Stem Cell Research 16 (2016) 331–333

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Stem Cell Line

Generation of a transgenic human embryonic stem cell line ectopically expressing the endosomal protein Asrij that regulates pluripotency in mouse embryonic stem cells: BJNhem20-Asrij

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ARTICLE INFO

Article history:
Received 31 December 2015
Accepted 31 December 2015
Available online 6 January 2016

ABSTRACT

Asrij is an endocytic protein expressed in mouse embryonic stem cells (mESCs) and is essential for maintenance of stemness of mESCs (Mukhopadhyay et al., 2003; Sinha et al., 2013). Its human ortholog named Ovarian Carcinoma Immuno-reactive Antigen domain containing protein 1 (OCIAD1) is 85% identical. We ectopically expressed Asrij in epiblast stage equivalent-human embryonic stem cells (hESCs) to test for induction of naive pluripotency in primed pluripotent cells. The construct pCAG-Asrij was introduced into hESCs by microporation. Ectopic expression of Asrij in BJNhem20 hESC line was performed by selecting for plasmid transfection, followed by stable cell line generation.

Resource table

Name of stem cell lines	BJNhem20-Asrij
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	22nd March, 2013
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, 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
Information in public databases	–
Ethics	Competent authority approval obtained

Resource details

BJNhem20-Asrij line was generated by transfection of BJNhem20 human embryonic stem cells (hESCs) with pCAG-Asrij construct using microporation technique with the conditions of 1100 V, 30 ms pulse width and 1 pulse. The pCAG-Asrij construct was generated by cloning Asrij Open Reading Frame amplicon from amino acids 1–247 between Xho1 and Not1 sites using appropriate primers. A stable hESC line was generated after subjecting these transfected cells to puromycin selection for two weeks.

Ectopic Asrij expression was validated by immunostaining and Western blotting 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 different markers such as AFP (endoderm), Brachyury (mesoderm) and β-III tubulin (ectoderm).

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-Asrij 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

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http://dx.doi.org/10.1016/j.scr.2015.12.044

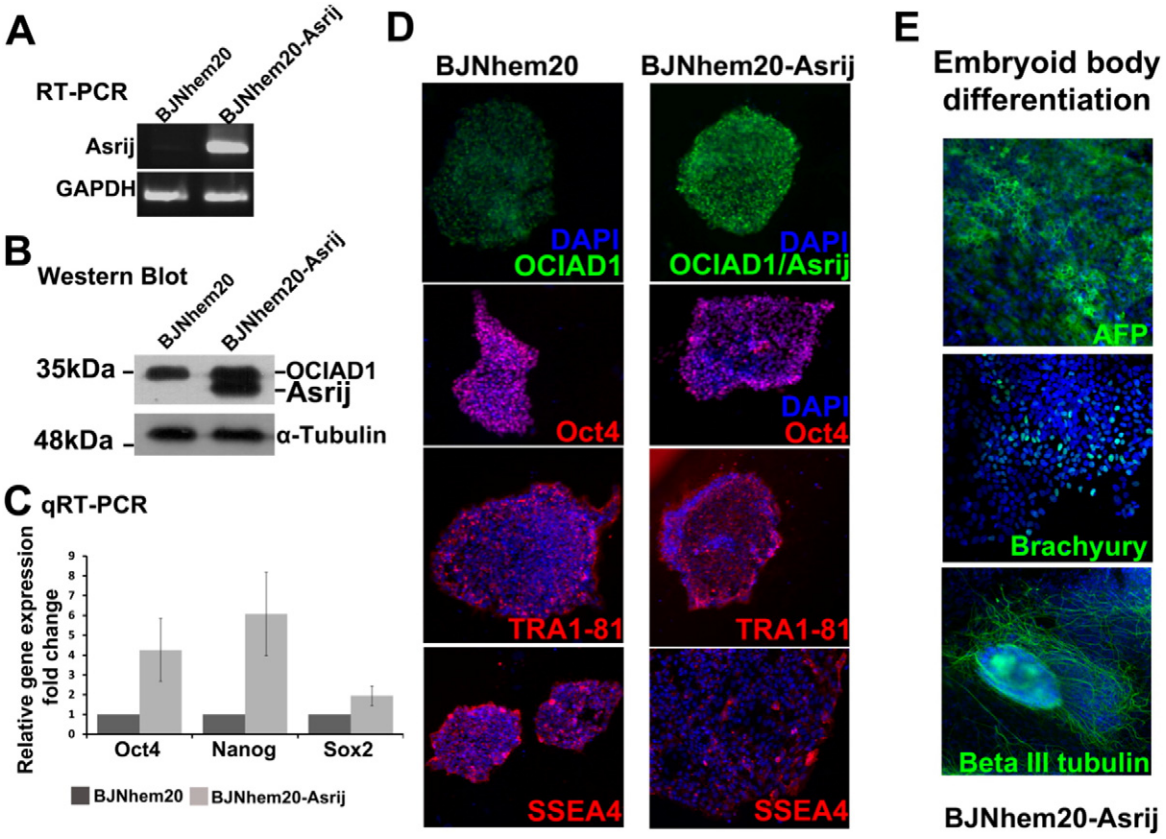

Fig. 1. Expression and pluripotency analysis of BJNhem20-Asrij hESC line: — (A) Reverse transcriptase PCR showing ectopic expression of Asrij in BJNhem20-Asrij compared to its parental line BJNhem20. (B) Western blot analysis showing overexpression of Asrij protein detected using OCIAD1 antibody (owing to its high sequence identity- 85%) as a lower molecular weight band below the endogenous OCIAD1 protein band. More than three biological replicates have been analyzed for Asrij expression. (C) qRT-PCR analysis showing expression of pluripotency marker genes Oct4, Nanog and Sox2 in Asrij overexpressing and wild type BJNhem20. (D) Immunostaining analysis of BJNhem20-Asrij for overexpression of Asrij and for pluripotency markers Oct4, SSEA4 and TRA1-81 as indicated, compared to parental line BJNhem20 hESC line. The OCIAD1 antibody detects both OCIAD1 and Asrij. (E) Differentiation analysis of BJNhem20-Asrij: Embryoid bodies stained to show differentiation to all the three germ layers by immunostaining for AFP, Brachyury and βIII-Tubulin marking the endoderm, mesoderm and ectoderm respectively.

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.

Microporation of hESCs to generate BJNhem20-Asrij cell line

For microporation, BJNhem20 cells were grown on Matrigel (Becton Dickinson Catalog No. 354277) coated dishes till they were 70% confluent. The cells were trypsinized with 1X 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 6 μg of DNA was mixed and the cells were transfected in a microporator (NeonMicroporation 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, puromycin (0.5 μg/ml Sigma Aldrich Pvt. Ltd. P8833) was added to the transfected culture and selection was continued for 2 weeks. Puromycin resistant individual clones of transgenic Asrij overexpressing hESCs were manually picked and passaged. BJNhem20-Asrij cells were subsequently cultured in both feeder free conditions on matrigel in the presence of mTESR medium and on feeders with regular hESC medium.

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 the tissue culture dishes pre-treated with 0.1% gelatin and allowed to attach and differentiate spontaneously till the time for harvest from day 6 onwards 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, 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 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 ab91574), Oct4 (BD Biosciences BD611203), TRA1-81 and SSEA4 monoclonal antibodies were a kind gift from Peter Andrews (University of Sheffield, UK), Brachyury (Santa Cruz Biotech Cat no.SC-17743), β-III tubulin, AFP (Sigma Chemical Pvt. Ltd. Cat no. A8452).

RNA isolation/cDNA synthesis and 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 following:

Primer name	Sequence
oct4_F	GAAGGTATTCAGCCAAACGAC
oct4_R	GTTACAGAACCACACTCGGA
nanog_F	TGCAAATGTCTTCTGCTGAGAT
nanog_R	GTTCAGGATGTTGGAGAGTTC
gapdh_F	GTCCATGCCATCACTGCCA
gapdh_R	TTACTCCTTGGAGCCATG
sox2_F	AGTCTCCAAGCGACGAAAAA
sox2_R	GCAAGAAGCCTCTCCTTGAA
mAsrijHx1_F	TTTGCAGAGTGCCATGAAGAGTGCTTCTGGTTC
mAsrijHPh_R	GACCGCAGTGGATCCCCAAGGGGGGAGTTTTC

Western blot

For Western blot analysis, cells were pelleted, washed with 1X 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 primary antibody OCIAD1 (Abcam; Cat no., ab91574) at 4 °C (overnight), washed, then probed with appropriate HRP-conjugated secondary antibody (Bangalore Genei) for 1 h at RT and developed using ECL chemiluminescence kit (Thermo Scientific, Rockford, IL, US).

Acknowledgments

This project was funded by the Department of Biotechnology, Government of India (Grant no. BT/PR5905/MED/31/171/2012) and the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore. We thank, Abhishek Sinha and Vinay K. S. for generating pCAG-Asrij plasmid construct.

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