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Lab Resource: Stem Cell Line

Generation of a constitutively expressing Tetracycline repressor (TetR) human embryonic stem cell line BJNhem20-TetR

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ABSTRACT

Human embryonic stem cell line BJNhem20-TetR was generated using non-viral method. The construct pCAG-TetRnls was transfected using microporation procedure. BJNhem20-TetR can subsequently be transfected with any vector harbouring a TetO (Tet operator) sequence to generate doxycycline based inducible line. For example, in human embryonic stem cells, the pSuperior based TetO system has been transfected into a TetR containing line to generate OCT4 knockdown cell line (Zafarana et al., 2009). Thus BJNhem20-TetR can be used as a tool to perturb gene expression in human embryonic stem cells.

Resource table

Name of Stem Cell lines	BJNhem20-TetR
Institution	Jawaharlal Nehru Centre for Advanced Scientific Research
Person who created resource	Ronak Shetty and Maneesha S. Inamdar
Contact person and email	inamdar@jncasr.ac.in
Date archived/stock date	10th March, 2011
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
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

To generate BJNhem20-TetR cell line, we transfected BJNhem20 by microporation at 1100 V, 30 ms pulse width and 1 pulse number. 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, TRA1-60 and TRA1-81 has been shown by immunostaining. Differentiation of BJNhem20-TetR to all the three germ layers was demonstrated by immunostaining for beta III tubulin (ectodermal), Brachyury (mesodermal) and AFP (endodermal). 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-TetR 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. The plasmid pCAG TetRnls was a kind gift from Peter Andrews (University of Sheffield, UK).

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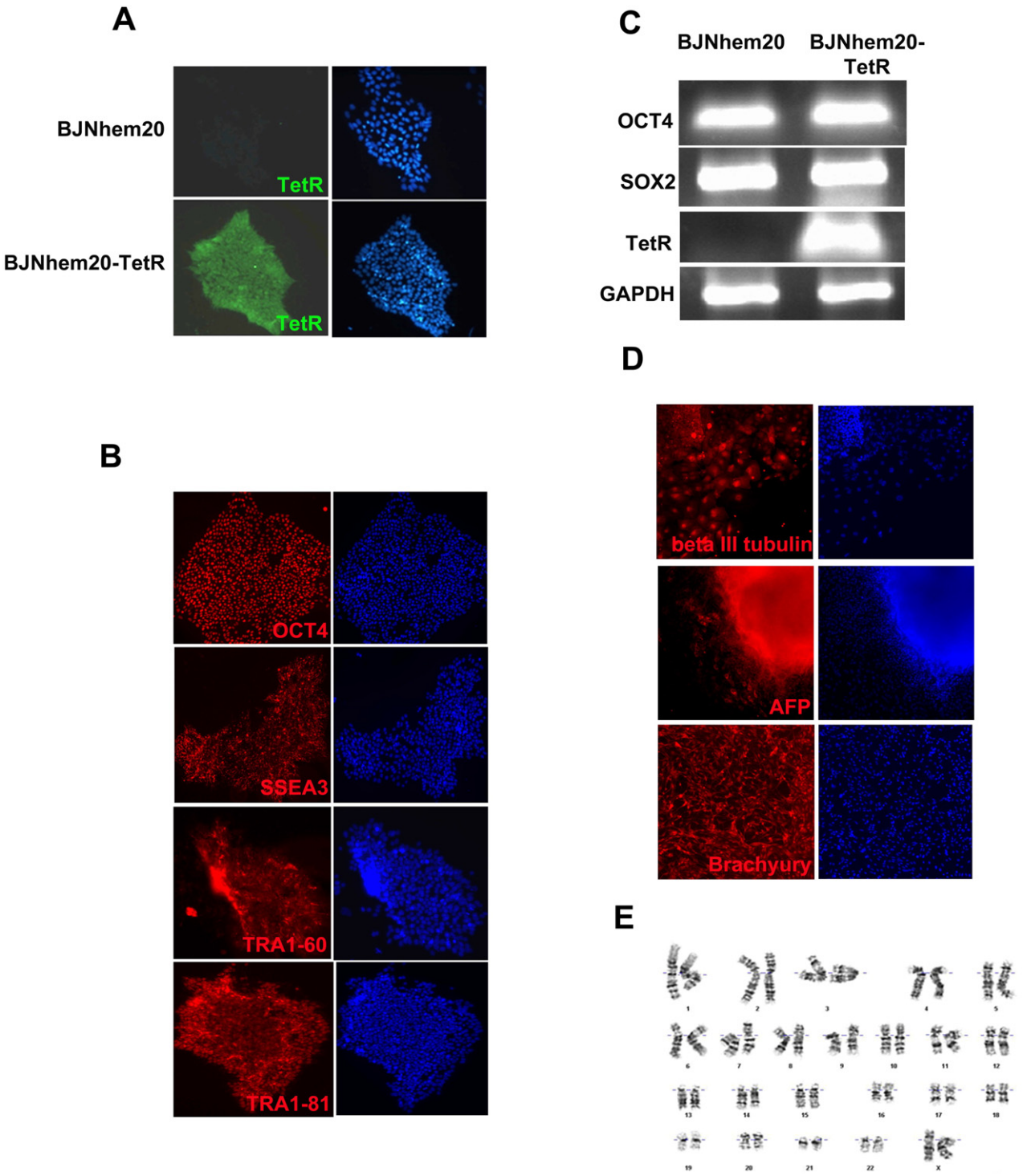

Fig. 1. Expression and karyotype analysis of BJNhem20-TetR line. A) Validation of BJNhem20-TetR cell line grown on matrigel by immunostaining with TetR antibody. B) Pluripotency marker expression in BJNhem20-TetR by immunostaining for OCT4, SSEA3, TRA 1-60 and TRA 1-81. C) Analysis of transcript levels of pluripotency marker genes by reverse transcription and polymerase chain reaction (RT-PCR) amplification. D) Differentiation analysis of BJNhem20-TetR: Embryoid bodies stained to show differentiation to all the three germ layers by immunostaining (red) for AFP, Brachyury and β III-Tubulin marking the endoderm, mesoderm and ectoderm respectively. E) Karyotype analysis of BJNhem20-TetR.

Microporation of hESCs to generate BJNhem20-TetR cell line

For microporation, BJNhem20 cells were grown on Matrigel (Becton Dickinson; Cat no. 354277) coated dishes till they were 60–70% 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-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.; Cat no. P8833) was added to the transfected culture and selection was continued for 10 days. Puromycin resistant individual clones were manually picked and passaged. BJNhem20-TetR 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; Cat no. 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 TetR (Clonetech; Clone 9G9 Cat no. 631131), Oct4 (BD Biosciences BD611203), TRA1-81 and SSEA3 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 follows:

Primer name	Sequence
OCT4_F	GAAGGTATTCAGCCAAACGAC
OCT4_R	GTTACAGAACCACACTCGGA
SOX2_F	AGTCTCCAAGCGACGAAAAA
SOX2_R	GCAAGAAGCCTCTCCTTGAA
GAPDH_F	GTCCATGCCATCACTGCCA
GAPDH_R	TTACTCCTTGGAGCCATG
TET_F	GGTTGCGTATTGGAAGAT
TET_R	TTCAAGGCCGAATAAGAA

Verification of karyotype

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

Acknowledgements

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