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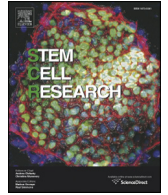
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Lab Resource: Stem Cell Line

Generation of a human induced pluripotent stem cell line via CRISPR-Cas9 mediated integration of a site-specific homozygous mutation in *CHMP2B*



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ABSTRACT

Frontotemporal dementia (FTD) is an early onset neurodegenerative disease. Mutations in several genes cause familial FTD and one of them is charged multivesicular body protein 2B (*CHMP2B*) on chromosome 3 (*FTD3*), a component of the endosomal sorting complex required for transport III (ESCRT-III). We have generated an induced pluripotent stem cell (iPSC) line of a healthy individual and inserted the *CHMP2B* IVS5AS G-C gene mutation into both alleles, resulting in aberrant splicing. This human iPSC line provides an ideal model to study *CHMP2B*-dependent phenotypes of FTD3.

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Resource table.

| | |
|---------------------------------|---|
| Name of stem cell line | CHMP2B IVS5AS GG-CC |
| Institution | University of Copenhagen |
| Person who created resource | Yu Zhang, Benjamin Schmid, Kristine Freude |
| Contact person and email | Yu Zhang, yu.zhang@sund.ku.dk Kristine Freude, kkf@sund.ku.dk |
| Date archived/stock date | June 5, 2016 |
| Origin | Human skin fibroblasts |
| Type of resource | Biological reagent: induced pluripotent stem cell (iPSC); genetically modified |
| Sub-type | Induced pluripotent stem cell (iPSC) |
| Key transcription factors | hOCT4, hSOX2, hKLF4, hL-MYC, hLIN28, and shRNA against TP53 (Addgene plasmids 27077, 27078 and 27080; Okita et al., 2011, all a gift from Shinya Yamanaka) |
| Authentication | Identity and purity of cell line confirmed by analysis of mutation sequencing, karyotyping, pluripotency markers and <i>in vitro</i> differentiation potential (Fig. 1) |
| Link to related literature | N/A |
| Information in public databases | N/A |
| Ethics | The study was approved by the Ethics Committee of the |

Capital Region of Denmark (H-4-2011-157) and written informed consent was obtained in all cases.

Resource details

Skin biopsy was obtained from a 63-year-old man (anonymized as H256) and an induced pluripotent stem cell (iPSC) line, H256 clone (C) 6, was generated using an episomal vector system carrying transcripts for human OCT4, SOX2, KLF4, L-MYC, LIN28, and small hairpin RNA for TP53 (Okita et al., 2011; Rasmussen et al., 2014).

We then generated a homozygous *CHMP2B* point-mutation (pathogenic G-to-C transition in the 5' acceptor splice site of exon 6) in this iPSC line via the CRISPR-Cas9 system (Ran et al., 2013). The mutation was validated by DNA sequencing (Fig. 1A).

Subsequently, we confirmed that the gene modified clone of H256 C6, termed *CHMP2B* IVS5AS GG-CC, remained truly pluripotent. This was demonstrated via expression analyses of key pluripotency markers on protein level (Fig. 1B). Additionally, *CHMP2B* IVS5AS GG-CC retained the potential to differentiate into cell types of all three germ layers upon embryoid body formation (Fig. 1C). More importantly, the gene editing process introduced no genetic chromosomal aberrations and the cells exhibit a normal karyotype (Fig. 1D).

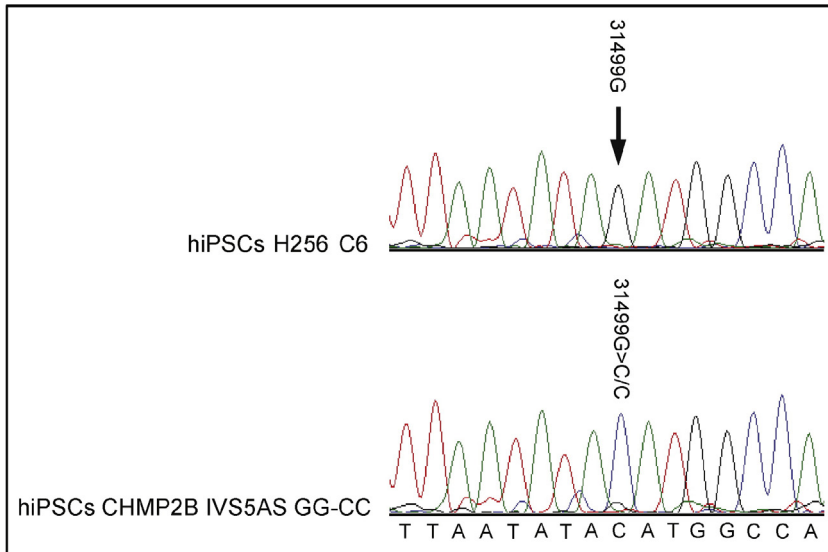
In summary, we have generated a disease-specific homozygous *CHMP2B* mutant human iPSC line. Together with one more heterozygous *CHMP2B* mutant iPSC line (Zhang et al., submitted to Stem Cell Research) and the original isogenic healthy control, they will serve as an

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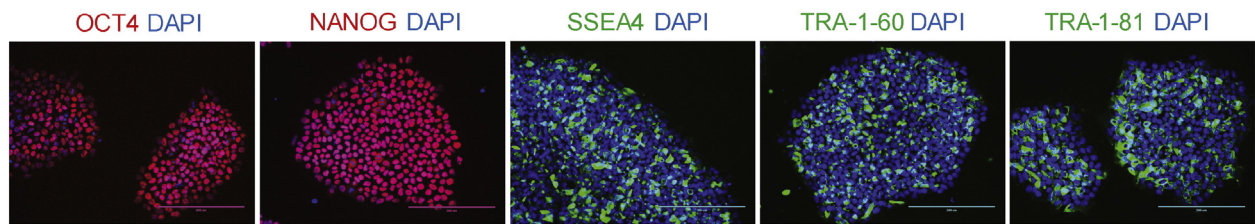
A

CHMP2B^{Intron 5} Point Mutation via CRISPR-Cas9



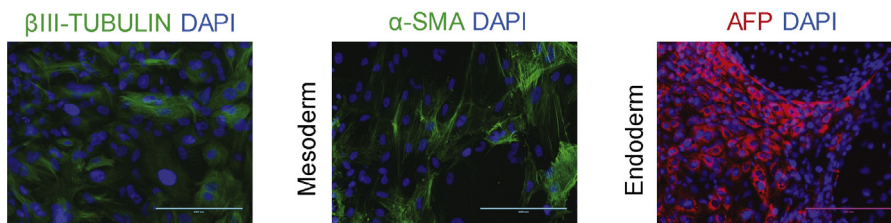
B

Pluripotency Markers



C

Ectoderm



D

Karyotype

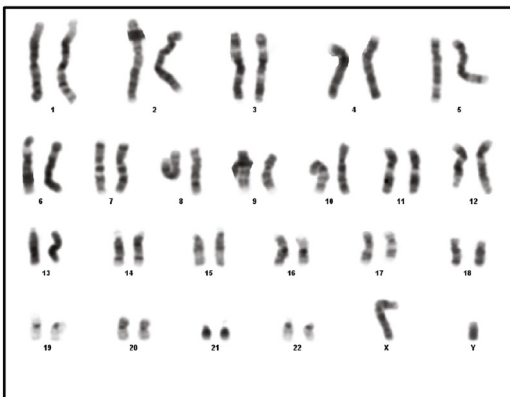


Fig. 1. Site-specific introduction of a homozygous mutation in *CHMP2B*. *CHMP2B* IVS5AS GG-CC is a bona fide human iPSC line with a normal karyotype. (A) DNA sequencing of both *CHMP2B* alleles shows a G-to-C transition in the 5' acceptor splice site of exon 6 in one allele, using the CRISPR-Cas9 system. (B) OCT4, NANOG, SSEA4, TRA-1-60 and TRA-1-81 immunofluorescence images of *CHMP2B* IVS5AS GG-CC counterstained with DAPI. Scale bars, 200 μ m. (C) Immunocytochemistry for marker proteins representative of the three germ layers, β III-TUBULIN (ectoderm), SMA (mesoderm), and AFP (endoderm), after *in vitro* differentiation of *CHMP2B* IVS5AS GG-CC by embryoid body formation. Scale bars, 200 μ m. (F) Chromosome analysis of *CHMP2B* IVS5AS GG-CC showing a normal 46, XY karyotype.

ideal study tool for in vitro disease modeling and pathological study of FTD3 (Skibinski et al., 2005), independent of the familial background and thereby completely focused on the biological effect of the particular CHMP2B mutation.

Materials and methods

CRISPR design

CHMP2B IVS5AS GG-CC was obtained using the CRISPR-Cas9 system in combination with ssODNs serving, as homologous templates covering the site where the mutation was integrated. CRISPRs were designed at <http://crispr.mit.edu/>. The CRISPRs were generated following the protocol from Ran et al. (Ran et al., 2013) in a single plasmid containing both sgRNA and the Cas9 (pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988)).

Transfection

iPSCs maintained on matrigel coated dishes (Corning Bioscience) in E8 medium (Gibco) were detached using Accutase (Gibco). 2×10^6 cells were co-transfected with 10 μ g of the CRISPR-Cas9 plasmid (Addgene, 62,988) and 1 μ L of the ssODNs. We used a 4D nucleofector (programme CA167) from Amaxa in combination with the P3 Primary Cell Kit for transfection. iPSCs were subsequently transferred back to a matrigel-coated dish in E8 medium supplemented with 1 mM ROCK inhibitor (Sigma). 24 h post-transfection, cells were subjected to puromycin selection for 48 h and allowed to recover for a week. Resistant colonies were picked and expanded for genotyping.

Genotyping

DNA for genotyping was extracted using the prepGEM™ kit (zyGEM, PT10500). PCR genotyping was performed using TEMPase Hot Start DNA Polymerase (Ampliqon) according to the manufacturer's instructions at an annealing temperature of 58 °C. The following primers were designed 444 base pairs upstream and 162 base pairs downstream of the CRISPR cutting site to detect successful gene editing: CHMP2B forward 5'-TGTTCACTGAGTTTGCCTTCTG-3' and CHMP2B reverse 5'-ACCAAGCACAGTGCAGATTTC-3'. Clones in which both wildtype alleles were successfully replaced with the CHMP2B mutant ssODNs were subjected to sequencing in order to ensure the inserted point mutation and exclude introduction of frameshifts or other mutations.

DNA sequencing

Sanger sequencing of a 606 base pair region around the 5' acceptor splice site of exon 6 region of the CHMP2B gene was carried out in an ABI PRISM 310 Genetic Analyzer.

Immunocytochemistry

iPSCs were fixed with 4% paraformaldehyde in DPBS for 15 min and immunocytochemistry was performed by standard immunofluorescence staining procedures. The following primary antibodies were used: Anti-OCT4 (Santa Cruz, sc-8628); anti-NANOG (Peprotech, 500-P236); anti-SSEA4 (BioLegend, 330402); anti-TRA-1-60 (BioLegend, 330602); anti-TRA-1-81 (BioLegend, 330702); anti- β III-TUBULIN (Millipore, MAB1637); anti-SMA (Dako, M0851), anti-AFP (Dako,

A0008); all 1:500. Secondary antibodies used were: Alexa Fluor 488 donkey anti-rabbit (A21207), donkey anti-goat (A11058), and goat anti-mouse (A11017), all 1:1000 (Molecular Probes).

Embryoid body differentiation

Embryoid body (EB) formation was performed by transferring EDTA-treated iPSCs to low attachment plates (Corning, NY, USA) in E8. After 2 days of culture, the medium was changed to DMEM/F12 containing 20% knockout serum replacement (Life Technologies), 1 \times non-essential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 1% PenStrep. After 7 days, the EBs were plated on 0.5 μ g/cm² vitronectin (Life Technologies) coated dishes and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% Pen/Strep for ecto-, meso- and endoderm induction up to three weeks. Subsequently, EBs were fixed for 15 min in 4% PFA for immunocytochemistry.

Karyotyping

iPSCs were treated for 45 min with KaryoMAX colcemid (Life Technologies), harvested in fresh fixative consisting of 25% acetic acid and 75% methanol and sent for G-band karyotyping (Cell Guidance Systems, UK).

Verification and authentication

Karyotyping was performed by Cell Guidance Systems (UK) and a minimum of 20 metaphases was analyzed. The results showed a normal 46, XY karyotype, without any detectable abnormalities (Fig. 1D). CHMP2B IVS5AS GG-CC iPSC line identity and purity was confirmed by sequencing of CHMP2B (Fig. 1A) and ICC for pluripotency markers expression (Fig. 1B).

Acknowledgments

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