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

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Alternative name(s) of stem cell line</td>
<td>NCS1-KO Clone 19</td>
</tr>
<tr>
<td>Institution</td>
<td>Technology Platform Pluripotent Stemcell, Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), 13125 Berlin, Germany</td>
</tr>
<tr>
<td>Contact information of the reported cell line distributor</td>
<td>Dr. Sebastian Diecke, Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC) <a href="mailto:sebastian.diecke@mdc-berlin.de">sebastian.diecke@mdc-berlin.de</a></td>
</tr>
</tbody>
</table>

Type of cell line

| hiPSC |

Origin

| Human |

Additional origin info (applicable for human ESC or iPSC)

| Age: N/A |
| Sex: Female |
| Ethnicity: Caucasian |
| Fibroblasts |
| Episomal |
| Clonal |

Method of reprogramming

| Clonality |

Evidence of the reprogramming transgene loss (including genomic copy if applicable)

| Absence of reprogramming vector is described in https://hpscreg.eu/cell-line/HMGU001-A |

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1 equal contribution (shared first/last authorship).

https://doi.org/10.1016/j.scr.2023.103253

Received 4 September 2023; Received in revised form 7 November 2023; Accepted 13 November 2023

Available online 14 November 2023

1873-5061/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
The cell culture system used Feeder-free culture with E8 medium and Geltrex-coated plates
Type of the Genetic Modification CRISPR/Cas9-mediated knock-out
Associated disease N/A

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

Fig. 1.
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). HMGU001-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, S3C). 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 Universitätsskinikums 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’- CUUGAAGGACCUCUUUGAC-3’ and 5’- UCGUCGCGUCCAGGAUCA A-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 (QiaGen). 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.

<table>
<thead>
<tr>
<th>Classification (optional italicized)</th>
<th>Test</th>
<th>Result</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Light microscopy</td>
<td>Normal hiPSC colony morphology</td>
<td>Fig. 1C</td>
</tr>
<tr>
<td>Pluripotency status for the described cell line</td>
<td>Qualitative analysis with immunofluorescence</td>
<td>Positive expression of undifferentiated stage markers</td>
<td>Fig. 1E</td>
</tr>
<tr>
<td></td>
<td>Quantitative analysis with Flow cytometry</td>
<td>OCT3/4: 98 %; Tra 1-60: 96 %; SSEA-4: 96 %; NANOG: 99 %</td>
<td>Fig. 1F</td>
</tr>
<tr>
<td>Karyotype</td>
<td>G-Banding</td>
<td>Normal female karyotype (46, XX)</td>
<td>Fig. 1G</td>
</tr>
<tr>
<td>Genotyping for the desired genomic alteration/allelic status of the gene of interest</td>
<td>PCR and Sanger Sequencing</td>
<td>lndels 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.</td>
<td>Fig. 1B, S2</td>
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<tr>
<td></td>
<td>Evaluation of the (homo-/hetero-/hemizygous status of introduced genomic alteration(s)</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td></td>
<td>Trangene-specific PCR (when applicable)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Verification of the absence of random plasmid integration events</td>
<td>N/A</td>
<td>NA</td>
<td>N/A</td>
</tr>
<tr>
<td>Parental and modified cell line genetic identity evidence</td>
<td>STR analysis</td>
<td>Identical alleles to parental hiPSC-WT at 10 STR Loci (<a href="https://hpscreg.eu/cell-line/HMGU001-A">https://hpscreg.eu/cell-line/HMGU001-A</a>)</td>
<td>N/A</td>
</tr>
<tr>
<td>Mutagenesis/ genetic modification outcome analysis</td>
<td>Western Blot</td>
<td>Demonstration of protein elimination in NCS1-KO in comparison to hiPSC-WT</td>
<td>Fig. 1D</td>
</tr>
<tr>
<td>Off-target nuclease activity analysis</td>
<td>PCR across top 6 predicted off-target sites (3 per gRNA)</td>
<td>No off-target Cas nuclease activity was observed in any of the 6 locations studied</td>
<td>Fig. S3B and S4</td>
</tr>
<tr>
<td>Specific pathogen-free status</td>
<td>Mycoplasma</td>
<td>Negative</td>
<td>Fig. S3A</td>
</tr>
<tr>
<td>Multilineage differentiation potential</td>
<td>Trilineage differentiation</td>
<td>Ability of hiPSC-NCS1-KO line to differentiate into derivatives of all 3 germ layers</td>
<td>Fig. S5 A, B, C</td>
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<tr>
<td>Donor screening</td>
<td>HIV 1 + 2, Hepatitis B, Hepatitis C</td>
<td>Negative</td>
<td>not shown but available</td>
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</tbody>
</table>

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 Mg2+ and Ca2+). 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 2x10^7 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® VYS (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 200 bands 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.
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 Vybe, 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

This work was supported by Animalfree Research Switzerland and Charité 3R — Replace — Reduce — Refine. CS, PH, and WB are members of the Clinician Scientist program funded by the Charité Universitätsmedizin Berlin and Berlin Institute of Health.

Appendix A. Supplementary data

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

References