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Lab Resource: Stem Cell Line

Generation and characterization of human iPSC line from CD34$^+$ cells isolated from umbilical cord blood belonging to Indian origin

Sophia Fernandes [a], Manasi Talwadekar [a], Raju Agarwal [b,1], Velu Nair [c,2], Vaijayanti Kale [a], Lalita Limaye [a,*]

[a] Stem Cell Laboratory, National Centre for Cell Science, NCCS Complex, University of Pune Campus, Ganeshkhind, Pune 411007, India
[b] Obstetrics and Gynecology at Obstetrics and Gynecology Department, AFMC, Pune 411040, India
[c] Medicine & Haematology, Armed Forces Medical College (AFMC), Pune 411040, India

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ABSTRACT

We describe here the reprogramming of CD34$^+$ cells isolated from umbilical cord blood obtained after full term delivery of a healthy female child of Indian origin. The cells were nucleofected by episomal vectors expressing Oct4, Sox2, L-Myc, Klf4, Lin28 and p53DD (negative mutation in p53). Colonies were identified by alkaline phosphatase staining and characterized for expression of pluripotency markers at protein level by immunofluorescence, flow cytometry and at transcript level by PCR. Genomic stability of the cell line was checked by G-banded karyotype. The ability to differentiate to endoderm, mesoderm and ectoderm in vitro was confirmed by immunofluorescence staining.

Resource Table

Name of Stem Cell line	**iP2/14**
Institution	Stem Cell Laboratory, National Centre for Cell Science, NCCS Complex, University of Pune Campus, Ganeshkhind, Pune-411007, India.
Person who created resource	Sophia Fernandes, Manasi Talwadekar, Vaijayanti Kale and Lalita Limaye
Contact person and email	Dr. Mrs. Lalita Limaye, lslimaye@nccs.res.in
Date archived/stock date	Sept 2014
Origin	Healthy human female umbilical cord blood (CB) derived CD34$^+$ cells from Indian ethnicity.
Type of resource	Biological reagent- Human iPSC line derived from CB CD34$^+$ cells of healthy female child of Indian ethnicity.
Sub-type	Cell line
Key transcription factors	Oct4, Sox2, L-Myc, Klf4, Lin28, dominant negative mutation ofp53 (p53DD)
Authentication	Identity of cell line was confirmed by pluripotency marker expression, maintenance of a normal karyotype and tri-lineage differentiation (Figs. 1 and 2)
Link to related literature	Nil
Information in public databases	Nil
Ethics	All the protocols were approved by both NCCS committee for Stem cell research (NCCS-IC-SCR) and AFMC-IC-SCR, both of these committees comply with the Declaration of Helsinki.

1. Resource details

The iP2/14 line was generated by nucleofecting CB-derived CD34$^+$ cells with episomal plasmids. CB was collected after full term delivery. The donor was a female child of Indian ethnicity. The episomal plasmids contained the factors Oct4, Sox2, Klf4, L-myc, Lin28 and mutated p53. These plasmids are non-integrative and are subsequently cleared out of the cells with rapid cell divisions, thus resulting into generation of "foot-print free" induced pluripotent stem cells (iPSCs). Advantage of this kind of a system is the clinical relevance of these cells as opposed to the conventional genomic integrative methods like retroviral transfection (Dowey et al., 2012 and Mack et al., 2011). After nucleofection by these plasmids, colonies emerged after 20–25 days with a reprogramming efficiency of 0.02%. The colonies showing cobblestone morphology and refractile edges (Fig. 1A) were passaged and maintained on a feeder-free system (vitronectin/geltrex) in Essential 8 media. The colonies stained positive for alkaline phosphatase (ALP) activity, which is a well-established marker for pluripotent stem cells (Fig. 1B). The cell line was further assessed for genomic stability, wherein it was found to maintain a normal female karyotype-46,XX with no distinct abnormalities or chromosomal anomalies (Fig. 1C). After passage

* Corresponding author.
E-mail address: lslimaye@nccs.res.in (L. Limaye).
[1] Present address- Professor and Head of Department, Obstetrics and Gynecology, Command Hospital, Lucknow- 226002, India.
[2] Present address- Director General Medical Services (Army) & Col Commandant, Dte Gen Medical Services (Army), Adjutant General's Branch Integrated HQ of MoD (Army), New Delhi-110001, India.

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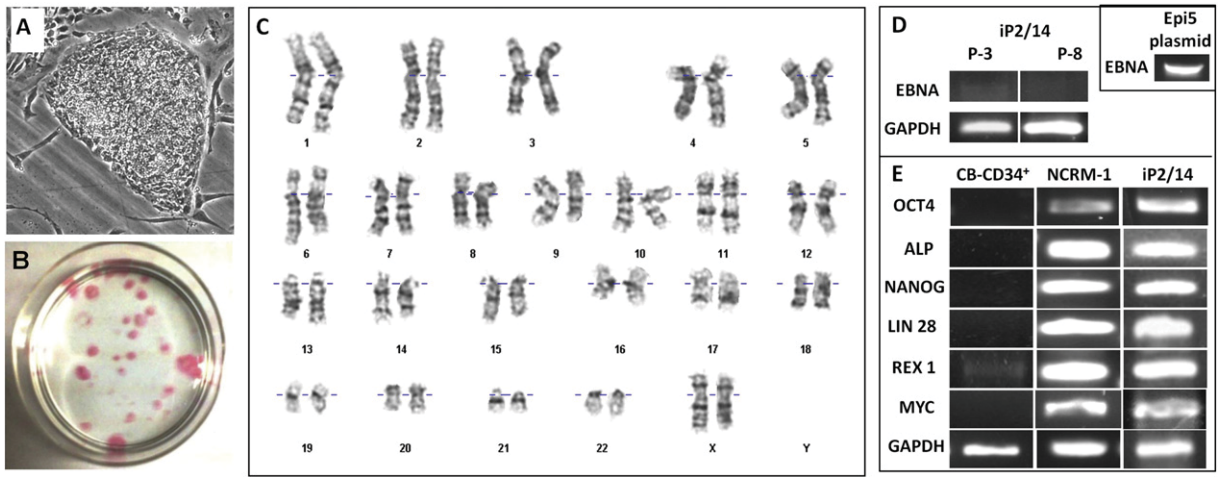

Fig. 1. Characterization of iPSC line iP2/14: Phase contrast image of iPSC colony at 20× magnification (A). ALP staining of colonies at passage 8 indicating pluripotent nature of the colonies (B). G-banded karyotype of cells in passage 19 demonstrating a normal human female karyotype (C). The loss of the plasmid with subsequent passages was assessed at different passages (P-3 and P-8) on the cell line iP2/14 by PCR with internal control GAPDH. Positive control- EBNA primer pair amplification on Epi5 plasmid is shown as inset (D). Transcript level expression of pluripotency markers-Oct4, ALP, Nanog, Lin28, Rex1 and myc on CB CD34$^+$ cells (left), NCRM-1 (middle) and iP2/14 (right). Equivalent expression was seen in NCRM and iP2/14 whereas HSCs showed no expression (E).

3, we checked for the retention of plasmids within the cells. We observed that the episomal plasmids were not detected by PCR suggesting their elimination which was again confirmed in passage 8 (Fig. 1D). Pluripotency markers were screened at transcript level using cDNA prepared from cells and the data were compared to that of NCRM-1, an established iPSC line (source-NIH CRM). The expression of pluripotency genes was similar in both the cell lines NCRM-1 and iP2/14 for Oct4, ALP, Nanog, Lin28, Rex-1 and myc, however CB derived CD34$^+$ cells did not show expression of pluripotency markers (Fig. 1E). In addition to the transcript level detection, the protein expression of the markers Oct4, Sox2, SSEA4 and Tra-1-60 were assessed by immunofluorescence (IF) (Fig. 2A). Pluripotency associated markers were also seen by flow cytometry, where the expression of Oct4, Sox2, SSEA4, Tra-1-60 and Tra-1-81 were detected (Fig. 2B). These cells lacked the expression of CD45 - a pan hematopoietic marker and CD34 - a HSCs marker (Fig. 2B). The cell line was maintained in vitro for over 20 passages with no distinct abnormality or contamination.

The ability of this line to differentiate in vitro to three lineages was checked by IF. The cells displayed expression for ectoderm marker-βIII tubulin, mesoderm marker-Brachyury and endoderm marker-FoxA2 (Fig. 2C). This indicated the ability of iP2/14 cell line to generate cells from all the three germ lineages further confirming that it is an iPSC cell line. To the best of our knowledge, this is the first report of an iPSC line generated from CB CD34$^+$ cells obtained from Indian ethnic population by use of non-integrative approach of reprogramming.

2. Materials and methods

2.1. Ethics approval

Informed consent was taken from the donor mother before collection of CB sample after full term delivery at AFMC, Pune. The consent form formats and protocols for collection and processing of sample were approved by NCCS Ethics Committee (NCCS-IEC), NCCS committee

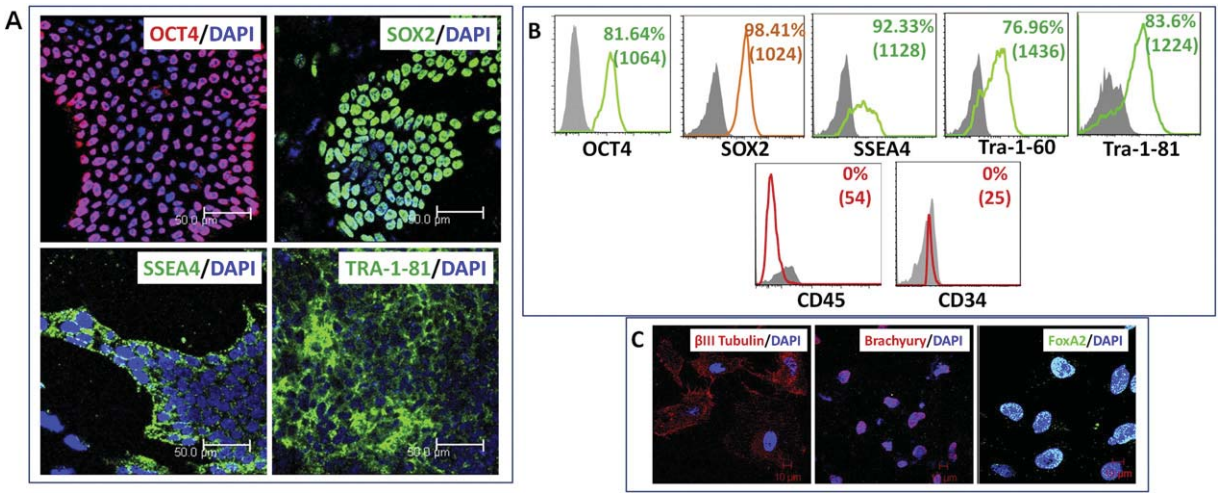

Fig. 2. Characterization and tri-lineage differentiation of iPSC line iP2/14: Immunofluorescence staining showing expression of pluripotency markers Oct4, Sox2, SSEA4 and Tra-1-60 on iP2/14 cells, scale bars = 50 μm (A). Flow cytometry overlays showing expression of Oct4, Sox2, SSEA4, Tra-1-60, Tra-1-81 whereas no expression of CD45-a pan hematopoietic marker and CD34- a HSC marker on iP2/14 cells. Filled grey peaks represent isotype controls and the colored unfilled peaks represent the marker. % expression is mentioned in the overlays with Mean Florescence Intensity (MFI) values in brackets (B). Tri-lineage differentiation ability of the cells was confirmed by staining with βIII tubulin (ectoderm), Brachyury (mesoderm) and FoxA2 (endoderm) by IF, scale bars = 10 μm (C).

for Stem cell research (NCCS-IC-SCR) and AFMC-IC-SCR. The use of plasmids and protocol for transfection were approved by NCCS-IEC and NCCS-Institutional Biosafety Committee (NCCS-IBSC). These committees are constituted in accordance with Declaration of Helsinki.

2.2. Reprogramming of $CD34^+$ cells

The CB was collected from AFMC, Pune and mononuclear cell fraction was separated by Ficoll-Hypaque (density 1.077 g/ml from Himedia, India) density gradient centrifugation. The $CD34^+$ fraction was isolated by magnetic bead method as per manufacturer's instructions (Dynabeads CD34 positive isolation kit, Invitrogen, Grand Island, NY, USA). Isolated $CD34^+$ cells were expanded for 3 days in StemPro-34 SFM (Invitrogen, Grand Island, NY, USA) serum free media with SCF, IL-6, TPO and Flt3L (25 ng/ml each, Peprotech, Princeton, NJ, USA). 10^6 expanded cells were nucleofected in Opti-MEM media (Gibco, Grand Island, USA) using 1 μg of Epi5 Episomal iPSC Reprogramming Kit plasmids (Invitrogen, Grand Island, NY, USA) by U-008 program in Amaxa Nucleofector (Lonza Walkersville, MD, USA). The cells were resuspended in StemPro-34 SFM containing same growth factors and set on geltrex (Invitrogen, Grand Island, NY, USA) coated plates for 24 h. After this, cells were maintained in DMEM/F-12 media containing 1% N2 supplement, 2% B27 supplement, 1% non essential amino acid (NEAA) (All from Invitrogen, Grand Island, NY, USA) and 100 ng/ml bFGF (Peprotech, Princeton, NJ, USA) for 8 days by demi-depopulation. On day 9, the spent media was replaced by Essential 8 (Invitrogen, Grand Island, NY, USA) with media change daily until colonies emerged. From here onwards, the cells were maintained in Essential 8 media. The colonies were scored for calculating transfection efficiency and manually picked after day 25 onto freshly coated plates and were passaged every 4–5 days.

2.3. Alkaline phosphatase staining

The 4–5 days old colonies were washed with 1× PBS, fixed with 4% Paraformaldehyde (PFA) for 3 min and washed with 1× Tris buffer saline (TBS). The colonies were stained with Sigma Fast; alkaline phosphatase substrate tablets set (Sigma Aldrich, St Louis, MO, USA) for 20 min at 37 °C. The colonies were washed with 1× TBS buffer to remove excess stain. The pluripotent colonies stained pink in color were observed.

2.4. G-banded karyotype

This was outsourced to Cytogenetics Department, Deenanath Mangeshkar Hospital, Pune. Cells in growing phase were assessed for chromosomal stability by karyotyping and >30 metaphases were counted.

2.5. Absence of episomal plasmids in the iP2/14 iPSC cell line

To check if the plasmid is excluded from the cells, primers against the EBNA region of plasmid backbone were used. PCR amplification was performed on cDNA of the iP2/14 cells at different passages (passage 3 and 8). The amplified products were run on 2% agarose gel and visualized under UV transilluminator. (Syngene Gel Doc, Frederick, MD, USA)

Table 1
List of sequences of primer pairs used for the above experiments.

Primer	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
Oct4	CGAGAGGATTTTGAGGCTG	CAGAGTGGTGACGGAGACAG
ALP	TCGCCTACCAGCTCATG	GCTCTTCCAGGTGTAAACGA
Nanog	AATGGTGTGACGCAGAAG	GGACTGGATGTTCTGGGT
Lin28	AAGGAGACAGGTGCTACAAC	AGGGTAGGGCTGTGGAT
Rex1	GAAGGTCATCCACAGCAC	TGGTCTTGGCGTCTTCT
v-myc	GAAAACCAGCAGCCTCC	AGGAGGGCGAGCAGAG
EBNA Backbone	ATCGTCAAAGCTGCACACA	CCAGGAGTCCCAGTAGTCA
Gapdh	CGGATTTGGTCGTATTG	GGAAGATGGTGATGGGA

2.6. Pluripotency marker expression by PCR

Total RNA was isolated from cells by using Trizol Reagent. cDNA was synthesized from 1 μg RNA using MMLV Reverse Transcriptase and 100 ng of random primers. Expression of genes as listed in Table 1 were checked by amplification with Taq Polymerase enzyme, 5 nM of desired primer pairs and 0.5 mM dNTPs (All from Invitrogen, Grand Island, NY, USA). Products were run on 2% agarose gel and visualized under UV (Syngene Gel Doc).

2.7. Pluripotency and differentiation marker expression by immunofluorescence

Cells were grown for 4–5 days on vitronectin (Stem Cell Technologies, Vancouver, Canada) coated coverslips. Cells were washed with 1× PBS and fixed for 5 min with 4% PFA. Wherever necessary, cells were permeabilized with 0.1% TritonX-100 solution for 8 min and blocked by 1% BSA in PBS for 1 h at room temperature (RT). Cells were incubated overnight at 4 °C in primary antibodies against Oct4, Sox2, SSEA4, Tra-1-60 (Abcam, Cambridge, UK). Staining of cells with secondary antibody (*anti*-mouse Alexa Fluor 488, 1:500, and anti-rabbit Alexa Fluor 594, 1:300; Invitrogen, Grand Island, USA) was done for 1 h at RT. Nucleus was counterstained with DAPI (10 ng, Sigma, St Louis, MO, USA) for 15mins. Images were acquired on Ziess LSM Meta 510 confocal microscope (Gottingen, Germany).

2.8. Pluripotency marker expression by flow cytometry

Cells were harvested, washed with 1× PBS and permeabilized using BD Fixation and Permeabilization (BD Pharmingen, CA, USA) kit as per manufacturer's instructions. Oct4 Alexa Fluor 488, Sox2 PE, CD34 APC, CD45 APC (BD Pharmingen, CA, USA) staining was carried out for 1 h at 4 °C. Staining with purified antibodies such as SSEA4, Tra-1-60, Tra-1-81 (Abcam, Cambridge, UK) was done for 1 h at 4 °C followed by incubation of cells with secondary antibody, *anti*-mouse Alexa Fluor 488 (Invitrogen, Grand Island, USA). The cells were washed in 1× PBS and acquired on FACS Canto II (BD, CA, USA). Respective isotype controls were used for the experiment. Analysis was carried out using Flow Jo software (LCC, Ashland OR, USA).

2.9. Differentiation to three lineages in vitro

Cells were harvested by enzymatic digestion using TrypLe solution (Invitrogen, Grand Island, USA). They were suspended in Essential 6 media (Invitrogen, Grand Island, USA) and set for embryoid body (EB) formation in low attachment plates (Sigma, St Louis, MO, USA) for 8 days with media change every alternate day. On day 9, the EBs were transferred on coverslips in DMEM/F-12 media (Lonza Walkersville, MD, USA) containing 15% FBS (Gibco, Grand Island, USA). After 7–10 days, cells were stained with markers for mesoderm (Brachyury from R&D Systems, Minneapolis, USA) and ectoderm (βIII tubulin from BD Pharmingen, CA, USA). For endoderm differentiation, the EBs were allowed to differentiate for 4 days in 2% KOSR (Invitrogen, Grand Island, USA) containing DMEM/F-12 media with 100 ng/ml Activin A (Peprotech, Princeton, NJ, USA) (D'Amour et al., 2005). Differentiation was assessed by staining cells for FoxA2 (BD Pharmingen, CA, USA). Cells were stained with secondary antibody Alexa Fluor 594 or Alexa Fluor 488 (Invitrogen, Grand Island, USA) and nucleus was counter stained with DAPI (Sigma, St Louis, MO, USA). Images were acquired on Ziess LSM Meta 510 confocal microscope (Gottingen, Germany).

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