluripotent stem cell colony morphology (Fig. 1A) and the clearance of exogenous Sendai virus RNA was determined with RT-PCR at passage 12 (Fig. 1B). Immunocytochemistry analyses demonstrated the protein expressions of pluripotency markers (OCT4, SOX2, TRA-1–60 & SSEA-4) in the colonies (Fig. 1C). Further pluripotency analyses using RT-qPCR demonstrated the expressions of pluripotency markers *POU5F1* (*OCT4*), *SOX2*, and *NANOG,* relative to corresponding PBMCs, as compared to positive control H9 human embryonic stem cells (Fig. 1D). METUi002-A was shown to have 100 % match with its corresponding PBMC by STR analysis and have a normal 46XY karyotype (Fig. 1E). Embryoid body formation and *in vitro* spontaneous differentiation were utilized for the assessment of differentiation capacity to the three germ layers and immunocytochemistry was utilized to examine the expression of AFP and FOXA2 (HNF-3β) for endoderm, SMA and TBX6 for mesoderm, and TUBB3 (TUJ1) and Pax6 for ectoderm (Fig. 1F). PCR based mycoplasma test showed no trace of mycoplasma (Fig. 1G). Finally, the mutation in the *SLC20A2* was confirmed by Sanger sequencing in the established iPSC line, and the electropherogram exhibits the heterozygous c.687dupT mutation in the exon 6 (Fig. 1H, indicated with an arrow).

3. Materials and methods

3.1. PBMC isolation, expansion, and reprogramming

PBMCs were isolated from blood using Histopaque-1077, cultured and expanded in StemSpan SFEM II with 1× StemSpan Erythroid Expansion Supplement, and transduced with CytoTune 2.0 Sendai Virus Reprogramming Kit at a MOI of 5:5:3. Grown under feeder dependent conditions (mitotically inactivated MEFs), single colonies were manually and clonally passaged, with 10 μM Y-27632, until passage 3. iPSCs were then enzymatically passaged using 1 U/mL dispase at a ratio of 1:3 and cultured at 37 °C with 5 % CO_2 in hiPSC medium that included Advanced DMEM/F12, 20 % KSR, 1 % GlutaMAX, 1 % Penicillin-Streptomycin, 50 μM 2-mercaptoethanol, and 4 ng/mL bFGF.

3.2. Karyotype and short tandem repeat (STR) analyses

METUi002-A (p12) was cultured without feeders for 24 h on matrigel using MEF-conditioned hiPSC medium. Following colcemid treatment, cells were incubated with hypotonic KCl solution, fixed using glacial acetic acid and methanol (1:3 ratio), and spread on a glass slide. G-banding was performed using Trypsin-Giemsa staining and at least 20 metaphase spreads were examined at a resolution of 550–600 (Mbgen-lab, Türkiye).

3.3. Gene expression analyses

Total RNA was extracted from passage 12 iPSCs using NucleoSpin RNA Kit, purity was determined with Nanodrop, and converted to cDNA using RevertAid First Strand cDNA Synthesis Kit and used as a template. Removal of Sendai vectors (SeV, KOS, c-Myc, and Klf4) was verified by RT-PCR, at passage 12, with parameters of initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and final elongation 72 °C for 2 min. PCR products were run on 2 % Agarose gel with GeneRuler 1 kb Plus DNA Ladder and imaged using Gel Imaging Station. Endogenous expression of pluripotency markers (*POU5F1*, *SOX2,* and *NANOG*) was measured using RT-qPCR using Corbett Rotor-Gene 6000 system. SsoAdvanced Universal SYBR Green

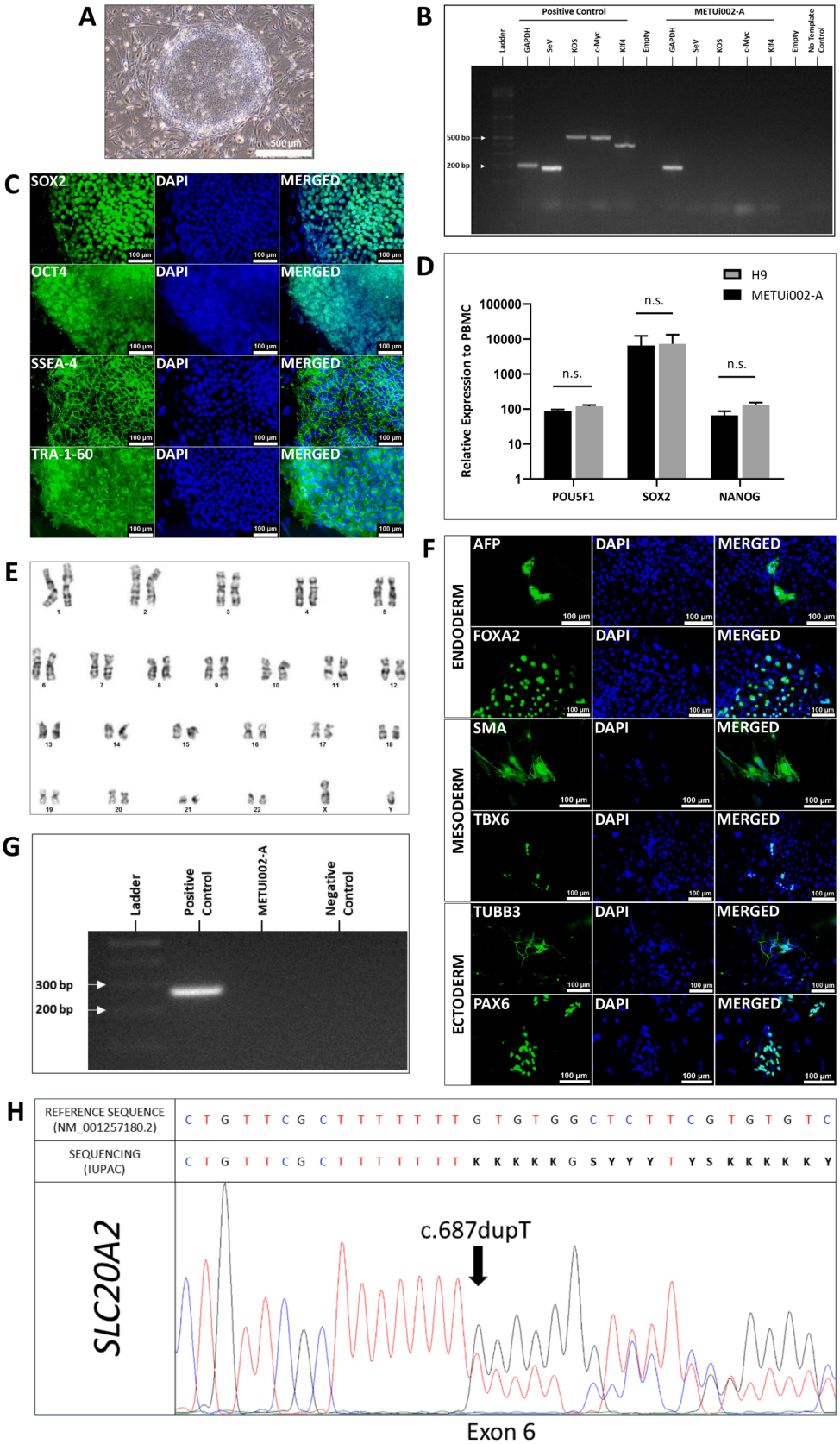

Fig. 1. Characterization of METUi002-A cell line.

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Mouse anti-Oct-3/4	1:100	Santa Cruz Biotechnology Cat# sc-5279	RRID: AB_628051
	Mouse anti-Sox-2	1:100	Santa Cruz Biotechnology Cat# sc-365,823	RRID: AB_10842165
	Mouse anti-TRA-1–60	1:100	Santa Cruz Biotechnology Cat# sc-21,705	RRID: AB_628385
	Mouse anti-SSEA-4	1:100	DSHB Cat# MC-813–70 (SSEA4)	RRID: AB_528477
Differentiation Markers	Mouse anti-AFP	1:100	Santa Cruz Biotechnology Cat# sc-8399	RRID: AB_626665
	Mouse anti-SMA	1:100	Santa Cruz Biotechnology Cat# sc-53142	RRID: AB_2273670
	Mouse anti-Beta III- Tubulin (TUBB3)	1:500	R&D Systems Cat# BAM1195	RRID: AB_356859
	Mouse anti-HNF-3β (FOXA2)	1:100	Santa Cruz Biotechnology Cat# sc-374376	RRID: AB_10989742
	Mouse anti-TBX6	1:100	Santa Cruz Biotechnology Cat# sc-517027	RRID: AB_3068012
	Mouse anti-Pax6	1:100	DSHB Cat# pax6	RRID: AB_528427
Secondary antibody	Goat antiMouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:200	Thermo Fisher Scientific Cat# A-11029	RRID: AB_2534088

	Primers		
	Target	Size of band	Forward/Reverse primer (5′-3′)
SeV-specific primers (RT-PCR)	SeV	181 bp	GGATCACTAGGTGATATCGAGC/ ACCAGACAAGAGTTTAAGAGATATGTATC
SeV-specific primers (RT-PCR)	hKOS	528 bp	ATGCACCGCTACGACGTGAGCGC/ ACCTTGACAATCCTGATGTGG
SeV-specific primers (RT-PCR)	hKlf4	410 bp	TTCCTGCATGCCAGAGGAGCCC/ AATGTATCGAAGGTGCTCAA
SeV-specific primers (RT-PCR)	hc-Myc	532 bp	TAACTGACTAGCAGGCTTGTCG/ TCCACATACAGTCCTGGATGATGATG
Targeted mutation analysis/ sequencing	*SLC20A2*	671 bp	CGCCAGATATAAGATGTCTTGAAC/ GGAATTGCAGGAACTTTGTACTCT
House-Keeping gene (RT-qPCR)	*GAPDH*	197 bp	GGAGCGAGATCCCTCCAAAAT/ GGCTGTTGTCATACTTCTCATGG
Pluripotency marker primers (RT-qPCR)	*POU5F1* (*OCT4*)	63 bp	GGGTTTTTGGGATTAAGTTCTTCA/ GCCCCCACCCTTTGTGTT
Pluripotency marker primers (RT-qPCR)	*SOX2*	63 bp	CAAAAATGGCCATGCAGGTT/ AGTTGGGATCGAACAAAAGCTATT
Pluripotency marker primers (RT-qPCR)	*NANOG*	190 bp	CCTGAAGACGTGTGAAGATGAG/ GCTGATTAGGCTCCAACCATA
Pluripotency marker primers (RT-qPCR)	*MGSO & GPO-3*	270 bp	TGCACCATCTGTCACTCTGTTAACCTC/ GGGAGCAAACAGGATTAGATACCCT

Supermix was used for qPCR reactions. Data were normalized against PBMCs, using *GAPDH* as a reference, and analyzed by the $2^{-\Delta\Delta Ct}$ method.

3.4. In vitro spontaneous differentiation

iPSCs at passage 12 were detached from feeders using dispase and cultured in hiPSC medium without bFGF for embryoid body induction. EBs were maturated for 3 weeks and seeded on 0.1 % gelatin-coated chamber slides for another week for differentiation and immunocytochemistry analyses.

3.5. Immunocytochemistry

Cells were fixed with 4 % PFA, permeabilized with 0.3 % Triton-X-100, and then blocked with 3 % BSA in PBS. Primary antibodies (Table 2) were incubated overnight at 4 °C in 0.3 % BSA, and secondary antibodies were applied at RT for 2 h. Slides were then mounted using ProLong Gold Antifade with DAPI, and imaged using FLoid Cell Documentation System.

3.6. Mutation analysis

For mutation verification, gDNA was purified from the established METUi002-A using GeneJet Genomic DNA Purification Kit, PCR was performed using primers targeting exon 6 of *SLC20A2* (Table 2), and the amplicon was sequenced by Sanger sequencing (Genoks).

3.7. Mycoplasma screening

PCR-based screening was utilized for the detection (Begentas et al., 2022), using MGSO & GPO-3 primers (Table2) targeting 16S rRNA of a wide range of *Mycoplasma* species.

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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