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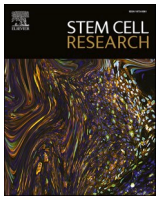

Lab Resource: Genetically-Modified Single Cell Line

Generation of an NCS1 gene knockout human induced pluripotent stem cell line using CRISPR/Cas9

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A B S T R A C T

NCS1 (Neuronal calcium sensor protein 1) encodes a highly conserved calcium binding protein abundantly expressed in neurons. It modulates intracellular calcium homeostasis, calcium-dependent signaling pathways as well as neuronal transmission and plasticity. Here, we generated a NCS1 knockout human induced pluripotent stem cell (hiPSC) line using CRISPR-Cas9 genome editing. It shows regular expression of pluripotent markers, normal iPSC morphology and karyotype as well as no detectable off-target effects on top 6 potentially affected genes. This newly generated cell line constitutes a valuable tool for studying the role of NCS1 in the pathophysiology of various neuropsychiatric disorders and non-neurological disease.

Resource Table

Unique stem cell line identifier	HMGUi001-A-22, https://hpscreg.eu/cell-line/HMGUi001-A-22
Alternative name(s) of stem cell line	NCS1-KO Clone 19
Institution	Technology Platform Pluripotent Stemcell, Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), 13125 Berlin, Germany.
Contact information of the reported cell line distributor	Dr. Sebastian Diecke, Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC) sebastian.diecke@mdc-berlin.de
Type of cell line	hiPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: N/A Sex: Female Ethnicity: Caucasian
Cell Source	Fibroblasts
Method of reprogramming	Episomal
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	Absence of reprogramming vector is described in https://hpscreg.eu/cell-line/HMGUi001-A

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[1] equal contribution (shared first/last authorship).

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(*continued*)

The cell culture system used	Feeder-free culture with E8 medium and Geltrex-coated plates
Type of the Genetic Modification	CRISPR/Cas9-mediateed knock-out
Associated disease	N/A

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Gene/locus	NCS1, chromosome 9q34.11
Method of modification/user-customizable nuclease (UCN) used,	CRISPR/Cas9

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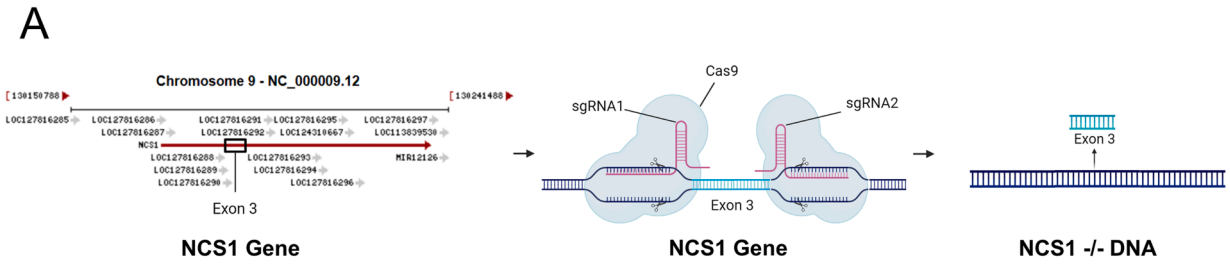

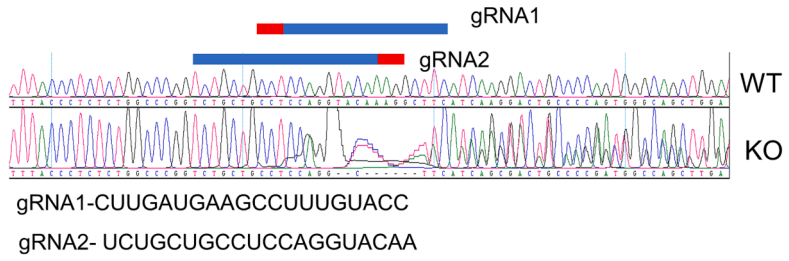

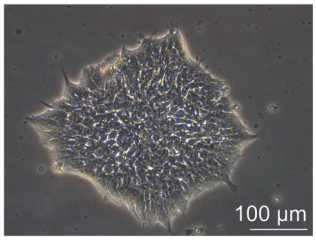

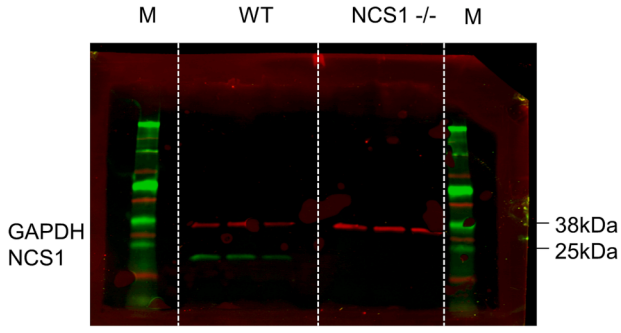

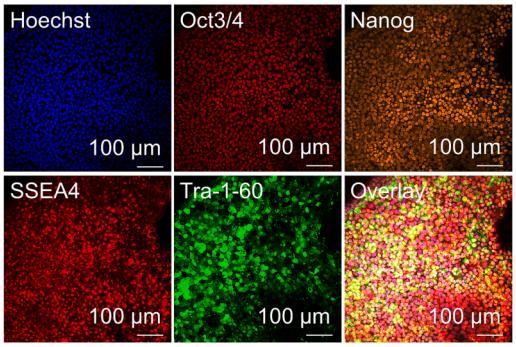

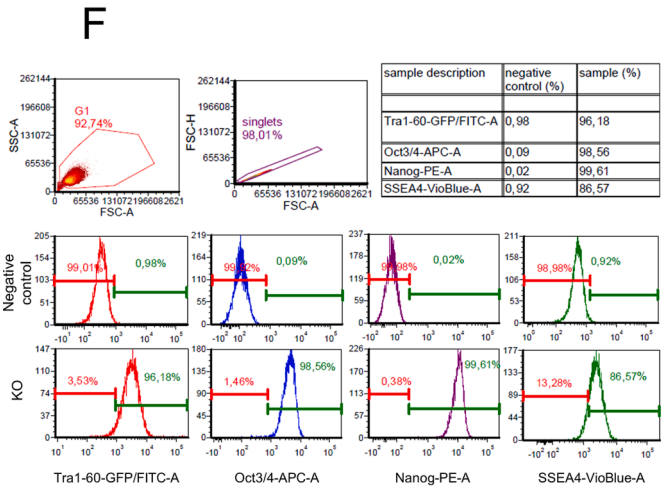

sample description	negative control (%)	sample (%)
Tra1-60-GFP/FITC-A	0,98	96,18
Oct3/4-APC-A	0,09	98,56
Nanog-PE-A	0,02	99,61
SSEA4-VioBlue-A	0,92	86,57

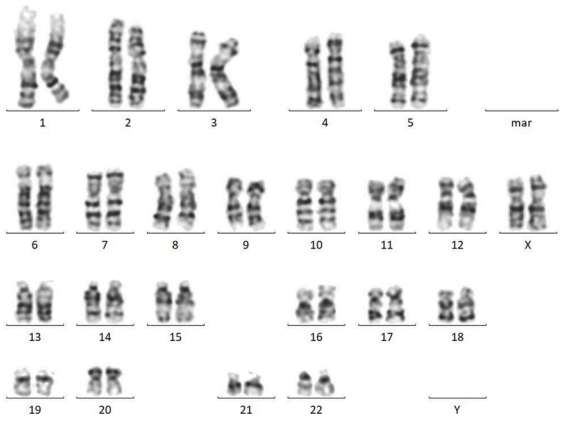

Fig. 1.

(*continued*)

the resource used for design optimization	
User-customizable nuclease (UCN) delivery method	Transfection with ribonucleoprotein (RNP) complexes
All double-stranded DNA genetic material molecules introduced into the cells	N/A
Analysis of the nuclease-targeted allele status	PCR of the targeted region followed by Sanger sequencing (.ab1 files in Supplementary material)
Method of the off-target nuclease activity prediction and surveillance	Targeted off-target site PCR followed by Sanger Sequencing
Descriptive name of the transgene	N/A
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	N/A
Inducible/constitutive expression system details	N/A
Date archived/stock creation date	17.03.2022
Cell line repository/bank	Technology Platform Pluripotent Stemcells, Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), 13,125 Berlin, Germany https://hpscreg.eu/cell-line/HMGUi001-A (parental cell line), https://hpscreg.eu/cell-line/HMGUi001-A-22 (NCS1-KO)
Ethical/GMO work approvals	Ethikkommission der Medizinischen Fakultät des Universitätsklinikums der Universität Tübingen, 130/2018BO2 (parental cell line)
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

1. The manuscript section expected contents clarification

1.1. Resource utility

NCS1 modulates intracellular calcium homeostasis and calcium-dependent signaling pathways responsible for neuronal development, synaptic transmission or apoptosis (Burgoyne, 2007). The hiPSC-NCS1-KO line may help elucidate the role of NCS1 in neuronal survival and degeneration, in neuro-psychiatric diseases such as bipolar or autism spectrum disorder, Parkinson's disease or chemotherapy-induced polyneuropathy (Boeckel and Ehrlich, 2018).

2. Resource details

The NCS1 knockout hiPSC line HMGUi001-A-22 (hiPSC-NCS1-KO) was generated using CRISPR-Cas9 gene editing in the parental hiPSC wildtype cell line HMGUi001-A (hiPSC-WT), generated at Helmholtz Zentrum Munich, Germany, https://hpscreg.eu/cell-line/HMGUi001-A. Specifically, two sgRNAs were designed to target an early, conserved exon 3 with at least 2 bp of mismatch to any other site in the human genome (Fig. 1A-B, S1). Subsequently, 300,000 cells were transfected with ribonucleoprotein (RNP) complexes. Editing efficiency in the pool was analyzed by Sanger Sequencing and Synthego ICE analysis 48 h after transfection. Then, single cell clones were generated using automated hiPSC single cell seeding and clonal expansion performed as described previously (Vallone et al., 2020). Clones identified with the desired out-of-frame indel modifications were identified with Sanger Sequencing and ICE analysis confirming a deletion of 50 bps with the occurrence of a STOP codon (Fig. 1B, S2). The generated HMGUi001-A-22 hiPSC clones showed typical undifferentiated hiPSC colony morphology (Fig. 1C). Absence of NCS1 protein was validated using western blot (Fig. 1 D). Expression of undifferentiated state markers octamer-binding transcription factor 3/4 (Oct3/4), Nanog, tumor rejection antigen (Tra-1–60) and stage specific embryonic antigen 4 (SSEA4) was confirmed in hiPSC colonies by immunofluorescent staining (Fig. 1E). Further confirmation and quantification of undifferentiated state markers was performed by Fluorescent-activated cell sorting (FACS): Tra-1–60 was expressed by more than 96 %, Oct3/4 by more than 98 %, Nanog by more than 99 % and SSEA4 by more than 86 %. Unstained cells served as negative gating control (Fig. 1F). Karyotype analysis with single nucleotide polymorphism (SNP) arrays and G-Banding showed normal female karyotype (46, XX) in the hiPSC-NCS1-KO (Fig. 1G). HMGUi001-A-22 line was tested negative for mycoplasma contamination (Fig. S3A). No off-target activity of CRISPR/Cas9 could be detected when the top 3 potential off-targets sites per gRNA (a total of 6 off-target sites with 2–4 mismatches) were sequenced (Fig. S3B, Fig. S4). Cell line identity was identical to the cells of origin validated with short tandem repeat analysis (STR) for 10 genomic loci (submitted in the archive with the journal). The cell line showed potency to differentiate into mesoderm, ectoderm and endoderm lineages as confirmed by trilineage differentiation assay and quantified by FACS (Fig. S5). Here, at least 68 % stained positive for the mesodermal markers CD140b+, <1% positive for CD144+, more than 98 % co-expressed the ectodermal markers Sox2+/Pax6 + and more than 93 % co-expressed the endodermal markers CD184+/Sox17+ (Fig. S5). Finally, a summary of KO validation and hiPSC quality control tests above described can be found in the Resource Table 1.

3. Materials and methods

The generation of the hiPSC-NCS1-KO cell line was approved by the local ethical committee (Ethikkommission der Medizinischen Fakultät des Universitätsklinikums der Universität Tübingen, 130/2018BO2 (parental cell line).

3.1. Culture of hiPSC

hiPSCs were cultured in Essential 8 (E8) medium in 6 well-plates coated with Geltrex and passaged every 4–5 days at a ratio of ~ 1:6 using 0.5 mM EDTA.

3.2. CRISPR-Cas9-mediated gene editing

The NCS1 gene was genetically engineered using two sgRNAs (5′-CUUGAUGAAGCCUUUGUACC −3′ and 5′- UCUGCUGCCUCCAGGUACAA −3′) targeting exon 3. Transfection was conducted using the Neon transfection system (Thermo Fisher Scientific) and Neon transfection 10 μL kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Single cell clones identified by Sanger Sequencing and Synthego ICE analysis 48 h after transfection were generated using the iota Sciences IsoCell platform and automated hiPSCs single cell seeding and clonal expansion as described previously (Vallone et al., 2020; Ludwik et al., 2023).

3.3. Knockout confirmation with western blot

hiPSC-WT and hiPSC-NCS1-KO were differentiated into hiPSC-derived sensory neurons (hiPSC-DSN) as described previously (Schinke, 2021) and matured until day 15. The loss of the protein expression of NCS1 was confirmed by semi-dry western blot. Membranes were imaged with the LI-COR Odyssey CLx.

3.4. Off-target analysis with PCR & Sanger sequencing

Genomic DNA from hiPSC-WT and hiPSC-NCS1-KO was extracted with the DNeasy blood and tissue Kit (Quiagen). Specific primers around off target regions were designed and used to amplify DNA fragments by PCR using the Phire Tissue Direct PCR Master Mix (ThermoFisher). Product specific size was confirmed by electrophoresis. PCR products

Table 1
Characterization and validation.

Classification (optional *italicized*)	Test	Result	Data
Morphology	Light microscopy	Normal hiPSC colony morphology	Fig. 1C
Pluripotency status evidence for the described cell line	Qualitative analysis with immunofluorescence	Positive expression of undifferentiated stage markers OCT3/4, NANOG, SSEA4, Tra1-60	Fig. 1E
	Quantitative analysis with Flow cytometry	OCT3/4: 98 %; Tra 1–60: 96 %; SSEA-4: 96 %; NANOG: 99 %	Fig. 1F
Karyotype	G-Banding	Normal female karyotype (46, XX)	Fig. 1G
	CNV using SNP arrays	No reportable genomic abnormalities detected when compared to parental line HMGUi001-A	Submitted in archive with journal
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR and Sanger Sequencing	Indels in Exon 3 of NCS1 gene in hiPSC-NCS1-KO were created. ICE analysis showed homozygous hiPSC-NCS1-KO clone, confirmed by ICE analyses and Western Blot.	Fig. 1B, S2
	Evaluation of the -(homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	N/A	N/A
	Transgene-specific PCR (when applicable)	N/A	N/A
Verification of the absence of random plasmid integration events	N/A	NA	N/A
Parental and modified cell line genetic identity evidence	STR analysis	Identical alleles to parental hiPSC-WT at 10 STR Loci (https://hpscreg.eu/cell-line/HMGUi001-A)	
Mutagenesis/ genetic modification outcome analysis	Western Blot	Demonstration of protein elimination in NCS1-KO in comparison to hiPSC-WT	Fig. 1D
Off-target nuclease activity analysis	PCR across top 6 predicted off-target sites (3 per gRNA)	No off-target Cas nuclease activity was observed in any of the 6 locations studied	Fig. S3B and S4
Specific pathogen-free status	Mycoplasma	Negative	Fig. S3A
Multilineage differentiation potential	Trilineage differentiation	Ability of hiPSC-NCS1-KO line to differentiate into derivatives of all 3 germ layers	Fig. S5 A, B, C
Donor screening	HIV 1 + 2, Hepatitis B, Hepatitis C	Negative	not shown but available upon request
Genotype - additional histocompatibility info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

were extracted from the gel and purified using the NucleoSpin ® Gel and PCR Clean Up Kit (Macherey-Nagel). Sanger sequencing was conducted on purified products by Azenta LifeSciences Genewiz.

3.5. Immunofluorescence staining of undifferentiated hiPSC markers

hiPSCs were cultivated in a CellCarrier-96 Black Imaging Plate (Perkin Elmer). hiPSCs were fixed for 10 min at room temperature with Roti®Histofix 4 % (Roth) reagent, followed by 30 min blocking and permeabilization with permeabilization/blocking buffer (1 % BSA; 0.2 % Saponin in PBS with Mg^{2+} and Ca^{2+}). Cells were incubated for 1 h at room temperature with fluorophore-conjugated antibodies (Oct3/4-APC, Nanog-PE, SSEA4-PerCpVio700 and Tra-1–60-Vio488) diluted in permeabilization/blocking buffer. Nuclei were stained with Hoechst 33,342 (1 µg/mL). Microscopic analysis was performed using Opera Phenix high content imaging system (Perkin Elmer).

3.6. FACS for undifferentiated markers

Single cells were harvested using TrypLE. Surface marker staining of $2x10^5$ hiPSCs was performed for 10 min at 4 °C in 100 µL surface staining solution (Table 2). Cells were washed 2 times with PBS, fixed and permeabilized using the FoxP3 Staining Buffer Set (Miltenyi Biotec) according to manufacturer's instructions. Intracellular markers were stained for 30 min at 4 ∘C in 100 µL inside permeabilization solution (Table 2). Stained cells were analysed using MACSQuant® VYB (Miltenyi Biotec).

3.7. Short tandem repeat (STR) analysis

The alleles of 10 short tandem repeat (STR) loci of the generated hiPSC-NCS1-KO cell line as well the parental hiPSC-WT cell line were analyzed with the GenePrint® 10 system (Promega). Nine ASN-0002 loci (TH01, TPOX, vWA, Amelogenin, CSF1PO, D16S529, D7S820, D13S317 and D5S818) as well as D21S11 were co-amplified and detected.

3.8. Karyotype analysis using g-banding

Karyotype analysis using G-banding was conducted by quantifying GTG stained metaphase chromosomes with an average resolution of at least 200bands per haploid chromosome set.

3.9. Karyotype analysis using SNP array

For karyotype analysis of hiPSC-WT and hiPSC-NCS1-KO, 20 metaphases were quantified using Illumina BeadArray and the Illumina Infinum Global Screening Array −24 BeadChip. The genotype analysis was performed in GenomeStudio V2.0.5.

3.10. Mycoplasma screening

hiPSC-WT and hiPSC-NCS1-KO cell line were tested for Mycoplasma contamination with the qPCR-based Venor®GeM qOneStep Kit according to the manufacturer's instructions.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Markers for undifferentiated iPSCs	SSEA-4 Antibody, anti-human, PerCpVio700, REAfinity™	1:10	Miltenyi Biotec, 130-105-053
	SSEA-4 Antibody, anti-human, VioBlue, REAfinity™	1:20	Miltenyi Biotec, 130-098-366
	Oct3/4 Antibody, anti-human/mouse, APC, REAfinity™	1:10/ 1:100	Miltenyi Biotec, 130-123-257
	Tra1-60 Antibody, anti-human, Vio488, REAfinity™	1:100/ 1:700	Miltenyi Biotec, 130-106-872
	Nanog (D73G4) XP Rabbit mAb (PE Conjugate)	1:100	Cell Signaling, 14955
Primary antibodies knockout-confirmation in immunofluorescence and western blot	Mouse anti-NCS1	1:1000	Santa Cruz, Cat #sc-376206, RRID: AB_11008074
	Rabbit anti-GAPDH	1:500	Merck, Cat#ABS16, RRID: AB_10806772
Secondary antibodies	Alexa Fluor Donkey anti-mouse 680	1:15,000	Thermo Fisher Scientific Cat# A-32788, RRID: AB_2762831)
	Alexa Fluor Donkey anti-rabbit 800	1:15,000	Thermo Fisher Scientific, Cat# A-32808, RRID: AB_2762837
Nuclear stain	Hoechst33342	1 μg/mL	Invitrogen™, Cat# H3570
Site-specific nuclease			
CRISPR-Cas9	Alt-R® S.p. HiFi Cas9 Nuclease V3, 500 μg	Integrated DNA technologies, 1,081,061	
Nucleofection	1200 V, 30 ms, 1 pulse	Neon/Invitrogen-TFS	
No selection	Single cell cloning using IsoCell	IotaSciences	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
e.g. Episomal Plasmids (qPCR or RT-PCR)	NA		
e.g. Pluripotency Markers (qPCR)	NA		
e.g. House-Keeping Genes (qPCR)	NA		
Genotyping (desired allele/transgene presence detection)	PCR specific for the targeted allele		
Targeted mutation analysis/sequencing	PCR specific for the targeted location followed by Sanger sequencing	Primer design: https://www.ncbi.nlm.nih.gov/tools/primer-blast/ Primers to amplify 500–600 bp having mutation in middle of product: NCS1-Fw1: ATGTCAGTTCGTAACCCCCT NCS1-Rev1: TGCATGTCAGTATGCACTCGT NCS1-Fw2: GCTCACTCTGTGTGCAGTATC NCS1-Rev2: CTAGAAGGGAACATCGGCAGG	
Potential random integration-detecting PCRs	Not relavant as we did not use any plasmids		
gRNA oligonucleotide/crRNA sequence	gRNA1:CUUGAUGAAGCCUUUGUACC gRNA2:UCUGCUGCCUCCAGGUACAA		
Genomic target sequence(s)	NCS1, Exon3	GRCh38 Chr9:130217834 and 130,217,835	
Bioinformatic gRNA on- and -off-target binding prediction tool used, specific sequence/outputs link(s)	Synthego gRNA design	https://design.synthego.com/#/validate/results?genome = homo_sapiens_gencode_26_primary&nuclease = cas9&guide = UCUGCUGCCUCCAGGUACAA https://design.synthego.com/#/validate/results?genome = homo_sapiens_gencode_26_primary&nuclease = cas9&guide = CUUGAUGAAGCCUUUGUACC	
Primers for top off-target mutagenesis predicted site sequencing	OT1 - RELN OT2 - UBR3 OT3 – PDZD2 OT4 - TRPM6 OT5 - TENM3 OT6 - CDH18	F: CCACTAGACGCTTCTCTTCTTC/ R: GCATCTAAGAGAGGGTTGGATT F: TGGTGGCCCATAACTGTAATC/ R: ACCCTTGAATTGCTTGACACTA F: CTGGAAATGGACCACAAAGGA/ R: GGTTAAGATGAGGATGGAGGAAAG F: TCACAACAAACGTTAGACGTAGAT/ R: CTGCCTCTTGTGCCGAATTA F: CTATTGAAGTGCCACCATATTGC/ R: CAGTGTTTGTTGGCATGGTATC F: GGCAGACCTTCAACCATCA/ R: GCCTTAAATGAGAGAATGAGAGAATG	
ODNs/plasmids/RNA molecules used as templates for HDR-mediated site-directed mutagenesis.	NA	N/A	

3.11. Bacteria/Yeast/Fungi testing

Cell cultures were maintained without the addition of antibiotics over 7 days and checked daily for the growth of fungi, bacteria or yeast with light microscopy.

3.12. Validation of pluripotent differentiation potential

Pluripotency was assessed analyzing differentiation potential using StemMACS Trilineage Differentiation Kit, human (MACS Miltenyi Biotec, Cat. No. 130-115-660). Resulting cell populations corresponding to endoderm, mesoderm and ectoderm lineages were analyzed by FACS (MACS Quant Vyb, Miltenyi) using specific lineage markers (Oct3/4-APC, Nanog-PE, SSEA4-VioBlue and Tra-1-60-Vio488). Additional

details on reagents used can be found in Table 2.

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.

Data availability

All relevant data are given in the Suppl. Material; STR file was submitted to the archive of the Journal.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103253.

References

Boeckel, G.R., Ehrlich, B.E., 2018. NCS-1 is a regulator of calcium signaling in health and disease. Biochimica et Biophysica Acta (BBA) – Mol. Cell Res. 1865 (11, Part B), 1660–1667.

Burgoyne, R.D., 2007. Neuronal calcium sensor proteins: generating diversity in neuronal Ca2+ signalling. Nature Rev. Neurosci. 8 (3), 182–193.

Ludwik, K.A., Telugu, N., Schommer, S., Stachelscheid, H., Diecke, S., 2023. ASSURED-optimized CRISPR protocol for knockout/SNP knockin in hiPSCs. STAR Protocols 4 (3), 102406.

Schinke, C., et al., 2021. Modeling chemotherapy induced neurotoxicity with human induced pluripotent stem cell (iPSC) -derived sensory neurons. Neurobiol. Dis. 155, 105391.

Vallone, V.F., Telugu, N.S., Fischer, I., Miller, D., Schommer, S., Diecke, S., Stachelscheid, H., 2020. Methods for Automated Single Cell Isolation and Sub-Cloning of Human Pluripotent Stem Cells. Current Protocols Stem Cell Biol. 55 (1).