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Lab Resource: Stem Cell Line

Generation of OCIAD1 inducible overexpression human embryonic stem cell line: BJNhem20-OCIAD1-Tet-On

Deeti K. Shetty [a], Ronak Shetty [a], Maneesha S. Inamdar [a,b,*]

[a] Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India
[b] Institute for Stem Cell Biology and Regenerative Medicine, Bengaluru, India

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ABSTRACT

Human embryonic stem cell line BJNhem20-OCIAD1-Tet-On was generated using non-viral method. The constructs pCAG-Tet-On and pTRE-Tight vector driving OCIAD1 expression were transfected using microporation procedure. pCAG-Tet-On cells can be used for inducible expression of any coding sequence cloned into pTRE-Tight vector. For example, in human embryonic stem cells, Tet-On system has been used to generate SOX2 overexpression cell line (Adachi et al., 2010).

Resource table

Name of stem cell lines	BJNhem20-OCIAD1-Tet-On
Institution	Jawaharlal Nehru Centre for Advanced Scientific Research
Person who created the resource	Deeti K. Shetty, Ronak Shetty and Maneesha S. Inamdar
Contact person and email	inamdar@jncasr.ac.in
Date archived/stock date	10th November, 2012
Origin	Human embryonic stem cell line BJNhem20
Type of resource	Biological reagent: genetically modified human embryonic stem cells
Sub-type	Cell line
Key transcription factors	Oct4, Sox2, Nanog
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://onlinelibrary.wiley.com/doi/10.1111/j.1365-2443.2010.01400.x/abstract;jsessionid=1140253906D2977382217462409DD32E.f03t03
Information in public databases	–
Ethics	Competent authority approval obtained

Resource details

To generate BJNhem20-OCIAD1-Tet-ON cell line, we transfected BJNhem20 with pCAG-Tet-On (pCAG-rtTA^{On}) and pTRE-Tight-OCIAD1 by microporation at 1100 V, 30 ms pulse width and 1 pulse number (see Fig. 1A). A stable hESC line was generated after subjecting these transfected cells to hygromycin and geneticin selection for ten days. The pTRE-Tight-OCIAD1 construct was generated by cloning OCIAD1 Open Reading Frame amplicon from amino acids 1 to 245 between EcoRI and NotI sites using appropriate primers. Induction of OCIAD1 expression was done using doxycyline (300 ng/ml).

Expression of pluripotent stem cell markers OCT4, SSEA4 and TRA1–81 has been shown by immunostaining and RT-qPCR analyses. Early differentiation of BJNhem20-OCIAD1-Tet-On was demonstrated by immunostaining for N-cadherin.

Materials and methods

Cell culture

hESC line BJNhem20 was derived from discarded Grade III embryos and tested for pluripotency (Inamdar et al., 2009). This cell line has been cultured continuously for over 200 passages without acquiring any karyotypic abnormality (Venu et al., 2010). The cell line was included in the analysis of genetic stability in the International Stem Cell Initiative project (ISCI2, 2011). Cultures of BJNhem20 and the transgenic line BJNhem20-OCIAD1-Tet-On 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

* Corresponding author.

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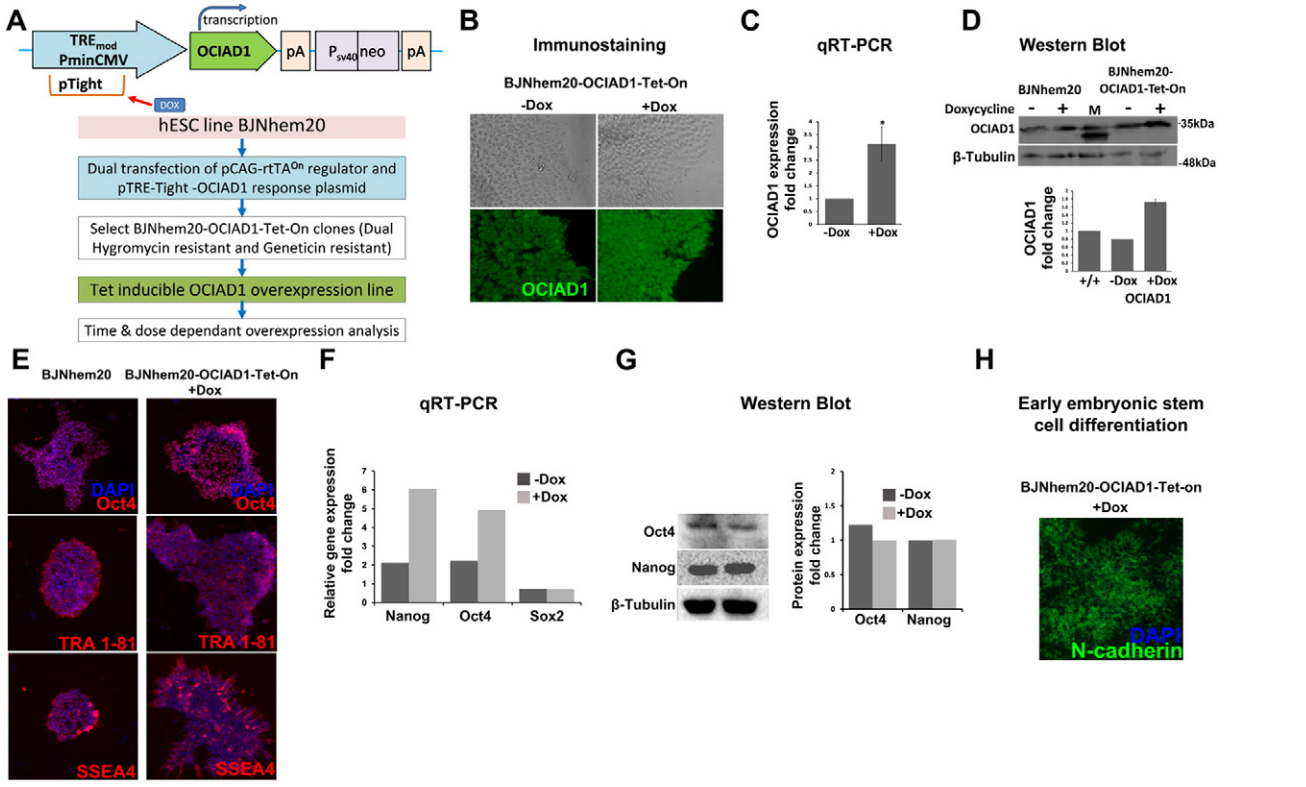

Fig. 1. Expression and karyotype analysis of BJNhem20-OCIAD1-Tet-On line:-. (A) Schematic representation of pTRE-Tight-OCIAD1 plasmid and strategy for generation of OCIAD1 inducible overexpression line. (B) Validation of OCIAD1 overexpression in BJNhem20-OCIAD1-Tet-On cell line grown on Matrigel and induced with Doxycycline, by immunostaining with OCIAD1 antibody. (C–D) Quantitative real-time PCR (C) and Western blot analysis (D) showing OCIAD1 overexpression upon Doxycycline induction. Graphs represent data from at least three biological replicates. (E) Pluripotency marker expression in BJNhem20-OCIAD1-Tet-On by immunostaining for OCT4, SSEA4 and TRA1–81. (F) Analysis of transcript levels of pluripotency marker genes by quantitative polymerase chain reaction (qPCR). (G) Tight regulation on pluripotency regulators expression upon Doxycycline induction of OCIAD1 analyzed by Western blot for Oct4 and Nanog. (H) Analysis of early differentiation (day 3.5) to mesodermal lineage upon Doxycycline induction, by immunostaining with N-cadherin antibody.

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.

Microporation of hESCs to generate BJNhem20-OCIAD1-Tet-On cell line

The plasmids pCAG-Tet-On (pCAG-rtTA^{On}) and vector pTRE-Tight were a kind gift from Norio Nakatsuji (Kyoto University, Japan). We generated pTRE-Tight-OCIAD1 construct by cloning OCIAD1 Open Reading Frame amplicon from amino acids 1 to 245 between EcoRI and NotI sites using appropriate primers. For microporation, BJNhem20 cells were grown on Matrigel (Becton Dickinson; cat no. 354277) coated dishes till they were 70% confluent. The cells were trypsinized with 1× TrypLE (Life Technologies; cat no. 12605-010) at 37 °C for 4 min. The cells were pelleted by centrifugation at 1000 rpm for 2 min and washed with phosphate buffered saline and enumerated. 0.5 million cells were resuspended in 10 μl of R buffer and 5 μg of both the plasmids was mixed and the cells were transfected in a microporator (Neon Microporation Kit-Life Technologies; cat no. MPK1096). Microporation conditions used were 1100 V, 30 ms and 1 pulse according to the manufacturer's instructions. The cells were then directly seeded onto Matrigel-coated dishes with mTESR media (Stem Cell Technologies; cat no. 05850) supplemented with ROCK inhibitor (10 μM Y-27632, Sigma Aldrich Pvt. Ltd.; cat no. Y0503) to aid the survival of single cells. mTESR medium without ROCK inhibitor was used after 12–16 h when the cells adhered. 48 h after transfection, hygromycin (10 μg/ml Sigma Aldrich Pvt. Ltd.; cat no.H-3274) and geneticin (100 μg/ml Life Technologies; cat. no. 11811-031) were added to the transfected culture and selection was continued for 10 days. Hygromycin and geneticin resistant individual clones were manually picked and passaged. BJNhem20-OCIAD1-Tet-On cells were subsequently cultured in both feeder free conditions on Matrigel in the presence of mTESR medium and on feeders with regular hESC medium.

Induction of OCIAD1 expression with Doxycycline

Wild type and OCIAD1-Tet-On cells were plated at a density of 30% confluence on Matrigel-coated plates in mTESR medium. After 24 h of seeding once the colonies have attached 300 ng/ml of Doxycycline (Sigma Aldrich Pvt. Ltd., cat no. D9891) was added to the medium and cells were cultured for 72 h replenishing with fresh Doxycycline containing medium every 24 h. To test for induction of OCIAD1 expression, control and test cell lines were harvested after 48 h for RNA extraction and 72 h for protein extraction or fixed for immunostaining.

Differentiation of embryonic stem cells

Differentiation of embryonic stem cells was carried out as described before (Evseenko et al., 2010). Briefly, colonies grown on Matrigel were mechanically cut and re-passaged onto Matrigel-coated dishes and grown in mTESR medium for 2 days. The medium was then changed to hematopoietic differentiation basal media (Stemline II) with growth factors BMP4, Activin A, VEGF and bFGF

(all Sigma Aldrich Pvt. Ltd) for 3.5 days. Cells were then fixed and immunostained as follows.

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, then were 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 the nuclei. The images were acquired using an epifluorescence microscope (IX-81 microscope system (Olympus)) fitted with a cooled CCD camera (CoolSnap, Roper Scientific). Primary antibodies used were against OCIAD1 (Abcam; cat no., Ab91574), Oct4 (BD Biosciences BD 611203), and TRA1–81 and SSEA4 monoclonal antibodies were a kind gift from Peter Andrews (University of Sheffield, UK), N-cadherin (BD Biosciences; cat no. BD 610921).

RNA isolation/cDNA synthesis and quantitative PCR

Total RNA was extracted from cells by 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). Quantitative real time PCR was performed using EvaGreen Master Mix (Biorad). Primer sequences used for cDNA amplification (5′–3′) were as follows:

Primer name	Sequence
*oct4*_F	GAAGGTATTCAGCCAAACGAC
*oct4*_R	GTTACAGAACCACACTCGGA
*sox2*_F	AGTCTCCAAGCGACGAAAAA
*sox2*_R	GCAAGAAGCCTCTCCTTGAA
*nanog*_F	TGCAAATGTCTTCTGCTGAGAT
*nanog*_R	GTTCAGGATGTTGGAGAGTTC
*gapdh*_F	GTCCATGCCATCACTGCCA
*gapdh*_R	TTACTCCTTGGAGCCATG
OCIAD1_F	AGGAACCATGGATGGGAGGGCTGACTTTC
OCIAD1_R	CATTACTCGAGCTCATCCCAAGTATCTCC

Western blot

For Western blot analysis, cells were pelleted, washed with 1× PBS, lysed in lysis buffer for 4 h at 4 °C and clarified by centrifugation at 10,000 rpm for 20 min at 4 °C. Total protein was estimated using Bradford reagent and 40 μg of lysate was loaded on a 12% SDS-PAGE gel. The gel was electro-blotted onto Nitrocellulose membrane (PALL corporation, USA) and incubated with OCIAD1 (Abcam; cat no., Ab91574), Oct3/4 (BD Biosciences; cat no. BD 611203), Nanog (Santacruz; cat no. sc-30328) and β tubulin (DSHB — Developmental Studies Hybridoma Bank; E7) primary antibodies at 4 °C (overnight), washed, then probed with appropriate HRP-conjugated secondary antibodies (Bangalore Genei) for 1 h at RT and developed using ECL chemiluminescence kit (Thermo Scientific, Rockford, IL, US).

Acknowledgments

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