Chapter 13

Generation of Transgene-Free iPSC Lines from Human Normal and Neoplastic Blood Cells Using Episomal Vectors

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Abstract

Human induced pluripotent stem cells (iPSCs) have become an important tool for modeling human diseases and are considered a potential source of therapeutic cells. Original methods for iPSC generation use fibroblasts as a cell source for reprogramming and retroviral vectors as a delivery method of the reprogramming factors. However, fibroblasts require extended time for expansion and viral delivery of transgenes results in the integration of vector sequences into the genome which is a source of potential insertion mutagenesis, residual expressions, and reactivation of transgenes during differentiation. Here, we provide a detailed protocol for the efficient generation of transgene-free iPSC lines from human bone marrow and cord blood cells with a single transfection of non-integrating episomal plasmids. This method uses mononuclear bone marrow and cord blood cells, and makes it possible to generate transgene-free iPSCs 1–3 weeks faster than previous methods of reprogramming with fibroblasts. Additionally, we show that this approach can be used for efficient reprogramming of chronic myeloid leukemia cells.

Key words Epstein–Barr virus, Episomal plasmids, Reprogramming, Induced pluripotent stem cells, Human bone marrow, Cord blood, Chronic myeloid leukemia

1 Introduction

The iPSC technology offers a novel opportunity for basic research, disease modeling, drug screening, and cell therapies. Conventional reprogramming methods rely on gamma retroviral or lentiviral vectors for the delivery of the reprogramming factors (1–3). Virus-based techniques cause insertion mutagenesis, residual expression, and reactivation of transgenes and are of low efficiency. Many approaches have been investigated to avoid or eliminate integration of transgenes in the reprogrammed cells including non-integrating adenoviral vectors (4), Sendai RNA viral vectors (5, 6), repeated transient transfection (7), protein transduction (8, 9), RNA transfection (10), Cre-LoxP excision system (11), PiggyBac transposon system (12, 13), and small molecules (14–20). However, these alternative approaches still have their limitations such as low efficiency, multiple rounds of transfection, additional expertise for

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RNA and protein preparations, instability of RNA and protein samples, extra steps for excision of pre-integrated sequences and subsequent screening, and incomplete removal of the exogenous sequences.

EBV-based plasmids exist in mammalian cells as an extrachromosomal entity. EBV-plasmids require only two viral elements for maintenance in the cells: (1) a short cis sequence which is the latent origin of plasmid replication (*oriP*), and (2) a transelement of EBNA1 (Epstein-Barr Nuclear Antigen 1) (21). Because up to 5% of the cells lose EBV plasmids during each cell division (22, 23), transgene-free cells can be obtained simply by passaging and subcloning (24, 25). For these reasons, many laboratories, including our own, favor the EBV-based episomal vector system for the generation of iPSCs free of foreign sequences (24–31). For the establishment of patient-specific iPSC line, starting cells are critical due to the varied accessibility and reprogrammability of cells. Although fibroblasts have been traditionally used as a source of cells for reprogramming, the efficiency of fibroblast reprogramming, especially adult fibroblasts, is relatively low. Moreover, fibroblasts require 4–6 weeks for isolation and expansion (32, 33). In contrast, mature blood cells and their progenitors are the most accessible sources of cells in our body.

We demonstrate that transgene-free human iPSCs can be obtained from human bone marrow, human cord blood, or purified human $CD34^+$ cells using the non-integrating episomal system (25). This protocol can reprogram blood cells previously frozen in liquid nitrogen for over 6 years. While many non-integrating protocols require multiple rounds of transfections, our protocol uses only one transfection and does not require purification of particular blood subset although the purification of $CD34^+$ progenitors can help reprogram cells with higher efficiency. Unlike other protocols for blood reprogramming that predominantly reprogram T cell populations (5, 34–36), the population reprogrammed with our method is neither T nor B cells and thus the reprogrammed genome is free of recombined genomic DNA that results from gene rearrangements following maturation of T or B cells. Additionally, we demonstrate that the episomal vector-based approach can be used to generate iPSCs from neoplastic bone marrow cells from patients with chronic myeloid leukemia (CML) to model leukemia development in vitro (25). The iPSC-based model provides numerous advantages for the study of neoplastic blood diseases. It can be used to examine leukemia stem cell potentials at various stages of differentiation for which it may be difficult to obtain samples from patients, for example, at the hemangioblast stage. It also provides a unique opportunity to explore the role of epigenetic changes in the activation of the oncogene-induced aberrant regulatory circuits and to identify cell subsets with distinct tumor-initiating potential and drug sensitivity.

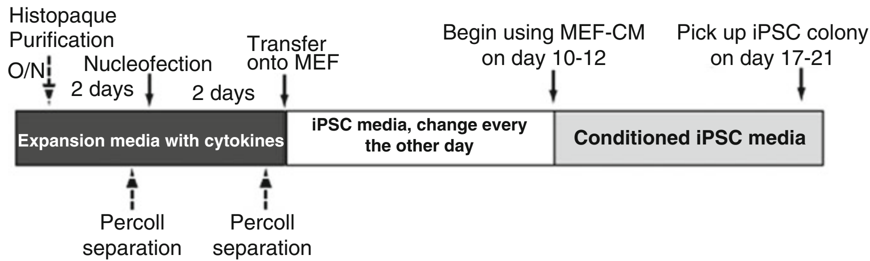

Fig. 1 Summary of protocols for reprogramming blood cells using episomal plasmids. *Arrows* indicate time points at which the indicated steps are carried out. *Broken arrows* designate the optional steps. O/N means overnight recovery by culturing of frozen whole bone marrow or whole cord blood overnight in hematopoietic expansion medium and fractioning for mononuclear cells with Histopaque gradient centrifugation. CM is conditioned media

In this chapter, we describe a detailed protocol for the reprogramming of archived normal and neoplastic blood cells with episomal constructs. Figure 1 outlines the major steps of the reprogramming protocol. The procedure starts with a short expansion of the mononuclear cells in the serum-free hematopoietic expansion media followed by nucleofection, an additional expansion for 2 days in the hematopoietic media and a transfer of cells onto mouse embryonic fibroblast (MEF) feeders. Reprogramming proceeds on MEF feeders in the standard iPSC medium for 10 days. Additional culture in MEF-conditioned media for another 10 days is required to grow typical iPSC colonies which can be handpicked for expansion and the final establishment of iPSC lines. Although we initially developed this method for reprogramming mononuclear cells from bone marrow and cord blood (25), we found that the same protocol works well for reprogramming of $CD34^+$ cells isolated from these sources.

2 Materials

All reagents should be cell-culture grade. Aseptic practice should be observed for all steps.

2.1 Reagents (See Note 1)

1. Recombinant human IL-3 (PeproTech).
2. Recombinant human IL-6 (PeproTech).
3. Recombinant human SCF (PeproTech).
4. Recombinant human Flt3L (PeproTech).
5. Recombinant human FGF-basic (PeproTech).
6. Zebra fish FGF-basic (gift from James Thomson, made in house).
7. StemSpan SFEM (Serum-Free Medium for Expansion of Hematopoietic cells) (Stemcell Technologies).
8. Histopaque-1077® (Sigma-Aldrich).
9. Percoll® (Sigma-Aldrich).

10. EX-CYTE® growth enhancement media supplement (Celliance).
11. Human cord blood mononuclear cells (AllCells, CA, USA).
12. Human bone marrow mononuclear cells (AllCells, CA, USA).
13. Human bone marrow cells from patient with CML (AllCells, CA, USA).
14. Human cord blood $CD34^+$ cells (AllCells, CA, USA).
15. Human bone marrow $CD34^+$ cells (AllCells, CA, USA).
16. DNase I (Promega).
17. L-Glutamine (Gibco).
18. γ-Irradiated MEF (WiCell).
19. Knockout serum replacement for ESC/iPSC (Gibco).
20. β-mercaptoethanol (Sigma).
21. D-MEM/F-12 (Gibco).
22. MEM nonessential amino acids solution (100×, 10 mM, HyClone®).
23. Penicillin-Streptomycin solution (100×, Cellgro®).
24. Hyclone® Fetal Bovine Serum (defined) (Thermo Scientific).
25. Collagenase type IV (Gibco).
26. Dimethyl sulfoxide (DMSO) (Sigma).
27. Gelatin (Sigma).
28. PBS (without calcium, without magnesium) (Hyclone®).
29. Sodium bicarbonate (Fisher Scientific).
30. Thiazovivin (Stemgent®).

2.2 Transfection Kit (See Note 2)

1. Amaxa® Human $CD34^+$ Cell Nucleofector® Kit (Lonza).

2.3 Plasmids

1. pEP4-EO2S-ET2K (Addgene).
2. pEP4-EO2S-EN2K (Addgene).
3. pCEP4-M2L (Addgene).

2.4 Key Equipment (See Note 2)

1. Nucleofector® II (Amaxa Biosystem).

2.5 iPSC Medium (See Note 3)

The iPSC medium is, in essence, ESC growth medium with a higher concentration of FGF2. It consists of 20% Knockout serum replacement (KOSR), 80% D-MEM/F-12, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1× nonessential amino acid (NEAA) (0.1 mM). We use 100 ng/ml of recombinant zebra fish FGF-basic, or 10 ng/ml of recombinant human FGF2. It can be stored for up to 2 weeks at 4°C.

2.6 Hematopoietic Expansion Medium

The hematopoietic expansion medium consists of StemSpan SFEM (serum-free expansion media) supplemented with 0.2% Ex-Cyte (Celliance), recombinant human IL-3 (10 ng/ml), recombinant human IL-6 (100 ng/ml), recombinant human SCF (100 ng/ml), and FMS-related tyrosine kinase-3 ligand (Flt-3 L, 100 ng/ml), 100 IU of penicillin, and 100 μg/ml of streptomycin. Upon arrival, StemSpan SFEM is aliquoted into 50-ml tubes, and stored at −20°C or −80°C. Cytokine, growth factors, lipid supplement, and antibiotics are added immediately before use.

2.7 MEF-Conditioned Medium (MEF-CM) (See Note 4)

For the preparation of MEF-conditioned medium, we use the modified method of Xu et al. (37). Add 15 ml of iPSC medium composed of 10% KOSR, 90% D-MEM/F-12, 0.5 mM L-glutamine, 0.5× NEAA, without FGF2 to a 10-cm tissue dish preseeded with MEF at a density of $2 \times 10^4/\text{cm}^2$. After 24 h of conditioning, medium is collected. The same dish of MEF can be used for up to 10 times. The collected conditioned media can be stored at −20°C for over 1 month. To make 500 ml of a working MEF-conditioned medium for reprogramming, combine the following: 450 ml of conditioned medium, 50 ml of fresh KOSR, 2.5 ml of 100 mM L-glutamine, 2.5 ml of 100× NEAA, 2 μl of β-mercaptoethanol, and appropriate amount of FGF to make a final concentration of 100 ng/ml for zebra fish FGF, or 10 ng/ml for recombinant human FGF2. The MEF-CM is sterilized by filtering through a 0.22-μm filter.

2.8 2× iPSC Freezing Medium

2× iPSC freezing medium is composed of 60% Hyclone® FBS, 20% DMSO, and 20% basal iPSC media. Prepare freshly when needed and keep chilled on ice.

2.9 0.1% Gelatin

Weigh 1 g of gelatin and put it into a 1-L Pyrex bottle; add 1 L of pure water. Autoclave to sterilize the solution. The gelatin can be stored at 4°C for over a year.

2.10 Preparation of Collagenase IV Solution

The working concentration of collagenase IV is 1 mg/ml in plain D-MEM/F-12 medium. Weigh 45 mg of collagenase IV; put it into a 50-ml tube; add 45 ml of D-MEM/F-12 medium into the 50-ml tube containing the collagenase IV; close the tube with a cap; mix well by shaking; remove the cap; sterilize the collagenase IV solution by filtration with a Steriflip® 50-ml filter (0.22 μm, Millipore). The solution can be stored for up to 14 days at 4°C.

2.11 MEF Preparation for Culture of Human Reprogrammed Cells (See Note 5)

Coat the 6-well plate or 10-cm dish with 0.1% gelatin (2 ml per well, and 10 ml per dish) overnight at 37°C. Next day, remove the plate/dish from the incubator and aspirate the gelatin solution from the plate/dish. Seed 2.5 ml of irradiated MEF at 0.75×10^5/ml into each well of a 6-well plate (around $2 \times 10^4/\text{cm}^2$), or 15 ml of the irradiated MEF (0.75×10^5 cells/ml) into one 10-cm tissue culture dish. Culture the MEF overnight at 37°C, 5% CO_2 before use.

2.12 Preparation of Percoll® Solution

5× Percoll solution: Mix 45 ml of Percoll® (Sigma, p1644) (sterile) with 5 ml of 10× PBS (sterilized by filtration), which results in 90% Percoll® in 1×PBS. Store the 5× Percoll stock solution at 4°C.

1× Percoll solution: Mix 10 ml of 5× Percoll® solution with 40 ml of sterile 1×PBS solution. The final Percoll® solution is 18% in 1× PBS. Smaller volume can be prepared similarly. The 1× Percoll solution can be prepared before use.

3 Methods

3.1 Preparation of Cells for Nucleofection (See Note 6)

1. Take one vial of blood mononuclear cells from liquid nitrogen tank, and thaw the cells quickly in a 37°C water bath; transfer the cells into the 15-ml tube containing 10 ml of plain SFEM medium (see Note 7).

Centrifuge the cells at 400×*g* for 8–10 min.

2. Aspirate the supernatant, add 10 ml of fresh plain SFEM to the cell pellet, and repeat the washing.
3. Resuspend cells in 1 ml of hematopoietic expansion medium and count the cells with trypan blue. Add appropriate volume of hematopoietic expansion medium to the cell suspension to make the final concentration 1–2 millions cells per ml (see Note 8).
4. Put the cells in one well of a 6-well plate if the cell suspension volume is 4 ml or less; culture the cells in a T-25 tissue flask if cell suspension volume is 5–10 ml.
5. Culture at 37°C and 5% CO_2 for 2 days.

3.2 Nucleofection of Blood Cells and Reprogramming

1. On day 2 (48 h of culture in hematopoietic expansion media), transfer the cell suspension into a 15 ml tube (see Note 9).
2. Underlay cell suspension with 1.0–1.5 ml of Percoll solution.
3. Centrifuge at 300×*g* for 20 min at room temperature.
4. During the centrifugation, take out the nucleofection kit from 4°C, and DNA from −80°C. Put 82 μl of nucleofection buffer into a sterile 1.5-ml tube and add 18 μl of the supplement (both are supplied as a components of Amaxa® Human CD34⁺ Cell Nucleofector® Kit) into the buffer; mix well. During this period of time, the buffer can be brought to room temperature (see Notes 10 and 11).
5. After centrifugation, aspirate the supernatant and interface containing dead cells and debris without disturbing cell pellet; resuspend the cell pellet with 10 ml of SFEM, and centrifuge at 400×*g* for 8 min (see Note 12).
6. During the washing, turn on the Nucleofector II. Set up the program to U-008 (U-08 for Nucleofector® I). Prepare one Amaxa cuvette and one Amaxa transfer pipette in the biosafety hood.

7. Add the following DNA plasmids into the buffer mixture prepared in step 4: 9 μg of pEP4-EO2S-ET2K, 9 μg of pEP4-EO2S-EN2K, and 6 μg of pCEP4-M2L. Mix well by gentle pipetting, being careful to avoid introducing bubbles. (See Note 13).
8. Carefully aspirate the entire media from cells in step 5 without disturbing cells (see Note 14).
9. Add buffer containing DNA (step 7) to the 15-ml tube containing the cells to be transfected (step 8); mix well by gentle pipetting, being careful to avoid introducing bubbles. Carefully transfer the cells and DNA in buffer into the transfection cuvette supplied with Amaxa® Nucleofector® Kit. Do not introduce any bubbles. Gently but quickly tap the cuvette five to ten times immediately to remove any bubbles.
10. Nucleofect the blood cells using program U-008 (see Note 15)
11. Add 500 μl of hematopoietic expansion medium into the cuvette; mix by one gentle pipetting motion, and transfer cells into dish or wells containing hematopoietic expansion medium (see Note 16).
12. Culture for 2 days in hematopoietic expansion medium at 37°C, 5% CO_2.
13. On day 2 after transfection, remove dead cells using Percoll® separation as described in steps 1–3 and 5. Transfer the cells onto a 10-cm dish preseeded with MEFs.
14. Change medium every other day for the first 10 days (see Note 17).
15. On day 10, start to use MEF-conditioned medium and change media every day.
16. iPSC colonies should appear between days 17 and 21. Under a microscope in the biosafety hood, scrape off and pick up the entire single iPSC colony (see Fig. 2) with a P200 tip. Transfer colony into a sterile 1.5-ml tube and separate cells by pipetting five times. Put one individual iPSC colony into one well of a 6-well plate. The first culture from this picked colony is considered a passage 1 iPSCs (P_1) (see Note 19).
17. Grow the P1 cells in iPSC medium at 37°C, 5% CO_2. Change the medium every day.
18. When new colonies reach the normal size, repeat step 16 if any incompletely reprogrammed colonies exist, and grow the P_2 iPSCs at 37°C, 5% CO_2 (see Note 20).
19. Between days 5 and 10 after culture, depending on the density and colony size, passage the new iPSC line using standard protocol for passaging human ESC/iPSCs on MEFs (now P_3).
20. Between days 5 and 7, freeze the newly established lines (see freezing protocol below). Passage the remaining wells

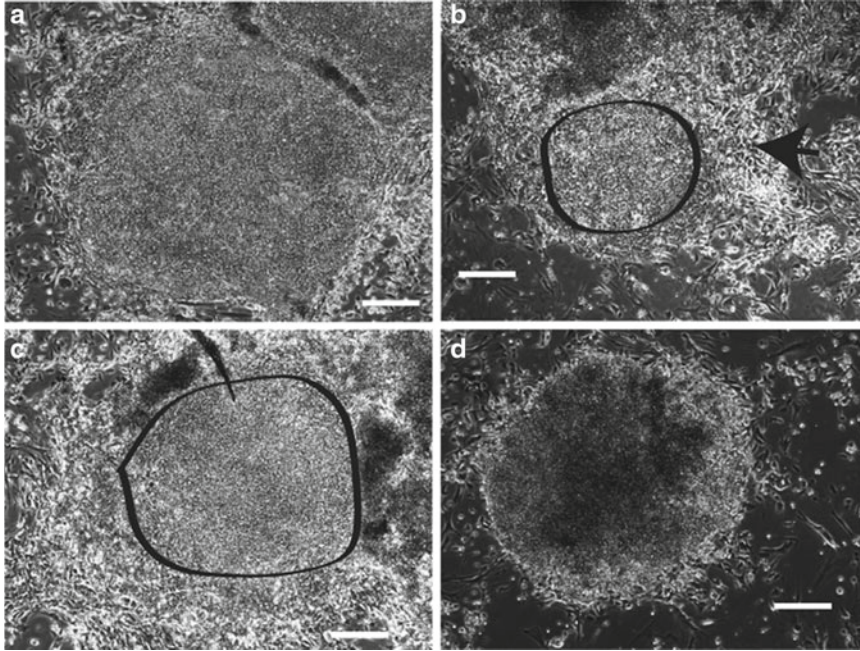

Fig. 2 Morphology of reprogrammed cell colonies. Panel (**a**) shows a high-quality colony. The colony is bright and composed of small, tightly packed cells. Only a few incompletely reprogrammed spindle cells are present at the periphery of the colony. Panels (**b**) and (**c**) are other examples of well-reprogrammed colonies. Completely reprogrammed cells within colonies are circled. However, more incompletely reprogrammed spindle-like cells can be seen at the periphery of colony (*arrow*). Panel (**d**) shows a colony at a less advanced stage of reprogramming. However, this colony can be picked up and used to establish iPSC lines after transferring to new MEFs to facilitate complete transition to pluripotency. Bars, 300 μm. Images of transfected human bone marrow mononuclear cells were taken on day 17 of culture on MEFs (day 19 after transfection)

for characterization and further freezing at higher passages. Established iPSC lines should be analyzed for expression of pluripotency markers by flow cytometry, immunofluorescence, RT-PCR, or gene profiling. Pluripotent potential of these cells should be evaluated using functional tests including teratoma generation, and in vitro differentiation into different lineages. The established lines should also be karyotyped to ensure genomic integrity. Characterization of iPSCs obtained from patient with chronic myeloid leukemia is shown in Fig. 3.

3.3 Freezing iPSCs

1. Warm up collagenase IV in a 37°C water bath for 10 min.
2. Take out the iPSC plate from the incubator.
3. Use 1.5 ml of collagenase IV solution for one well of iPSCs of a 6-well plate.
4. Incubate at 37°C for 5 min.
5. Aspirate the collagenase IV solution.

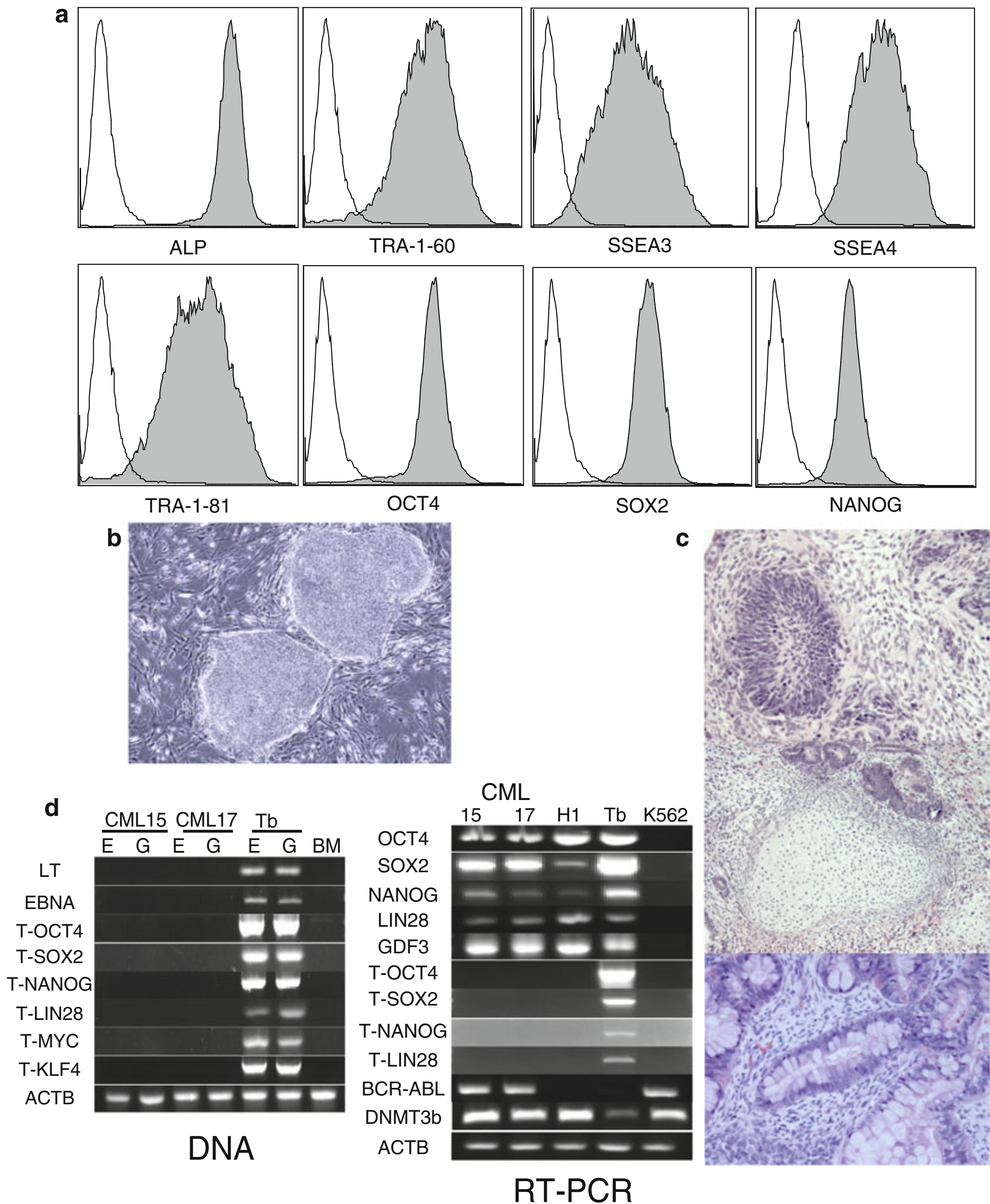

Fig. 3 Demonstration of pluripotency of iPSC lines generated from bone marrow of a patient with chronic myeloid leukemia (CML) in chronic phase. (**a**) Flow cytometric analysis of hESC-specific marker expression in CMLiPSC15 line (surface staining is shown for ALP, TRA-1-60, SSEA3, SSEA4, and TRA-1-81; intracellular staining is shown for OCT4, SOX2, and NANOG). (**b**) Typical morphology of CMLiPSC15 colony growing on MEFs. (**c**) H&E staining of teratoma from CMLiPSC15 line. Neural rosettes (*upper panel*), cartilage (*middle panel*), and intestine-like structure (*lower panel*) are shown. (**d**) PCR analysis of genomic (G) and episomal (E) DNA demonstrates that CMLiPSCs are transgene-free. Vector (EBNA)- and transgene (T)-specific primers were used as indicated. RT-PCR analysis of expression of transgenes (T) and endogenous pluripotency genes and BCR-ABL in CMLiPSC lines 15 and 17. H1 is human embryonic stem cell line H1. BM is bone marrow mononuclear cells. K562 is myeloid leukemia line established from a patient with chronic myeloid leukemia in blast crisis. Tb is positive controls, BM cells transfected with the same reprogramming plasmids

6. Add 3 ml of iPSC medium and gently wash off the iPSC colonies from the MEF feeder with a 5-ml pipette.
7. Put the iPSCs into a 15-ml tube and centrifuge at $200 \times g$ for 5 min.
8. Aspirate the washing medium.
9. Add 0.5 ml of iPSC media (ice cold), and add 0.5 ml of iPSC freezing media (ice cold) to cell pellet.
10. Resuspend cells and transfer cells into a cryotube (1.8 ml). Avoid excessive pipetting during cell transfer.
11. Put the cryotube in the freezing container, and start the freezing process immediately in −80°C freezer; the following day, transfer the iPSCs into a liquid N_2 storage tank for the long-term storage.

4 Notes

1. All stock solutions of cytokines are prepared at 1,000×, aliquoted and kept at −80°C.
2. Blood progenitors are difficult to transfect using the traditional nonviral transfection methods due to their quiescent nature and suspension growth (38). Nucleofection is critical for successful reprogramming of blood progenitor cells. Nucleofection directly delivers the DNA into the nuclei of cells and results in early and strong expression of transgenes.
3. Recombinant zebra fish FGF-basic can be used instead of human FGF2. However it is less stable at 37°C and must be used at concentration of 100 ng/ml. For commercial recombinant human FGF2, we recommend a concentration of 8–12 ng/ml.
4. Standard protocol for preparation of MEF-conditioned media requires high density of MEF to be plated in T-75 flask ($5.4 \times 10^4/\text{cm}^2$). We found that the routine $2 \times 10^4/\text{cm}^2$ density of MEF used for ESCs culturing works well for preparation of MEF-conditioned media and simplifies the procedure.
5. The complete MEF preparation protocol can be found in WiCell protocols: SOP-CC-003B; SOP-CC-006D; SOP-CC-031D; SOP-CC-021A; SOP-CC-009A; SOP-CC-013A (https://www.wicell.org).
6. We grow bone marrow and cord blood mononuclear cells for 2 days before transfection to amplify hematopoietic progenitors. We also recommend a 2-day expansion before transfection for $CD34^+$ cells isolated using magnetic beads. The electroporator creates an electromagnetic field in the cell solution which could affect viability of the purified $CD34^+$ cells

when magnetic beads attached to them. However, after 2 days of culture, the beads detach and degrade so that the cells can be safely electroporated.

7. If unprocessed bone marrow or cord blood is used, a standard Histopaque gradient centrifugation step should be carried out to obtain the mononuclear cell fraction. The current protocol is based on mononuclear cells.
8. For bone marrow mononuclear cells and $CD34^+$ cells, $1–2 \times 10^6$ is typically sufficient for reprogramming. However, for cord blood mononuclear cells, $5–10 \times 10^6$ cells are required for reprogramming.
9. Mature blood cells die in hematopoietic expansion medium and result in cell clumps. If cell clumping occurs, treat the cells with DNase I at a concentration of 200 units/ml for 30 min before Percoll® purification. DNase I treatment removes the DNA released by the dead cells and alleviate the clumping.
10. The shelf life of buffer after mixture with supplement is 3 months. We usually mix these two parts of the reaction just before transfection to ensure high transfection efficiency.
11. The optimal volume of the reaction buffer for the Nucleofector device is 100 μl. Significant changes to the reaction volume will result in transfection failure.
12. If purified $CD34^+$ cells are used for reprogramming, Percoll® separation is not required due to the low number of dead cells.
13. Concentration of plasmid DNA in stock solution should be >1.5 μg/μl to avoid significant dilution of nucleofection buffer following DNA addition. Excessive dilution of nucleofection buffer decreases transfection efficiency.
14. Washing medium must be completely removed before adding nucleofection buffer. This should be done very carefully to avoid aspiration and loss of cells. Tilt the tube so that the bottom is elevated to let liquid to flow down toward the mouth of the tube. The liquid can be collected by keeping the tip of the running vacuum pipette immediately downstream of the flow. This method allows for effective liquid removal and avoids disruption of the cell pellet.
15. Do not keep cells in the transfection buffer longer than 20 min. Extended contact time with buffer reduces the viability of cells and the gene transfer efficiency. After adding buffer, proceed with electroporation as quick as possible.
16. After nucleofection procedure, cells become friable. It is critical to use a pipette with larger bore size supplied with the nucleofection kit for gentle handling of cells.
17. Blood cells grow in suspension and transfected cells gradually become adherent. However, during the first few days following

reprogramming, many cells still grow in suspension. To avoid loss of cells following medium change, aspirated medium can be centrifuged to collect floating cells and reseeded back onto the same MEF dish.

18. MEF cultures should be prepared fresh for plating transfected cells. Because reprogramming requires extended cultures, the use of 3-day or older MEF plates will compromise the experiment due to the deterioration of the MEFs.
19. Sometimes no typical colonies appear following the first round of transfected cells on MEFs (P_0). Passaging P_0 cells onto new MEFs may produce high-quality iPSCs colonies. The culture ratio may vary from 1:3 to 1:6 depending on the density of the colonies in the P_0 dishes/plates. The reprogramming efficiency can be significantly increased by adding thiazovivin (1 μM) to reprogramming cultures. Thiazovivin can be added as early as the transfected cells are transferred onto MEF feeders or after the first passage.
20. The second picking will eliminate the contaminated incompletely reprogrammed cells from the first culture. Multiple colonies can be cultured in the second round of culture.

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