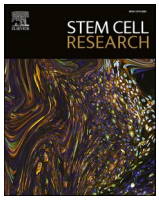
Stem Cell Research 76 (2024) 103363

Contents lists available at ScienceDirect

Stem Cell Research

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



An iPSC model for POLR3A-associated spastic ataxia: Generation of three unrelated patient cell lines

Kalaivani Manibarathi [a,b,c,d,f,g], Tam Pham [b,c,e], Holger Hengel [b,c], Matthis Synofzik [b,c], Maike Nagel [b,c,d], Rebecca Schüle [a,b,c,*]

[a] Division of Neurodegenerative Diseases, Department of Neurology, Heidelberg University Hospital and Faculty of Medicine, Heidelberg, Germany
[b] Center for Neurology and Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany
[c] German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany
[d] Graduate School of Cellular and Molecular Neuroscience, University of Tübingen, Tübingen, Germany
[e] Interfaculty Institute of Biochemistry (IFIB), Tübingen, Germany
[f] Faculty of Biosciences, University of Heidelberg, Heidelberg, Germany
[g] Interdisciplinary Center for Neurosciences (IZN), Heidelberg, Germany

A B S T R A C T

Spastic Ataxias (SA) are a group of neurodegenerative disorders with combined pyramidal and cerebellar system affection, leading to an overlap phenotype between Hereditary Spastic Paraplegias (HSP) and Cerebellar Ataxias (CA). Here we describe the generation of iPSCs from three unrelated patients with an ultra-rare subtype of SA caused by compound heterozygous mutations in *POLR3A*, that encodes the largest subunit of RNA polymerase III. iPSCs were reprogrammed from normal human dermal fibroblasts (NHDFs) using episomal reprogramming with integration-free plasmid vectors: HIHRSi004-A, derived from a 44 year-old male carrying the mutations *c.1909 + 22G > A/c.3944_3945delTG*, HIHRSi005-A obtained from a 66 year-old male carrying the mutations *c.1909 + 22G > A/c.1531C > T*, and HIHRSi006-A from a 27 year-old male carrying the mutations *c.1909 + 22G > A/c.2472_2472delC* (ENST00000372371.8).

Resource Table:

Unique stem cell lines identifier	*HIHRSi004-A* *HIHRSi005-A* *HIHRSi006-A*
Alternative name(s) of stem cell lines	*iPSC SP-184 4.1 (Unique cell line 1 name)* *iPSC LD-12 5.3 (Unique cell line 2 name)* *iPSC SP-76 1.3 (Unique cell line 3 name)*
Institution	*Hertie Institute for Clinical Brain Research, Tübingen, Germany; German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany;* *Division of Neurodegenerative Diseases, Department of Neurology, Heidelberg University Hospital and Faculty of Medicine, Heidelberg, Germany.*
Contact information of distributor	*Rebecca Schüle* *rebecca.schuele@uni-heidelberg.de*
Type of cell lines	*iPSC*
Origin	*Human*
Additional origin info required	*Age: 49,74,36 44, 66, 27* *Sex: Male, Male, Male*

(*continued on next column*)

(*continued*)

Unique stem cell lines identifier	*HIHRSi004-A* *HIHRSi005-A* *HIHRSi006-A*
	Ethnicity if known: Western European, Middle European, Western European
Cell Source	*Dermal fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Episomal reprogramming*
Genetic Modification	*No*
Type of Genetic Modification	*N/A*
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*PCR for episomal vectors*
Associated disease	*Spastic ataxia (ORPHA: 685), Hereditary Spastic Paraplegia (ORPHA: 316240)*
Gene/locus	*POLR3A*
Date archived/stock date	*Jan 2024*
Cell line repository/bank	*TreatHSP Biobank (https://www.treathsp.net)*
Ethical approval	*199/2011BO1, 423/2019BO1, S-439/2023*

* Corresponding author at: Division of Neurodegenerative Diseases, Department of Neurology, Heidelberg University Hospital and Faculty of Medicine, Heidelberg 69120, Germany.
E-mail address: rebecca.schuele@uni-heidelberg.de (R. Schüle).

https://doi.org/10.1016/j.scr.2024.103363
Received 19 December 2023; Received in revised form 17 February 2024; Accepted 24 February 2024
Available online 25 February 2024

1. Resource utility

POLR3A spastic ataxia is an ultra-rare neurodegenerative disorder with combined pyramidal and cerebellar system affection. Majority of cases are caused by a specific recurrent intronic variant in *POLR3A* (*c.1909 + 22G > A*) occurring *in trans* with a second, variable loss of function allele. The iPSC lines reported here can be differentiated into relevant cellular lineages to study the pathophysiology of *POLR3A*-associated spastic ataxia and the effects of its therapeutic interventions.

2. Resource details

Human dermal fibroblasts obtained from three unrelated individuals with spastic ataxia caused by mutations in *POLR3A* were reprogrammed using episomal reprogramming vectors that encodes for human *OCT4*, *KLF4*, *L-MYC*, *SOX2* and *LIN28* (Okita et al., 2011). All three lines share an intronic variant introducing a cryptic splice site in *POLR3A* (*c.1909 + 22G > A*), compound heterozygous with a second, variable *POLR3A* variant (Minnerop et al., 2017). Fibroblasts for the line iPSC SP-184 4.1 were obtained from a 44 year-old male of western European origin (*c.1909 + 22G > A/c.3944_3945delTG,* p.Y637Cfs*14/p.V1315fs*7). Fibroblasts for the line iPSC LD-12 5.3 were derived from a 66 year-old male of middle European origin (*c.1909 + 22G > A/c.1531C > T,* p.Y637Cfs*14/p.Q511*). Fibroblasts for the line iPSC SP-76 1.3 were acquired from a 27 year-old male of western European origin (*c.1909 + 22G > A/c.2472_2472delC,* p.Y637Cfs*14/p.S825Qfs*18). Details on the clinical phenotypes of all three patients have been published before (Minnerop et al., 2017).

8–10 days after nucleofection of the fibroblast lines, iPSC-like colonies became visible and were picked manually after 24–28 days, and further expanded for culturing and cryopreservation. The iPSC lines thus obtained exhibited an embryonic stem cell like morphology (Fig. 1E). By passage 8–10, the iPSC lines were transgene-free (Fig. 1F) as well as mycoplasma-free (Supplementary Fig. 1B). The cell lines were then subjected to SNP genotyping to confirm genomic integrity (Fig. 1A) and Sanger sequencing to confirm the presence of the patient mutations as in the fibroblast lines (Supplementary Fig. 1A).

The pluripotency of the three iPSC lines was confirmed at the protein expression level with immunocytochemistry for the markers TRA-1–81, and OCT4 (Fig. 1B), and with an alkaline phosphatase (ALP) staining which is a classical marker for iPSCs (Fig. 1D). On transcript level, expression of the markers *OCT4*, *SOX2*, *KLF4*, *C-MYC, NANOG*, *DNMT3B*, *TDGF1* were analysed with a RT-qPCR: Marker expression profiles in iPSC lines were clearly distinct from the source fibroblast lines but demonstrated similarities to the human embryonic stem cell line I3, thus confirming stemness of the cell lines (Fig. 1G). The differentiation potential of the three iPSC lines was assessed using an embryoid body based spontaneous differentiation method producing the three germ layers: ectodermal, mesodermal, and endodermal cell lineages which were probed for lineage-specific markers (Fig. 1B, scale bar 100 μm). The differentiation potential and the pluripotency tests together confirms the stem cell characteristics of the generated iPSC lines (see Table 1).

3. Materials and methods

3.1. Cell culture and reprogramming

Human skin fibroblasts were cultured in fibroblast culture medium: DMEM (Sigma-Aldrich) with 10 % FCS (Life Technologies) to around 80 % confluency. For reprogramming, fibroblasts (3*10^5 cells) were resuspended in 82 μl of fibroblast nucleofector solution (Lonza), 18 μl of supplement solution (Lonza) and 3 μg of each episomal plasmid as described (Okita et al., 2011) (pCXLE-hUL, ID: #27080; pCXLE-hSK, ID: #27078 and pCXLE-hOCT3/4, ID: #27076 purchased from Addgene), and transferred to an electroporation cuvette and nucleofected by using the Amaxa 2b nucleofector program P-022 (Human Dermal Fibroblast Nucleofection Kit VPD-1001 [Lonza]). The reprogrammed cells were then seeded onto Matrigel® coated (1:60 in DMEM, Corning) 6-well plates in fibroblast culture medium. On the second day, medium was changed to fibroblast culture medium supplemented with 2 ng/ml FGF2 (Peprotech) and 1 % P/S (Gibco). Media was further switched to the in-house E8 medium as described previously (Nagel et al., 2019) along with 100 μM sodium butyrate (Sigma-Aldrich) and 0.1 % P/S, and subsequently changed every other day. After 3 to 4 weeks, the reprogrammed iPSC colonies were picked manually assisted with P26 gauge needles and placed separately onto Matrigel® coated (1:60) 24-well plates. At around 80 % confluency, iPSCs were passaged at a ratio of 1:12, using 0.2 % (w/v) EDTA (AppliChem) in PBS (Sigma-Aldrich), and plated onto Matrigel® coated (1:60) 6-well plates, using E8 medium, supplemented with Rock inhibitor Y-27632 (10 μM in DMSO, Abcam Biochemicals), which was also used at further passaging. iPSCs were frozen into cryostocks for maintenance using 50 % E8, 40 % KO-SR (Life Technologies), 10 % DMSO (Sigma-Aldrich) and 10 μM Y-27632. A PCR based mycoplasma testing (PanReac AppliChem kit) was performed for each generated line following the manufacturer's recommendations. All cells described were maintained at 37 °C and 5 % CO_2.

3.2. Analysis of genomic integrity

To confirm that the iPSC lines were transgene-free after reprogramming, genomic DNA was extracted after the 10th passage by using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) and were analysed via touchdown PCR with primers specific to the reprogramming factors (Table 2) in a Bio-Rad T100 Thermal Cycler (annealing temperature, TA: 65 °C, decreasing by 1 °C each cycle to 55 °C, repeat: 25 cycles at 55 °C, elongation time, TE: 30 s). Presence of the respective *POLR3A* variants were confirmed by conventional Sanger sequencing in the iPSCs along with their fibroblasts on a Genetic Analyzer 3130xl (Applied Biosystems) using BigDye® Terminator v3.1 chemistry and primers as listed (Table 2).

Genomic integrity was further analysed using a whole-genome SNP genotyping method using the HumanOmni2.5Exome-8 BeadChip v1.5 (Illumina) (Fig. 1A). To independently confirm the identity of the reprogrammed cells and to validate the parentage of the iPSC lines to their corresponding fibroblasts, an STR analysis was performed. For this, six short tandem repeats loci (D12S1648, D6S265, D12S345, D8S323, D12S1687, D12S85) were amplified via touchdown-PCR (16 cycles, TA 65 °C to 55 °C, TE 30 s, 19 cycles, TA 55 °C, TE 30 s, final TE 5 min) and the resulting fragments were analysed with a Genescan™ size standard (Applied Biosystems), using the Gene Mapper generic software (Applied Biosystems).

3.3. Analysis of stem cell characteristics

The stem cell characteristics of the three generated iPSC lines were investigated with the following experiments:

1) Pluripotency markers: To examine the expression of pluripotency markers through immunocytochemistry, iPSC cell lines were fixed with 4 % paraformaldehyde (PFA) (Merck) for 15 min at RT, followed by permeabilization with 0.1 % Triton-X (Carl Roth), and blocked with 5 % BSA/PBST (Thermo Fisher scientific) for 1 h at RT. They were further incubated with specific antibodies for pluripotency markers (Table 2) over night at 4 °C. These primary antibodies were detected via Alexa fluorophore conjugated secondary antibody staining for 1 h at RT (Table 2) and are counterstained with DAPI (1:10,000 in PBS, 5 min at RT, Invitrogen). The slides were further

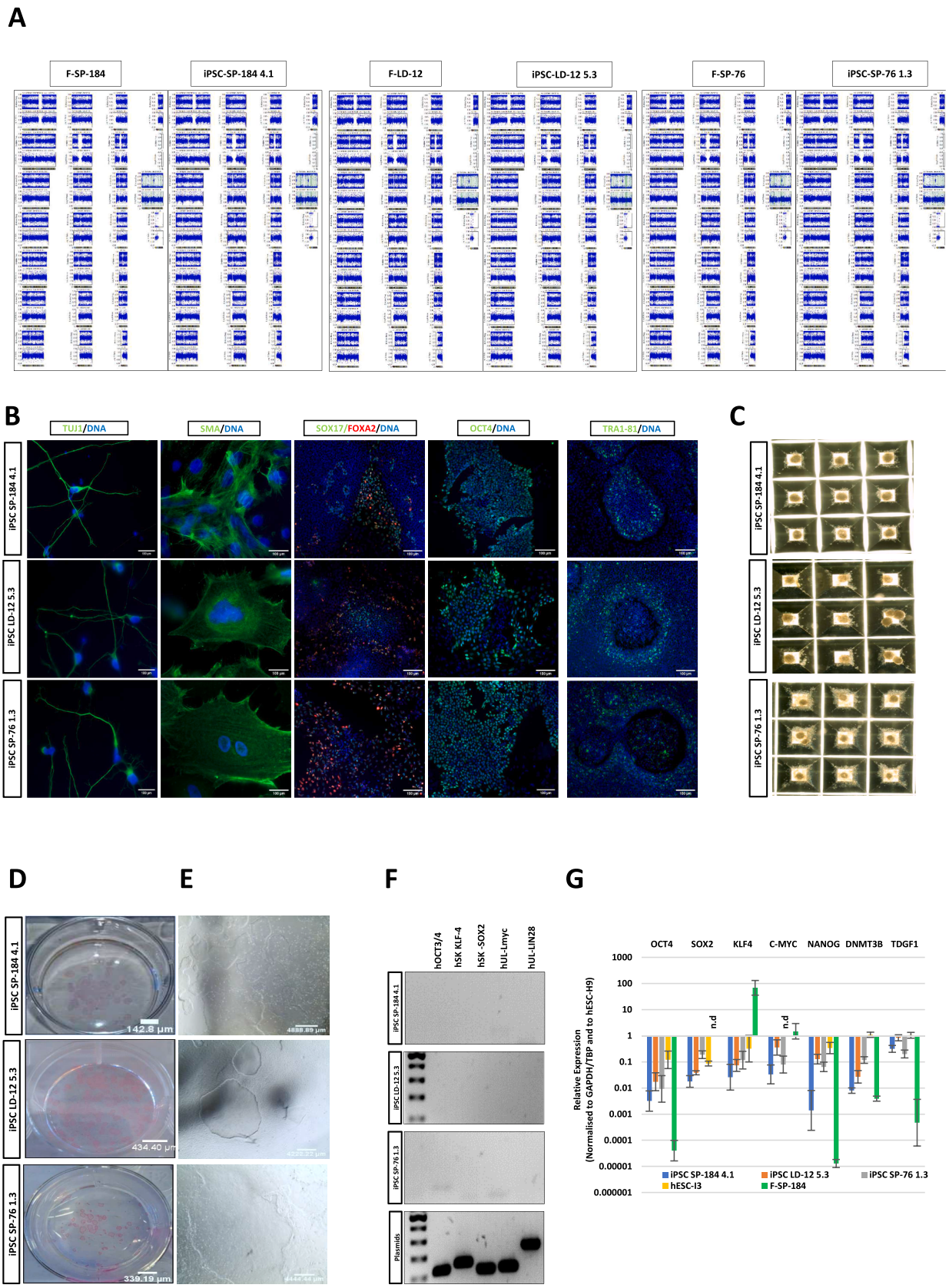


Fig. 1. Characterisation of iPSC lines HIHRSi004-A, HIHRSi005-A, HIHRSi006-A.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1E
Phenotype	Qualitative analysis	i) Expression of pluripotency markers: OCT4, TRA 1–81 through immunocytochemistry. ii) Expression of alkaline phosphatase (ALP) through staining	Fig. 1B and D
	Quantitative analysis	RT-qPCR for expression of *OCT4*, *NANOG*, *KLF4*, *c-MYC*, *SOX2*, *DNMT3B* and *TDGF1*	Fig. 1G
Genotype	Whole genome SNP genotyping with Infinium OmniExpressExome-8 BeadChip (Illumina) Spacing (kb): Mean: 3,03; Median: 1,36	No larger chromosomal aberrations or copy number variations were observed upon reprogramming	Fig. 1A
Identity	Microsatellite PCR (mPCR)	Not performed	N/A
	STR analysis	6 sites tested, all matched	submitted in archive with journal
Mutation analysis	Sanger sequencing	Known *POLR3A* mutations confirmed	Supplementary Fig. 1A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma test	Mycoplasma testing by PCR: Negative	Supplementary Fig. S1B
Differentiation potential	Embryoid body formation	Formation of embryoid bodies in aggrewell plates are demonstrated	Fig. 1C
List of recommended germ layer markers	Expression of specific germ layer markers after spontaneous differentiation of the embryoid bodies by immunocytochemistry	Ectoderm: TUJ1 Endoderm: SOX17, FOXA2, Mesoderm: SMA	Fig. 1B
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*

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency Markers	mouse anti-OCT4	1:100	SantaCruz	AB_628051
	mouse anti-Tra-1–81	1:500	Merck_Millipore	AB_177638
Differentiation Markers	mouse anti-TUJ1	1:1000	Sigma Aldrich	AB_477590
	mouse anti-SMA	1:100	Dako	AB_2223500
	goat anti-SOX17	1:250	R&D Systems	AB_355060
	rabbit anti-FOXA2	1:300	Millipore	AB_390153
Secondary antibodies	Alexa Fluor 568 donkey anti-goat IgG	1:1000	Invitrogen	
	Alexa Fluor 488 Goat anti-rabbit IgG	1:1000	Invitrogen	
	Alexa Fluor 488 Goat Anti-mouse IgG	1:1000	Invitrogen	
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids	OCT3/4_Plasmid	124 bp	CATTCAAACTGAGGTAAGGG/TAGCGTAAAAGGAGCAACATAG	
	SOX2_Plasmid	111 bp	TTCACATGTCCCAGCACTACCAG/ TTTGTTTGACAGGAGCGACAAT	
	KLF4_Plasmid	156 bp	CCACCTCGCCTTACACATGAAG/ TAGCGTAAAAGGAGCAACATAG	
	L-MYC_Plasmid	122 bp	GGCTGAGAAGAGGATGGCTAC/TTTGTTTGACAGGAGCGACAAT	
	LIN28_Plasmid	251 bp	*AGCCATATGGTAGCCTCATGTCCGC/* *TAGCGTAAAAGGAGCAACATAG*	
Pluripotency Markers (qPCR)	OCT4		GGAAGGTATTCAGCCAAACG/CTCCAGGTTGCCTCTCACTC	
	SOX2		AGCTCGCAGACCTACATGAA/CCGGGGAGATACATGCTGAT	
	KLF4		CCATCTTTCTCCACGTTCGC/CGTTGAACTCCTCGGTCTCT	
	C-MYC		ATTCTCTGCTCTCCTCGACG/CTGTGAGGAGGTTTGCTGTG	
	NANOG		CAAAGGCAAACAACCCACTT/TGCGTCACACCATTGCTATT	
	DNMT3B		ACGACACAGAGGACACACAT/AAGCCCTTGATCTTTCCCCA	
	TDGF1		GGTCTGTGCCCCATGACA/AGTTCTGGAGTCCTGGAAGC	
House-Keeping Genes (qPCR)	GAPDH		TCACCAGGGCTGCTTTTAAC/GACAAGCTTCCCGTTCTCAG	
	TBP		CTTCGGAGAGTTCTGGAATTG/CACGAAGTGCAATGGTCTTTAG	
Genotyping	*N/A*			
Targeted mutation analysis/sequencing	Primer1: HIHRSi004-A	233 bp	AGTAGGCAGCGTCAAAGAGA/CTTTCACACCTGACGTTGGG	
	Primer2: HIHRSi005-A	224 bp	TGGCGTTGTTAGTTGTGGTG/CCCACCGGACCTTCAGATTT	
	Primer3: HIHRSi006-A	201 bp	GAAATTGGCCAACGGTCTT/ACAGATGATTGCCTGTGTGG	
	Primer4: HIHRSi004-A, HIHRSi005-A, HIHRSi006-A	158 bp	TGAATTTGCTTGCTTCGCGA/GAACCAAGGGCAAGCAGTAC	

embedded in mounting medium (DAKO) for detection with fluorescence microscopy.

2) Alkaline phosphatase staining: To assess the expression of alkaline phosphatase (ALP), a marker of iPSCs, iPSC lines were fixed with 4 % PFA for 1 min at RT, washed thrice with PBS, then incubated in the staining solution: 20 µl naphthol AS-MX phosphate alkaline solution (Sigma-Aldrich) with 500 µl Fast Red (1 mg/ml, Sigma-Aldrich), for 30 min at RT and imaged with a camera.
3) RT-qPCR: To confirm the expression of iPSC markers at transcript level, RT-qPCR was performed for all cell lines. RNA was extracted

from the generated iPSCs and fibroblasts using the RNAeasy mini-isolation Kit (Qiagen) and transcribed to cDNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-qPCR was performed with the Light Cycler 480 SYBR Green I Master kit (Roche) for the pluripotency markers with primers as mentioned (Table 2). The pluripotency gene expression was compared to that of the embryonic stem cell line I3 and fibroblasts, and the expression values were normalized to housekeeping genes *GAPDH* and *TBP*, and the embryonic stem cell line H9, using the $2^{-\Delta\Delta}$Ct method in the Light Cycler 480 software.

4) Spontaneous differentiation to germ layers: To demonstrate the differentiation potential of the generated iPSC lines into cells of all 3 germ layers, an embryoid body (EB) based protocol was used as described (Nagel et al., 2019). For ectodermal differentiation, the EBs were plated in 3 N medium in house (50 % DMEM/F12 medium (Life Technologies), 50 % Neurobasal medium (Life Technologies), 1 % B27 (Life Technologies), 1 mM Glutamine (Gibco), 1 % NEAA (Sigma Aldrich), 50 µM ß-MeOH (Sigma Aldrich), 1 % Penicillin/Streptomycin (P/S) (Gibco), 160 mg/ml Glucose (Carl-roth)) and for mesodermal differentiation, EBs were plated in mesodermal differentiation medium (82 % DMEM high glucose (Sigma), 16 % FCS (Gibco), 1 % P/S (Gibco), 1 % NEAA (Sigma Aldrich), 55 mM ß-MeOH (Sigma Aldrich), 0.0004 % Thioglycerol (Sigma-Aldrich)). Endodermal differentiation of the EBs was performed as described (Carpentier et al., 2014). Expression of differentiation markers at protein level was validated by immunofluorescence staining for specific markers of the three germ layers: endodermal markers - SOX17 and FOXA2, mesodermal marker - SMA and ectodermal marker - TUJ1 (Table 2). Images for both the immunostainings were acquired with Axio Imager Z1 (Zeiss) and were processed with Zen lite 2.0.

CRediT authorship contribution statement

Kalaivani Manibarathi: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Tam Pham:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Holger Hengel:** Writing – review & editing, Resources, Data curation. **Matthis Synofzik:** Writing – review & editing, Resources, Data curation. **Maike Nagel:** Writing – review & editing, Supervision, Methodology, Data curation. **Rebecca Schüle:** Conceptualization, Funding acquisition, Writing – review & editing, Validation, Supervision, Resources.

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.

Acknowledgement

cDNAs of the embryonic stem cell lines I3 and H9 were provided by the kind courtesy of the Institute of Reconstructive Neurobiology, Bonn, Germany. We acknowledge the support of Jishu Xu, University hospital Tübingen, in verification of analysis of the SNP genotyping and Karyotyping results. The work was supported by the Bundesministerium für Bildung und Forschung (BMBF) through funding for the TreatHSP network (grant 01GM2209A to RS), the European Joint Programme on Rare Diseases for the PROSPAX consortium (grant 441409627 to RS), the National Institute of Neurological Disorders and Stroke (NINDS) and the National Institutes of Health (NIH) under Award Number R01NS072248 (RS), and the Clinician Scientist Programme PRECISE.net funded by the Else Kröner-Fresenius-Stiftung (RS). RS is a member of the European Reference Network for Rare Neurological Diseases – Project ID 739510.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103363.

References

Carpentier, A., Tesfaye, A., Chu, V., Nimgaonkar, I., Zhang, F., Lee, S.B., Thorgeirsson, S. S., Feinstone, S.M., Liang, T.J., 2014. Engrafted human stem cell-derived hepatocytes establish an infectious HCV murine model. J. Clin. Investig. 124, 4953–4964. https://doi.org/10.1172/JCI75456.

Minnerop, M., Kurzwelly, D., Wagner, H., Soehn, A.S., Reichbauer, J., Tao, F., Rattay, T. W., Peitz, M., Rehbach, K., Giorgetti, A., Pyle, A., Thiele, H., Altmüller, J., Timmann, D., Karaca, I., Lennarz, M., Baets, J., Hengel, H., Synofzik, M., Atasu, B., Feely, S., Kennerson, M., Stendel, C., Lindig, T., Gonzalez, M.A., Stirnberg, R., Sturm, M., Roeske, S., Jung, J., Bauer, P., Lohmann, E., Herms, S., Heilmann-Heimbach, S., Nicholson, G., Mahanjah, M., Sharkia, R., Carloni, P., Brüstle, O., Klopstock, T., Mathews, K.D., Shy, M.E., De Jonghe, P., Chinnery, P.F., Horvath, R., Kohlhase, J., Schmitt, I., Wolf, M., Greschus, S., Amunts, K., Maier, W., Schöls, L., Nürnberg, P., Zuchner, S., Klockgether, T., Ramirez, A., Schüle, R., 2017. Hypomorphic mutations in POLR3A are a frequent cause of sporadic and recessive spastic ataxia. Brain 140, 1561–1578. https://doi.org/10.1093/brain/awx095.

Nagel, M., Reichbauer, J., Böhringer, J., Schelling, Y., Krägeloh-Mann, I., Schüle, R., Ulmer, U., 2019. Generation of two iPSC lines derived from two unrelated patients with Gaucher disease. Stem Cell Res 35. https://doi.org/10.1016/j.scr.2018.10.021.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., Shibata, T., Kunisada, T., Takahashi, M., Takahashi, J., Saji, H., Yamanaka, S., 2011. A more efficient method to generate integration-free human iPS cells. Nat. Methods 8, 409–412.