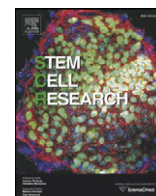


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Lab Resource: Stem Cell Line

Generation of a heterozygous knockout human embryonic stem cell line for the *OCIAD1* locus using CRISPR/CAS9 mediated targeting: BJNh20-OCIAD1-CRISPR-39

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ABSTRACT

Ovarian carcinoma immuno-reactive antigen domain containing 1 (*OCIAD1*) single copy was knocked out generating an *OCIAD1* heterozygous knockout human embryonic stem line named BJNh20-OCIAD1-CRISPR-39. The line was generated using CRISPR-Cas9D10A double nickase knockout strategy (Mali et al., 2013).

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Resource table

Name of stem cell lines	BJNh20-OCIAD1-CRISPR-39
Institution	Jawaharlal Nehru Centre for Advanced Scientific Research
Person who created resource	Deeti K. Shetty and Maneesha S. Inamdar
Contact person and email	inamdar@jncasr.ac.in
Date archived/stock date	23rd April, 2015
Origin	Human embryonic stem cell line BJNh20
Type of resource	Biological reagent: genetically modified human embryonic stem cells
Sub-type	Cell line
Key transcription factors	Oct4, Nanog, Sox2
Authentication	Identity of the cell line confirmed (see Fig. 1)
Link to related literature	http://online.liebertpub.com/doi/abs/10.1089/scd.2008.0131 http://link.springer.com/article/10.1007%2Fs11626-010-9277-3 http://link.springer.com/protocol/10.1007%2F978-1-61779-794-1_9 http://www.stembook.org/node/1438.html
Information in public databases	–
Ethics	Competent authority approval obtained

Resource details

BJNh20-OCIAD1-CRISPR-39 line heterozygous knockout for *OCIAD1* was generated by targeting exon3 of *OCIAD1* using CRISPR-Cas9 mediated knockout strategy (Fig. 1A). The method is based on an earlier report (Peters et al., 2013). BJNh20 cells were co-transfected with pCas9D10A-GFP and *OCIAD1* sgRNA expression constructs targeting two sites on exon3 for double nickase activity. Cas9D10A-GFP expressing cells were sorted and plated clonally on mTESR containing Matrigel-coated dishes. Forty individual colonies were picked and expanded for screening. Genomic DNA was isolated and screened by high fidelity PCR amplification followed by sequencing of exon3 of *OCIAD1* at the targeted region. Seven heterozygous knockouts were obtained and one of them is BJNh20-OCIAD1-CRISPR-39, which was characterized further.

OCIAD1 depletion was validated by immunostaining and Western blot analysis for *OCIAD1* levels, in three biological replicates.

Expression of pluripotent stem cell markers OCT4, SSEA3/4, and TRA1-81 has been shown by immunostaining. Differentiation of these hESC lines to all the three germ layers was demonstrated by immunostaining for markers such as AFP (endoderm), Brachyury (mesoderm) and β -III tubulin (ectoderm). Karyotype was also checked in these cells and found to be normal.

Materials and methods

Cell culture

hESC line-BJNh20 was derived from the discarded Grade III embryos and tested for its pluripotency (Inamdar et al., 2009). This cell line has been cultured continuously for over 200 passages without acquiring karyotypic abnormality (Venu et al., 2010). The cell line was included in the analysis of genetic stability in the International Stem Cell

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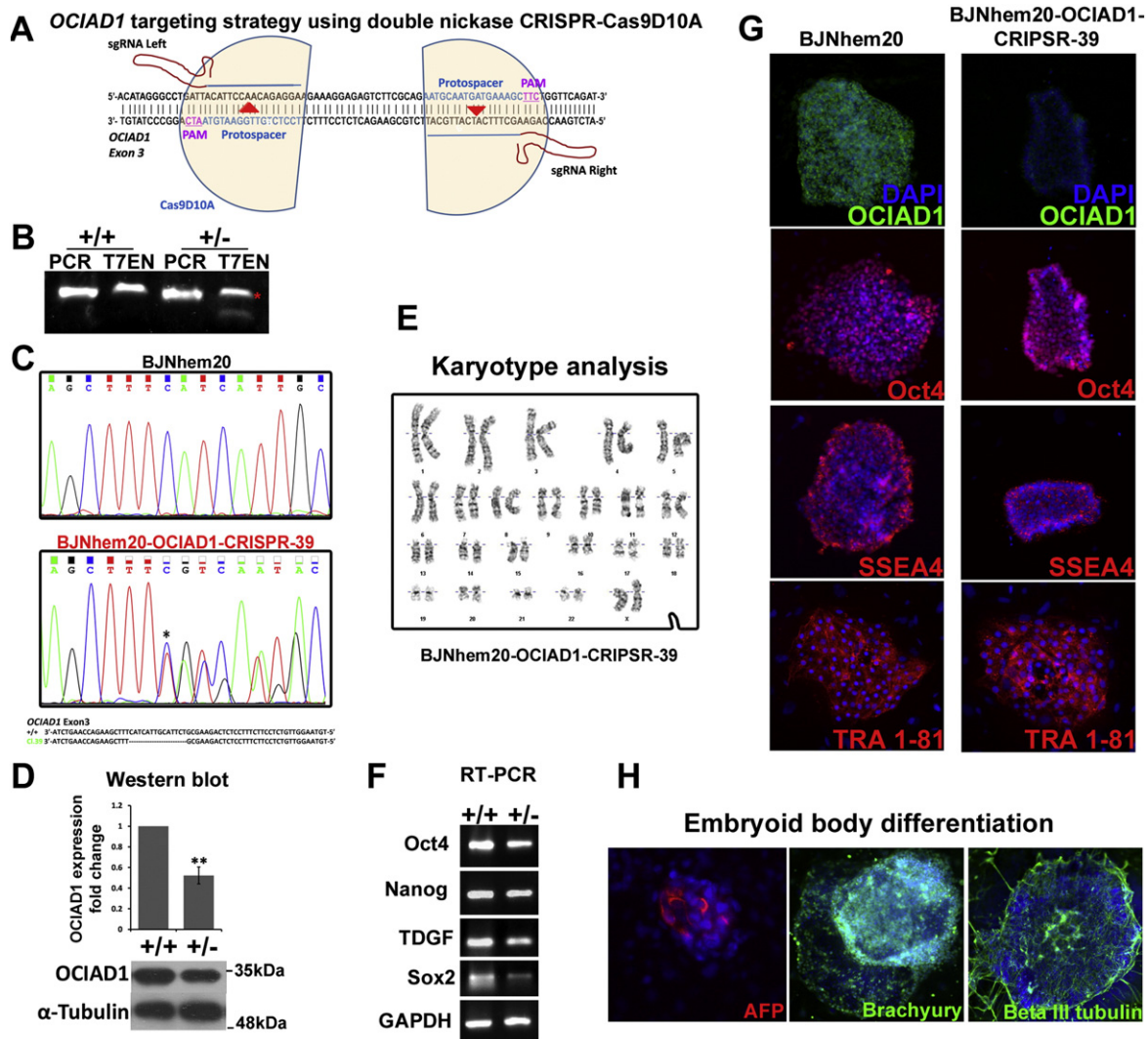


Fig. 1. (A) Strategy for targeting *OCIAD1* exon3 by CRISPR-CAS9. (B) Genomic targeting of *OCIAD1* exon3 of BJNh20-OCIAD1-CRISPR-39 identified by T7 endonuclease mediated digestion of high fidelity exon 3 amplicon detecting deletions in this clone. (C) Chromatograms showing sequence analysis to confirm heterozygous mutation in the targeted region of *OCIAD1*. (D) Western blot analysis showing decreased *OCIAD1* levels in BJNh20-OCIAD1-CRISPR-39. Graph represents standard error mean after analysis of three biological replicates using single factor ANOVA (P value 0.001). (E) Karyotype analysis of BJNh20-OCIAD1-CRISPR-39. (F) Analysis of transcript levels of pluripotency marker genes *Oct4*, *Nanog*, *TDGF* and *Sox2* by reverse transcription and polymerase chain reaction (RT-PCR) amplification. *GAPDH* was used to normalize transcript levels. (G) Immunostaining analysis of BJNh20-OCIAD1-CRISPR-39 for depletion of *OCIAD1* and for pluripotency markers *Oct4*, *SSEA4* and *TRA1-81* as indicated, compared to parental hESC line BJNh20. (H) Differentiation analysis of BJNh20-OCIAD1-CRISPR-39: embryoid bodies stained to show differentiation to all the three germ layers by immunostaining for *AFP*, *Brachyury* and β -III tubulin marking the endoderm, mesoderm and ectoderm respectively.

Initiative project (ISCI2, 2011). Cultures of BJNh20 and the transgenic line BJNh20-OCIAD1-CRISPR-39 were maintained on mouse embryonic fibroblast feeder layers in hESC medium composed of KnockOut Dulbecco's modified Eagle medium (KO-DMEM-Life Technologies Cat no. 10829-018) supplemented with 20% KnockOut Serum replacement (KOSR-Life Technologies Cat no. 10828-028), 1% GlutaMAX (Life Technologies Cat no. 35050-061), 1% non-essential amino acids (MEM-NEAA-Life Technologies Cat no. 11140-050), 0.1% β -mercaptoethanol (Life Technologies Cat no. 21985-023) and 8 ng/ml human recombinant basic fibroblast growth factor (human bFGF-Sigma Aldrich Pvt. Ltd. Cat no. F-0291). These cells were routinely passaged by mechanical cutting of undifferentiated colonies.

Electroporation of hESCs to generate BJNh20-OCIAD1-CRISPR-39 cell line

For electroporation, BJNh20 cells were grown on Matrigel-coated (Becton Dickinson Cat. No. 354277) dishes in mTESR medium (Stem Cell Technologies Cat. No. 05850), until the culture was 80% confluent.

To aid survival of single cells post transfection the cultures were pre-treated with ROCK inhibitor (10 μ M Y-27632, Sigma Aldrich) for 3 h prior to electroporation. The cells were trypsinized using 1X TrypLE (Life Technologies Cat. No. 12605010) at 37 $^{\circ}$ C for 4 min. The cells were pelleted by centrifugation at 1000 rpm for 2 min, washed with phosphate buffered saline and enumerated. 1 million cells were resuspended in 400 μ l cold OptiMEM medium and mixed with plasmids pCAS9-D10A-GFP (Addgene ID 44720) 5 μ g, *OCIAD1* CRISPR Left and Right Exon3 targeting sgRNA expression constructs (Custom generated by Genocopia) 2.5 μ g each. Electroporation was performed in a 0.4 cm Biorad electroporation cuvette at 250 mV, 500 μ F for 10 ms. Immediately after electroporation 0.5 ml of cold mTESR1 medium was added and the cells were transferred to a 15 ml centrifugation tube and spun down at 1000 rpm for 2 min. The cells were resuspended in "post-FACS recovery media" which was made using pre-conditioned mTESR and fresh mTESR1 (1:1), 8 ng/ml bFGF, supplemented with ROCK inhibitor (10 μ M Y-27632) and seeded on Matrigel-coated dishes. 16 h after electroporation the medium was replaced with mTESR1 medium without ROCK inhibitor.

Sorting of hESCs to generate BJNhem20-OCIAD1-CRISPR-39 cell line

GFP expression was monitored 48 h post transfection and after 72 h cells were harvested as mentioned above for sorting GFP+ population. The cells were resuspended in cold OptiMEM, passed through a 40 µm pore size cell strainer and then sorted for GFP expression using BD FACS ARIA II cell sorter. Sorted cells were collected in 15 ml centrifuge tubes containing 5 ml of OptiMEM with Antibiotic–Antimycotic (Life technologies Cat no. 15240-062). Post-sort the cells were incubated at 4 °C for 10 min, pelleted by centrifugation at 1000 rpm for 2 min and resuspended in 2 ml of post-FACS recovery medium. 200 GFP positive cells were seeded in 35 mm Matrigel-coated dish in post-FACS recovery medium with 10 µM ROCK inhibitor and 1X Antibiotic–Antimycotic Medium was replaced with fresh post-FACS recovery medium (without ROCK inhibitor) every 24 h.

Screening of BJNhem20 OCIAD1-CRISPR and Cas9D10A transfected cultures to generate BJNhem20-OCIAD1-CRISPR-39 cell line

Four days after FACS sorting, undifferentiated hESC colonies were clearly visible and cultures were switched to regular mTESR medium. Colonies were picked on day 8 and seeded individually on Matrigel-coated wells of 24 well plate for clonal expansion. After passaging to obtain duplicates, a single well of each clone was harvested and screened for in-del mutations as described before (Peters et al., 2013). Briefly, genomic DNA was isolated and PCR amplified using high-fidelity DNA polymerase (KAPA HiFi) and primers spanning OCIAD1 exon3 (OCIAD1 Intron2F: 5'gggtgctcttggtaagtgttaggc3'; OCIAD1 Intron3R: 5'cagacagacctgtttcaactcca3'), followed by PCR Cleanup (Machery Nagel Kit), sequencing (Sanger's Sequencing) and in-del detection using T7endonuclease (New England Biolabs) for the digestion of high-fidelity PCR amplicon (Fig. 1B).

Embryoid body differentiation

Differentiation of embryoid bodies was carried out as described before (Inamdar et al., 2009). Briefly, colonies grown on MEFs were mechanically cut and pieces cultured in human embryoid body media (10% FBS-Hyclone-SH30070 with β-mercaptoethanol and GlutaMAX in DMEM) on low attachment plates. These hESC aggregates were allowed to grow in suspension for 3 days after which the colonies formed embryoid body-like structures and were transferred to tissue culture dishes pre-treated with 0.1% gelatin and allowed to attach and differentiate spontaneously till the time for harvest for immunostaining analyses.

Immunostaining

hESC and hEBs were fixed in 2% paraformaldehyde (in PBS), permeabilized with Triton-X 100 (0.1% for cells and 0.3% for EBs) and then blocked with 4% FBS for 1 h at RT (Room Temperature). Post blocking, cells were incubated with the appropriate primary antibodies overnight at 4 °C, washed with PBS at RT and incubated with secondary antibody conjugated to either Alexa Fluor 488 or 568 for 1 h at RT (Dilution 1:400

Invitrogen). The cells were washed and stained using DAPI (1:500 in M1 buffer Invitrogen) to visualize nuclei. The images were acquired using an epifluorescence microscope (IX-81 (Olympus)) fitted with a cooled CCD camera (CoolSnap, Roper Scientific). Primary antibodies used were against OCIAD1 (Abcam Ab91574), Oct4 (BD Biosciences BD611203), TRA1-81 and SSEA4 (kind gift from Peter Andrews, University of Sheffield, UK), Brachyury (Santa Cruz Biotech Cat no. SC-17743), β-III tubulin (Santacruz SC-51670), AFP (Sigma Chemical Pvt. Ltd. Cat no. A8452).

RNA isolation, cDNA synthesis and PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies Cat no. 15596-026) following the manufacturer's instructions. 2 µg of DNase treated RNA was converted to cDNA by performing reverse transcription using Superscript II (Life Technologies Cat no. 18064-014). Primer sequences used for cDNA amplification (5'–3') were as follows:

Primer name	Sequence
oct4_F	gaaggattcagccaaacgac
oct4_R	gttacagaaccacactcgga
nanog_F	tgcaaatgtcttctgctgagat
nanog_R	gttcaggatgttggagagttc
gapdh_F	gtccatgccatcaactgcca
gapdh_R	ttactccttggagccatg
tdgf_F	tccttctacggacggaactg
tdgf_R	agaaatgcctgaggaaagca

Verification of karyotype

Standard G banding of metaphase spreads was performed and analyzed using CytoVision software. For each sample, at least 50 metaphases were counted and 10 were analyzed.

Acknowledgments

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