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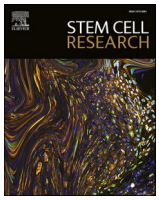

Lab Resource: Single Cell Line

Generation of the human iPSC line AKOSi010-A from fibroblasts of a female FAHN patient, carrying the compound heterozygous mutation p.Gly45Arg/p.His319Arg

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A B S T R A C T

Fatty acid hydroxylase-associated neurodegeneration (FAHN) is a rare childhood onset neurodegenerative disease caused by mutations in the FA2H gene. Patients display abnormal myelination, cerebellar atrophy and some have iron deposition in the central nervous system. Here we describe the generation of AKOSi010-A, a human induced pluripotent stem cell (hiPSC) line derived from fibroblasts of a female patient carrying the compound heterozygous p.Gly45Arg/p.His319Arg, using non-integrating Sendai virus. The generated iPSCs express pluripotency markers, can differentiate into cell types of the three germ layers and show a normal karyotype. This cell line displays a unique source to study the pathophysiology of FAHN.

1. Resource Table

Unique stem cell line identifier	AKOSi010-A
Alternative name(s) of stem cell line	iPSC FAHN1-S43
Institution	Translational Neurodegeneration Section "Albrecht Kossel", Department of Neurology, University Medical Center Rostock, 18147 Rostock, Germany
Contact information of distributor	Dr. Moritz J. Frech; moritz.frech@med.uni-rostock.de
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 24 years Sex: Female
Cell Source	Fibroblasts
Clonality	Clonal
Associated disease	Fatty acid hydroxylase-associated neurodegeneration (FAHN)
Gene/locus	*FA2H* / 16q23.1
Date archived/stock date	April 2021
Cell line repository/bank	https://hpscreg.eu/cell-line/AKOSi010-A
Ethical approval	Children's Hospital of Eastern Ontario, Ontario, Canada, Ottawa, ON, K1H 8L1

2. Resource utility

Fatty acid hydroxylase-associated neurodegeneration (FAHN) is a rare disease caused by mutations in the fatty acid 2-hydroxylase (*FA2H)* gene belonging to the disease family of neurodegeneration with brain iron accumulation (NBIA). The pathophysiological mechanisms of FAHN are poorly understood and iPSC lines from FAHN patients are not yet available. Thus, the generated iPSCs represent a valuable tool to gain a better understanding of the pathophysiology of FAHN.

3. Resource details

Neurodegeneration with brain iron accumulation (NBIA) comprises a group of progressive rare neurodegenerative diseases characterized by iron accumulation mainly in the basal ganglia of the central nervous system. A rare form of NBIA is due to mutations in the FA2H gene, and patients carrying such mutations suffer from a condition called fatty acid hydroxylase neurodegeneration (FAHN, OMIM #611026, (Hinarejos et al., 2020). FA2H encodes an enzyme that catalyzes the hydroxylation of 2-hydroxy fatty acids. These fatty acids display a precursor for ceramides, which are in turn main components of sphingolipids and

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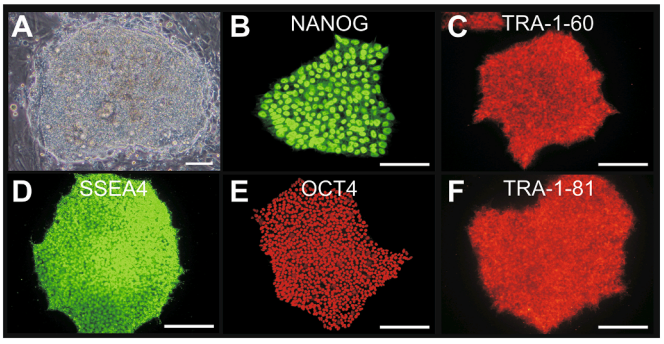
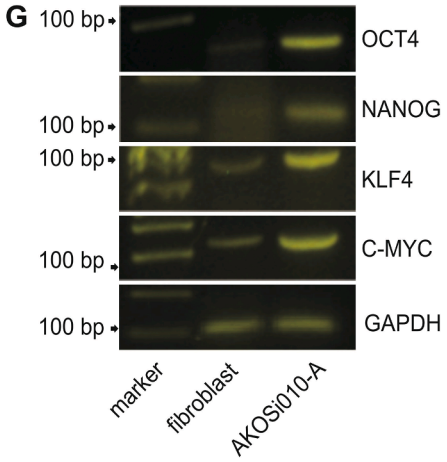
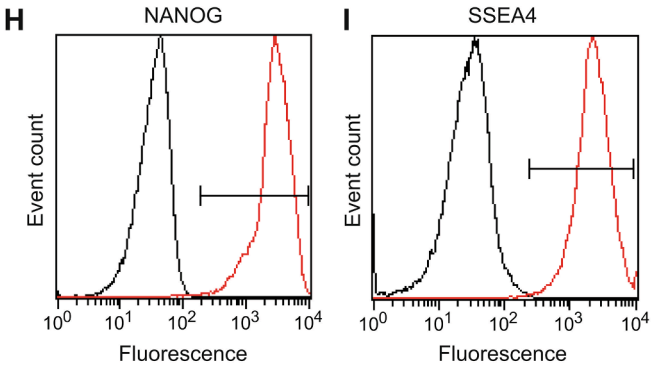
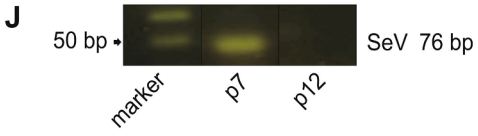
myelin. Consequently, abnormal myelination can be observed in certain parts of the CNS of FAHN patients. However, little is known about the pathophysiology underlying the observed myelin abnormalities or iron deposition. This may be due to the fact that only a low number of patients with FAHN have been described worldwide to date (Kolarova et al., 2022). Therefore, it is important to develop cellular model systems that can contribute to a better understanding of the pathophysiological processes of FAHN. Against this background, we describe here the development of an iPSC line derived from patient-specific fibroblasts from a symptomatic FAHN patient, carrying the compound heterozygous FA2H mutation p.Gly45Arg/p.His319Arg.

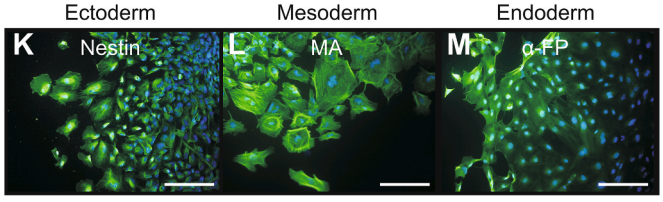
Using a non-integrating Sendai virus encoding KLF4, OCT 3/4, SOX2, and C-MYC factors, patient-derived fibroblasts were reprogrammed into iPSCs. The obtained iPSC colonies displayed stem cell-like morphology (Fig. 1A, all scale bars = 200 μm). Pluripotency was proven by immunofluorescence staining of the pluripotency markers NANOG, TRA-1–60, SSEA4, OCT4 and TRA-1–81 (Fig. 1B-F, all scale bars = 100 μm) after passage 12 of iPSCs. The expression of pluripotency-related genes

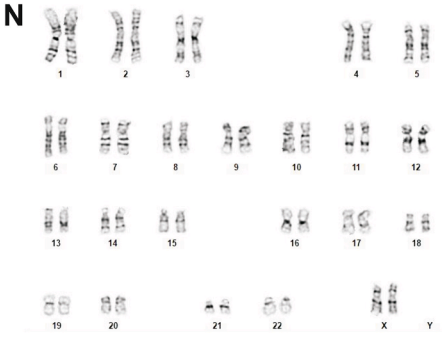












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	Gene	Amino Acid Change	Allele Coverage	Allele Ratio	Frequency %
Fibroblasts	FA2H	p.Gly45Arg	C=980 T=1013	C=0.4917 T=0.5083	50.83
	FA2H	p.His391Arg	T=984 C=1016	T=0.492 T=0.5083	50.80
AKOSi-010A	FA2H	p.Gly45Arg	C=963 T=1032	C=0.4827 T=0.5173	51.73
	FA2H	p.His391Arg	T=1026 C=974	C=0.513 T=0.487	48.70

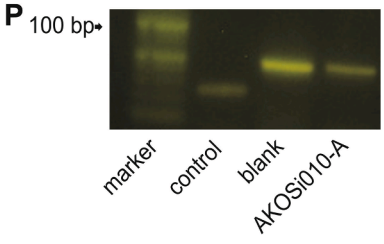

Fig. 1. Characterization of AKOSi010-A iPSC line.

OCT4, NANOG, KLF4, and C-MYC was shown using RT-PCR (Fig. 1G) after passage 12. Quantitative analysis of the pluripotency markers NANOG and SEEA4 was done by flow cytometry (Fig. 1H, I) after passage 12. RT-PCR was performed to confirm the presence or absence of the Sendai virus backbone. As an example the analysis of passage 7 is shown, in which the backbone was still detectable. After passage 12, the backbone was no longer detectable. (Fig. 1J). To confirm the differentiation capability, embryoid body (EB) formation was used and the expression of specific markers for the ectoderm (Pax6, Nestin), the endoderm (α-FP, Sox17) and the mesoderm (MA, Pax3/Pax7) was proven by immunocytochemistry after passage 12. Examples of immunofluorescence staining are shown for Nestin (Fig. 1K, scale bar = 100 μm), muscle actin (MA, Fig. 1L, scale bar = 100 μm) and α-feto-protein (α-FP, Fig. 1M, scale bar = 100 μm). A normal female karyotype (46, XX) was observed in G-banding karyotyping analysis (Fig. 1N) after passage 12. The compound heterozygous mutation p.Gly45Arg/p.His319Arg was confirmed, both in the parental fibroblasts and the iPSC line, using targeted NGS sequencing (Fig. 1O). Comparison of short tandem repeat analysis of 18 genomic loci between parental fibroblast cells and iPSCs confirmed that iPSCs originated from the patient specific fibroblasts (Table 1). Mycoplasma infection was excluded by PCR analysis (Fig. 1P). In conclusion, we report the successful establishment of the iPSC line AKOSi010 carrying the compound heterozygous FA2H mutation p.Gly45Arg/p.His319Arg.

4. Materials and methods

4.1. Reprogramming of fibroblasts

Fibroblasts were cultured in high glucose DMEM (Gibco) supplemented with 10 % fetal bovine serum (GE Healthcare) and 1 % penicillin–streptomycin (10000 U/ml, Gibco). For reprogramming, the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) was used. Fibroblasts were transduced with KLF4, OCT 3/4, SOX2 and C-MYC. iPSC colonies were picked and maintained on Matrigel coated dishes in E8 medium (Thermo Fisher Scientific) with daily medium changes and passaged every 5–7 days. Cells were incubated at 37 °C in a humidified 5 % CO2 incubator as previously described (Völkner et al., 2020).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1 A
Phenotype	Qualitative analysis	Immunofluorescence: NANOG, TRA-1-60, SSEA4, OCT4, TRA-1-81	Fig. 1 B-F
	Quantitative analysis	FACS: NANOG: 98 % OCT4: 94 % SSEA4: 97 % % TRA-1-60: 94 % TRA-1-81: 93 %	Example for NANOG and SSEA4 Fig. 1 H and I
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 400 – 450 bands	Fig. 1 N
Identity	STR analysis	18 STR loci tested, all matched	with author
Mutation analysis	N/A	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR was negative	Fig. 1P
Differentiation potential	Embryoid body formation	Ectoderm: Nestin, PAX6 Mesoderm: MA, Pax3 Endoderm: α-FP, SOX17	Example of Nestin Fig. 1K MA Fig. 1L α-FP Fig. 1M
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

4.2. Alkaline phosphatase staining

iPSC colonies were fixed with ice-cold methanol for 10 min and incubated for 15 min with staining solution containing 75 % distilled water, 10 % 1 M sodium chloride, 10 % 1 M Tris (pH 9.8), 5 % 1 M magnesium chloride, 1:50 NBT/BCIP stock solution (Roche).

4.3. Immunofluorescence

For immunofluorescence staining, the iPSC colonies were maintained on matrigel-coated glass cover slips and fixed in 4 % paraformaldehyde for 15 min. Blocking was carried out using PBS containing 10 % normal goat serum and 0.1 % Triton-X 100 for 45 min. Primary antibodies were incubated overnight at 4 °C and secondary antibodies for 1 h at room temperature (RT). DAPI was added for 5 min at room temperature and cover slips were then mounted on glass slides with Fluoromount-G® (SouthernBiotech). Images were acquired using a Keyence BZ-8000 K microscope (Keyence). For antibodies and dilutions please refer to Table 2.

4.4. Flow cytometry

$1x10^6$ cells were collected for each sample using Gentle Cell Dissociation Reagent (STEMCELL Technologies). For the analysis of the pluripotency markers NANOG and SSEA4, cells were prepared with the True-NuclearTM Transcription Factor Buffer Set (Biolegend). Fluorophore-conjugated antibodies were incubated for 1 h at RT in the dark (Table 2). $5x10^4$ cells were measured with FACSCalibur (BD) and analysis was done with the FCSalyzer software version 0.9.18-alpha.

4.5. Embryoid body formation

For spontaneous formation of embryoid bodies (EBs), colonies were treated with 0.5 mM ETDA/PBS and detached with a cell scraper and subsequently transferred to a low attachment plate. EBs were cultured in suspension for 5 days in mTeSR1 supplemented with 4 mg/ml polyvinylalcohol. After 5 days EBs were seeded onto gelatin-coated cover slips and maintained in EB-medium (78 % Knockout DMEM, 0.1 mM MEM non-essential amino acids, 1 % GlutaMax, 0.1 mM 2-mercaptoethanol, 0.25 % penicillin–streptomycin (all Gibco) and 20 % FBS (GE Healthcare)) for 10 days. EBs were fixed in 4 % paraformaldehyde for 15 min and then subjected to immunofluorescence.

4.6. Targeted sequencing

Genomic DNA was extracted using the Quick-DNA™ Miniprep Kit (Zymo Research). Targeted sequencing library construction was performed using a custom designed Ion AmpliSeq™ FA2H Panel (Thermo Fisher Scientific). 10 ng of genomic DNA were used for library construction covering the complete coding sequence. Sequencing was carried out on an Ion Torrent™ Personal Genome Machine™ System, using an Ion Torrent 318 V2 chip. Sequence analysis was performed using the hg19 assembly of the human genome using Torrent Suite™ software and the variant caller plugin version 5.12.V2 (Thermo Fisher Scientific).

4.7. RT-PCR

Total RNA was extracted with the Quick-RNA Miniprep kit (Zymo Research). An Eppendorf 5331 MasterCycler Gradient Thermal Cycler

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency Marker (IF)	Rabbit anti-OCT4	1:100	Stemgent Cat# 09-0023	RRID: AB_2167689
Pluripotency Marker (IF)	Rabbit anti-NANOG	1:100	Stemgent Cat# 09-0020	RRID: AB_2298294
Pluripotency Marker (IF)	Mouse anti-SSEA4	1:100	Stemgent Cat# 09-0006	RRID: AB_1512169
Pluripotency Marker (IF)	Mouse anti-TRA-1-60	1:100	Stemgent Cat# 09-0010	RRID: AB_1512170
Pluripotency Marker (IF)	Mouse anti-TRA-1-81	1:100	Stemgent Cat# 09-0011	RRID: AB_1512171
Pluripotency Marker (FC)	Alexa Fluor 488 anti-OCT4, mouse IgG2b	1:20	BioLegend Cat# 653705	RRID: AB_2562250
Pluripotency Marker (FC)	Alexa Fluor 647 anti-NANOG, mouse IgG1	1:50	BioLegend Cat# 674210	RRID: AB_2650619
Pluripotency Marker (FC)	Alexa Fluor 647 anti- SSEA-4, mouse IgG3	1:500	BioLegend Cat# 330407	RRID: AB_1089201
Pluripotency Marker (FC)	PE anti-human TRA-1-60, mouse IgM	1:20	BioLegend Cat# 330609	RRID: AB_1279447
Pluripotency Marker (FC)	Alexa Fluor 488 anti-TRA-1-81, mouse IgM	1:20	BioLegend Cat# 330709	RRID: AB_2561741
Differentiation Marker (IF)	Mouse anti-Muscle actin	1:50	Agilent Dako Cat# M0635	RRID: AB_2242301
Differentiation Marker (IF)	Mouse anti-Nestin	1:100	R and D Systems Cat# MAB1259	RRID: AB_2251304
Differentiation Marker (IF)	Mouse anti-α fetoprotein	1:20	R and D Systems Cat# MAB1368	RRID: AB_357658
Differentiation Marker (IF)	Anti-PAX6 antibody	1:50	Abcam Cat# EPR15858	RRID: AB_195045
Differentiation Marker (IF)	PAX3 Antibody	1:50	Thermo Fisher Scientific Cat# 38-1801	RRID: AB_2533359
Differentiation Marker (IF)	Anti-SOX17 antibody		Abcam Cat# EPR20684	RRID: AB_224637
Secondary antibody	Alexa Fluor 488, Goat anti-mouse IgG	1:500	Thermo Fisher Scientific Cat# A-11029	RRID: AB_2534088
Secondary antibody	Alexa Fluor 568, Goat anti-mouse IgM	1:500	Thermo Fisher Scientific Cat# A-21043	RRID: AB_2535712
Secondary antibody	Alexa Fluor 488, Goat anti-rabbit IgG	1:500	Thermo Fisher Scientific Cat# A-11034	RRID: AB_2576217

	Primers		
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**
Pluripotency Marker (RT-PCR)	*C-MYC*	325 bp	GCGTCCTGGGAAGGGAGATCCGGAGC/TTGAGGGGCATCGTCGCGGGAGGCTG
Pluripotency Marker (RT-PCR)	*NANOG*	128 bp	TGTGTTCTCTTCCACCCAGC/ACCAGGTCTTCACCTGTTTGT
Pluripotency Marker (RT-PCR)	*OCT4*	144 bp	GACAGGGGGAGGGGAGGAGCTAGG/CTTCCCTCCAACCAGTTGCCCCAAAC
Pluripotency Marker (RT-PCR)	*KLF4*	397 bp	ACGATCGTGGCCCCGGAAAAGGACC/TGATTGTAGTGCTTTCTGGCTGGGCTCC
Sendai reprogramming vector (RT-PCR)	*SeV*	181 bp	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTT AAGAGATATGTATC
House-Keeping Gene (RT-PCR)	*GAPDH*	112 bp	CATGTTCCAATATGATTCCACCC/GGGATCTCGCTCCTGGAAGAT
House-Keeping Gene (RT-PCR)	*G6PD*	76 bp	TGCCCCCGACCGTCTAC/ATGCGGTTCCAGCCTATCTG

was used to perform a One-step RT-PCR (QIAGEN). 50 ng of total RNA were used for each reaction. Cycle number and annealing temperatures were optimized for each primer (Table 2). PCR products were run on TBE agarose gels.

4.8. Transgene expression silencing

RNA of iPSCs was obtained using the Quick-RNA Miniprep kit (Zymo Research). cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen). Absence of transgene was detected by PCR using LightCycler® FastStart DNA MasterPLUS SYBR Green I Kit (Roche) and transgene-specific primers (Table 2).

4.9. Karyotyping

Karyotype analysis was performed commercially by WiCell (Madison, Wisconsin, USA) at passage 12. G-banding of 20 metaphases spreads, with a resolution of 400 – 450 bands per haploid, set was performed.

4.10. STR analysis

Short Tandem Repeat (STR) Analysis of 18 STR loci was carried out the ATCC FTA Sample Collection Kit for Human Cell Authentication Service (ATCC, Manassas, Virginia, USA). Samples of fibroblasts and iPSCs, containing $1x10^6$ cells/ml PBS, were collected on Whatman® FTA® cards according to the manufacturer's protocol.

4.11. Mycoplasma detection

Absence of mycoplasma was proven after passage 12 with the PCR Mycoplasma Test Kit I/C (PromoCell) according to manufacturer's instructions.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Fatima Efendic reports financial support was provided by NBIA Disorders Association. Andreas Hermann reports financial support was provided by Hermann and Lilly Schilling Foundation. Christin Voelkner reports financial support was provided by State Office for Health and Social Services Mecklenburg West Pomerania.

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