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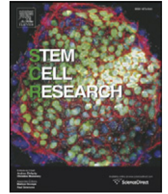

Lab Resource: Stem Cell Line

Generation of human embryonic stem cell line expressing a red fluorescent protein: BJNhem20-pCAG-tdTomato

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ABSTRACT

Human embryonic stem cell line BJNhem20-pCAG-tdTomato was generated using non-viral method. The construct pCAG-tdTomato was transfected using microporation procedure. This fluorescent hESC line can help to study heterogeneity within individual cells in hESC colonies by enabling live tracking of their growth, migration and differentiation properties. This cell line also serves as a resource for additional transgene introduction/knock-out/knock-in generation in a fluorescent background and allows ease of analysis in studies involving cell mixing.

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Resource table

Name of stem cell lines	BJNhem20-pCAG-tdTomato
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	28th May, 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, 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_9Information in public databases–EthicsCompetent authority approval obtained

Resource details

BJNhem20-pCAG-tdTomato line was generated by transfection of BJNhem20 human embryonic stem cells (hESCs) with pCAG-tdTomato construct using microporation technique with the conditions of 1100 V, 30 ms pulse width and 1 pulse. A stable hESC line was generated after subjecting these transfected cells to puromycin selection for two weeks.

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).

Karyotype was also checked in these cells and found to be normal.

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-pCAG-tdTomato 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.

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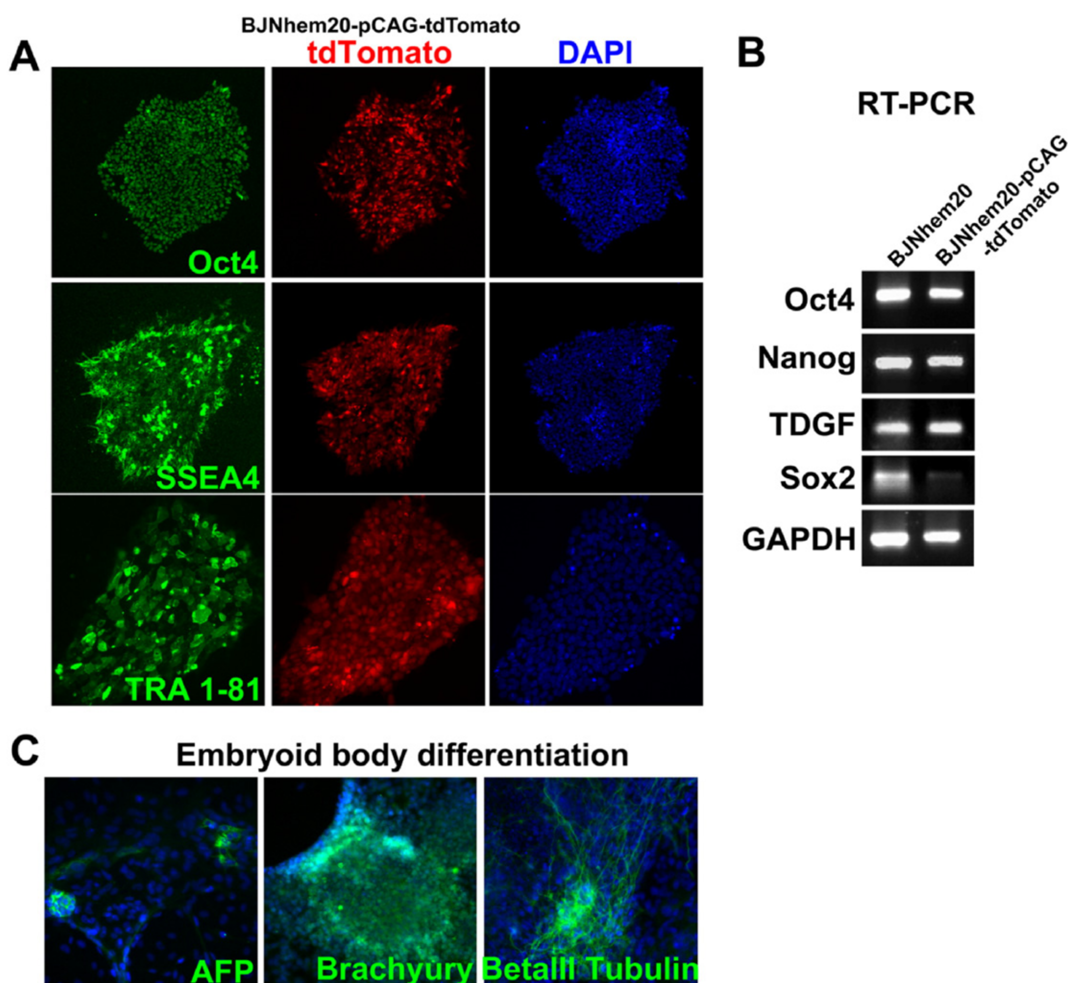

Fig. 1. Expression analysis of BJNhem20-pCAG-tdTomato hESC line:—(A) Validation of tdTomato expression in undifferentiated hESCs and immunostaining analysis for expression of pluripotency markers Oct4, SSEA4 and TRA1-81 of BJNhem20 hESC line. (B) Analysis of transcript levels of pluripotency marker genes Oct4, Nanog, TDGF and Sox2 by reverse transcription and polymerase chain reaction (RT-PCR) amplification. GAPDH was used to normalize transcript levels. (C) Differentiation analysis—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.

Microporation of hESCs to generate BJNhem20-pCAG-tdTomato cell line

For microporation, BJNhem20 cells were grown on Matrigel-coated (Becton Dickinson Cat No. 354277) 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 (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-27,632, 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 two weeks. Puromycin resistant individual clones of transgenic tdTomato expressing hESCs were manually picked and passaged. BJNhem20-pCAG-tdTomato 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-mercapthoethanol 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 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). 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
tdgf_F	TCCTTCTACGGACGGAACTG
tdgf_R	AGAAATGCCTGAGGAAAGCA
sox2_F	AGTCTCCAAGCGACGAAAAA
sox2_R	GCAAGAAGCCTCTCCTTGAA

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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References

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