Generation of induced pluripotent stem cells from human cord blood cells with only two factors: Oct4 and Sox2

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Induced pluripotent stem cells (iPSC) provide an invaluable resource for regenerative medicine as they allow the generation of patient-specific progenitors with potential value for cell therapy. However, in many instances, an off-the-shelf approach is desirable, such as for cell therapy of acute conditions or when the patient's somatic cells are altered as a consequence of a chronic disease or aging. Cord blood (CB) stem cells appear ideally suited for this purpose as they are young cells expected to carry minimal somatic mutations and possess the immunological immaturity of newborn cells; additionally, several hundred thousand immunotyped CB units are readily available through a worldwide network of CB banks. Here we present a detailed protocol for the derivation of CB stem cells and how they can be reprogrammed to pluripotency by retroviral transduction with only two factors (OCT4 and SOX2) in 2 weeks and without the need for additional chemical compounds.

INTRODUCTION

Human induced pluripotent stem cells (iPSC) derived from adult somatic cells could be used in regenerative medicine to repair tissues damaged by disease or injury. Although human iPSC have been generated from various type of somatic cells, such as skin fibroblasts¹, keratinocytes², neural stem cells (NSCs)³, blood cells^{4,5} and cord blood-derived endothelial cells (CBEC)⁶, scientists are still debating the best sources from which to derive iPSC. Further studies have shown that the age, origin and cell type used has a profound impact on the reprogramming efficiency as well as the quality of iPSC generation^{2,7}. A desirable protocol should aim to not only optimize all these parameters, but also to reduce both the number of factors used and the time required for reprogramming. A key observation in this regard is the recent study by Kim *et al.*³ where iPSC were generated from adult NSCs by direct reprogramming with either two transcription factors (OCT4 and KLF4) or one transcription factor (OCT4)⁸. However, NSCs are usually rare and difficult to access, representing a complicated target for reprogramming. Recently, the possibility of reprogramming blood cells to pluripotency has suggested an alternative source of somatic cells to generate iPSC. In particular, iPSC have been generated from mobilized peripheral blood (mPB)⁴ or bone marrow (BM)⁵ CD34⁺ derived cells by ectopic expression of OCT4, SOX2, KLF4 and c-MYC. Although these cells are certainly an accessible and widely used source, they may have accumulated genomic alterations as a result of aging or disease, and the isolation of hematopoietic stem cells could represent a health risk for the donor⁹.

Cord blood (CB) stem cells, currently widely used as a source of hematopoietic stem cells for transplantation, could overcome these problems. The main practical advantages of using CB stem cells are the relative ease of procurement, the absence of risks for the donors and the ability to store fully tested and HLA-typed samples in public banks¹⁰, available for immediate use. Another relevant consideration is that CB cells are young and are expected to carry minimal somatic mutations¹¹.

We have demonstrated that CB CD133⁺ cells can be reprogrammed to pluripotency faster than fibroblasts and keratinocytes by ectopic expression of only two transcription factors (OCT4 and SOX2)¹². CB CD133⁺ cells express a subset of pluripotency-associated genes (*OCT4*, *SOX2*, *NANOG* and *CRIP1*), albeit at much lower levels than human ES cells (hESCs) (data not shown). On the other hand, the endogenous levels of c-MYC and KLF4 are higher in CB CD133⁺ cells compared with fibroblasts and keratinocytes (Fig. 1). The combination of low levels of pluripotency markers with the high levels of KLF4 and c-MYC may allow for enhanced reprogramming of CB CD133⁺ cells. Cord blood iPS (CBiPS) cells are generated with only two factors offer new possibilities for investigating the molecular mechanisms that underline reprogramming to pluripotency.

Furthermore, and from a more practical point of view, CB cells could represent, in the future, an alternative, safer source of iPSC amenable to worldwide banking and distribution.

Here, we present a detailed protocol for the derivation of CB CD133⁺ cells and how to reprogram them to pluripotency by retroviral transfection of OCT4 and SOX2 in 2 weeks without the need for additional chemical compounds.

Experimental design

Derivation of CB CD133⁺ cells. Our protocol first focuses on the importance of selecting the correct cell subpopulation, within CB, for reprogramming. CD133 antigen is known as a stem cell marker for hematopoietic stem and progenitor cells. In contrast to CD34, the most widely used marker for hematopoietic stem cells (HSC) enrichment, CD133 is not found in late progenitors, providing a selection of a more homogeneous population enriched in HSC. CD133⁺ cells, which represent a small fraction of total nucleated cells in human CB (0.1–0.5%), are positively selected using the standard immunomagnetic separation system.

Retrovirus production. We produce retroviral vectors using the Phoenix Amphotropic packaging cell line. Retroviral vectors based on the Moloney murine leukemia virus (MuLV) have been extensively used as the primary tool for gene delivery into hematopoietic cells; however, they have also shown low transduction efficiencies. We could improve the gene transfer of MuLV-based vectors into CD133+ cells using a retronectin-facilitated protocol.

Transduction of CD133+ cells. The standard protocol used for fibroblast reprogramming needs to be modified for the transduction of CD133+ cells, which are in a quiescent state and grow in suspension. First, because the integration and expression of retroviral constructs requires mitotic division of the target cells, it is important to culture the CD133+ cells *in vitro* in the presence of SCF, Flt3-ligand, TPO and IL-6 cytokines for 24 h. Second, to increase the transduction efficiency, CD133+ cells are seeded over retronectin-coated plates pre-absorbed with the viral particles. CD133+ cells are infected three times every 12 h; on day 3 post-infection, cells are transferred to 6-well plates containing irradiated human fibroblasts (HFF) and hES medium and cultured until iPS colonies appear.

MATERIALS

REAGENTS

- Umbilical CB unit providing umbilical CB **▲ CRITICAL** Informed consent must be obtained from the parents. Extraction must conform to national and institutional regulations.
- Retroviral vectors expressing the reprogramming transgene (see REAGENT SETUP)
- Phoenix amphotropic 293 cells (ATCC, cat. no. SD 3443)
- Mitotically inactivated human foreskin fibroblasts (ATCC, cat. no. CRL-2429)
- Phosphate-buffered saline (PBS) without calcium and magnesium (Invitrogen, cat. no. 2531)
- EDTA disodium 0.5 M (Sigma-Aldrich, cat. no. E7889)
- Lympholyte-H (Cederlane, CL5016) **! CAUTION** Toxic by skin contact; wear gloves and lab coat when handling.
- CD133 microbead kit (Miltenyi Biotec, cat. no. 130-050-801)
- DMEM (Invitrogen, cat. no. 11965-092)
- OPTIMEM (Invitrogen, cat. no. 31985-062)
- Knockout (KO)-DMEM (Invitrogen, cat. no. 10829-018)
- Heat-inactivated FBS (Invitrogen, cat. no. 10270-106)
- KO serum replacement (KOSR; Invitrogen, cat. no. 10828-028)
- 20% human serum albumin solution (HSA) (Instituto Grifols, SA, cat. no. 670612)
- GlutaMAX (Invitrogen, cat. no. 35050-038)
- Penicillin–streptomycin (Invitrogen, cat. no. 15140-122)
- Nonessential amino acid solution (Invitrogen, cat. no. 11140-050)
- 50 mM 2-mercaptoethanol (Invitrogen, cat. no. 31350-010) **! CAUTION** Toxic by inhalation and skin contact.
- Recombinant human stem cell factor (SCF, PeproTech, cat. no. 300-07) **▲ CRITICAL** Dilute and store all the growth factors in appropriate aliquots according to the manufacturer's recommendations at -20°C (see REAGENT SETUP).
- Recombinant human Flt3-ligand (Flt3, PeproTech, cat. no. 300-19) (see REAGENT SETUP)
- Recombinant human interleukin-6 (IL-6, PeproTech, cat. no. 200-06) (see REAGENT SETUP)
- Recombinant human thrombopoietin (TPO, PeproTech, cat. no. 300-18) (see REAGENT SETUP)
- Recombinant human fibroblast growth factor, basic (bFGF, PeproTech, cat. no. 100-18B) (see REAGENT SETUP)
- Retronectin (Takara, cat. no. T100A)
- 0.05% Trypsin–EDTA (Invitrogen, cat. no. 25300-054)
- Gelatin 0.1% solution (Millipore, cat. no. ES-006-B)

Figure 1 | Quantitative RT-PCR analysis for pluripotent markers and the two transcription factors c-MYC and KLF4. Comparison of endogenous levels of OCT4, SOX2, NANOG, CRIPTO, c-MYC and KLF4 among cord blood (CB) CD133+ cells, fibroblast and keratinocytes. Transcript levels were normalized to GAPDH levels. Error bars indicate the s.d. generated from triplicates.

CBiPS cells. As early as 9 d post-transduction with OCT4 and SOX2 (OS), small colonies start to appear, and after 15 d, some of the colonies exhibit a typical hESC morphology.

- DMSO (Sigma, cat. no. D4540) **! CAUTION** Toxic by inhalation and skin contact.
 - Trypan Blue stain (Invitrogen, cat. no. 15250-061)
 - FuGENE 6 transfection reagent (Roche Applied Science, cat. no. 1181509001)
 - Polybrene (10 mg ml⁻¹) (Chemicon, cat. no. TR-1003-6)
 - hES medium (see REAGENT SETUP)
 - Freezing medium (see REAGENT SETUP)
 - Mouse IgG1 anti-human CD133–PE (Miltenyi Biotec, cat. no. 130-080-801)
 - Mouse IgG1, k anti-human CD45–APC (Becton Dickinson, cat. no. 555485)
- EQUIPMENT**
- Class-II cabinet with aspirator for tissue culture (Bio-II-A, Telstar)
 - Class-II cabinet with aspirator for tissue culture and space for stereomicroscope (Bio-II-A/G, Telstar)
 - Aspirator tube assembly (Sigma, cat. no. A5177)
 - Stereomicroscope (SZX12, Olympus)
 - Low-end color video camera (TKC1481BEG, JVC)
 - Low-end 8-inch LCD display (TV304, BOMAN)
 - Inverted tissue culture microscope with phase contrast and epifluorescence, with $\times 5$, $\times 10$, $\times 20$ and $\times 40$ objectives (Leica DMIL, Leica)
 - Thermostated tissue culture centrifuge and swinging rotor with adapters for 15- and 50-ml tubes and microplates (Allegra X-12R centrifuge with SX4750A rotor, Beckman Coulter)
 - Cell-culture incubator set at 37°C , 5% CO₂ (REVC0, cat. no. RCO3000D-9-VBC)
 - Microcentrifuge (Eppendorf, cat. no. 5424)
 - Tissue culture dish, 100 mm
 - Tissue culture plates, 24 well and 6 well
 - Mini-Macs separator (Miltenyi Biotec, cat. no. 130-090-312)
 - MS column (Miltenyi Biotec, cat. no. 130-041-301)
 - Pre-separation filters (Miltenyi Biotec, cat. no. 130-041-407)
 - Syringe, 2.5 ml (PentaFerte, cat. no. 08L01)
 - Stripper micropipette (Mid Atlantic, cat. no. MXL3-STR)
 - Stripper tips, 150 μm
 - Conical tubes, 15 and 50 ml
 - Cryovials (Sigma, cat. no. V7634-500EA)
 - Cryo 1°C freezing container, 'Mr. Frosty' (Nalgene, cat. no. 5100-0001)
 - Slide flask (Nunc, cat. no. 170920)
 - Bottle-top filter system, 0.22 μm , 500 ml (Millipore, cat. no. SCGPU05RE)
 - Storage bottle, 500 ml (Corning, cat. no. 430282)
 - Filter, Millex-HV PVDF, 0.45 μm (Millipore, cat. no. SLHV033RS)
 - Cell counter or hemocytometer
 - Moflo Cell Sorter (Dako Cytomation)

REAGENT SETUP

PBS–EDTA buffer Add 2 mM EDTA to 500 ml PBS (vol/vol) and cool the solution at 4 °C before use. Store at 4 °C and use within 2 weeks.

iPS cell-freezing medium Mix 10% DMSO (vol/vol) and 90% FBS (vol/vol). Use immediately.

Complete DMEM media for 293 Phoenix Amphotropic cells or human fibroblasts High glucose DMEM, 10% FBS, GlutaMAX 200 mM, penicillin–streptomycin (100 U ml⁻¹, 100 µg ml⁻¹). To prepare 500 ml of medium, remove 60 ml of DMEM from a new bottle and add 50 ml FBS, 5 ml GlutaMax and 5 ml penicillin–streptomycin. Store at 4 °C in the dark and use within 2–3 weeks.

bFGF Spin the lyophilized bFGF vial briefly to bring the contents down. Prepare HSA 0.2% in PBS in a sterile tube (1:50 dilution): 9.8 ml PBS + 0.2 ml HSA. Dissolve bFGF with PBS at 0.2% HSA to obtain a final concentration of 100 µg ml⁻¹. Make 50 µl aliquots in screw-cap microcentrifuge tubes and store at –20 °C.

SCF, IL-6, TPO and Flt-3 ligand Centrifuge the lyophilized SCF, IL-6, TPO and Flt-3 ligand vials for 1 min so that all the content will fall down to the bottom. Prepare HSA 0.2% in PBS in a sterile tube (1:50 dilution). Dissolve SCF, IL-6, TPO and Flt-3 ligand with PBS 0.2% HSA to obtain a final concentration of 5 ng ml⁻¹. Prepare 50 µl aliquots in screw-cap microcentrifuge tubes. Store at –20 °C.

hES cell medium To prepare 500 ml of hES medium, mix 387.5 ml of KO-DMEM with 100 ml of KOSR, 5 ml of Glutamax (1 mM), 5 ml of penicillin–streptomycin, 5 ml of nonessential amino acids (100 µM), 500 µl of 2-mercaptoethanol (100 µM) and 50 µl of bFGF (10 ng ml⁻¹). Filter the medium with a bottle-top 0.22-µm filter and store at 4 °C (maximum 1 week).

Gelatin-coated culture dishes Add 0.1% gelatin solution to cover the bottom of the dish. Incubate the dish for at least 30 min at 37 °C. Aspirate and leave to dry for at least 10 min in the tissue culture hood. Use immediately.

Retroviral vectors pMSCV-based retroviral vectors expressing FLAG-tagged OCT4, SOX2, KLF4 and c-MYC are available from Addgene (e.g. 20072, 20073, 20074 and 20075, respectively).

PROCEDURE

Umbilical CB collection ● TIMING ~5 min

1| Collect umbilical CB into an integral plastic blood collection bag by venipuncture (**Fig. 2a**).

▲ **CRITICAL STEP** Patients' informed consents must be obtained before the delivery.

▲ **CRITICAL STEP** The CB should be stored at 4 °C during the transport and before separation. Processing of the CB should be carried out as quickly as possible to prevent the blood hemolysis or coagulum formation. Do not use samples older than 15 h.

Isolation of umbilical CB mononuclear cells using Lympholyte-H density gradient ● TIMING ~2 h

2| All subsequent steps should be carried out in a tissue culture hood. Dilute the CB 1:3 with sterile PBS–EDTA in a sterile 500-ml bottle.

3| Layer 35 ml of diluted blood slowly on 15 ml of Lympholyte-H layer (ratio 3:1) in a 50-ml tube (**Fig. 2b**).

▲ **CRITICAL STEP** Do not mix the tube; make sure the layers are not disturbed.

4| Centrifuge for 25 min at 400g at room temperature (20–22 °C).

▲ **CRITICAL STEP** Acceleration and deceleration should be kept at speeds as low as possible to avoid any disturbance to cells in the white layer (**Fig. 2c**).

5| Collect the interphase cells (white layer) in a 50-ml tube using a plastic pipette.

▲ **CRITICAL STEP** Some cells might attach to the side of the tube in the form of a pellet; 'rub' them gently using the end of the pipette and collect them.

6| Dilute the cell suspension with PBS–EDTA (ratio 1:1) and centrifuge for 5 min at 300g at room temperature.

7| Pipette off the supernatant and pull together the pellets, resuspending the pellets in 50 ml of PBS–EDTA.

8| Count the total number of mononuclear cells using a hemacytometer and assess the viability using Trypan Blue exclusion method (as described in **Box 1**).

Figure 2 | Umbilical cord blood collection and separation. (a) An average of 75 ml of umbilical cord blood is collected into a plastic blood bag by venipuncture. (b) Before centrifugation, two distinct layers can be clearly distinguished: the diluted blood (B) over the Lympholyte-H (F). (c) After centrifugation, there should be a well-defined white layer (W) at the interface, red blood cells on the bottom below the Lympholyte (R) and plasma with platelets on the top (P).

BOX 1 | CELL COUNTING

The following steps are used to count the total number of mononuclear cells in 10 μ l of cell suspension:

1. Place 10 μ l of mononuclear cell suspension in an appropriate tube containing 90 μ l of Trypan Blue and mix gently.
2. Place 10 μ l of stained cells in a hemocytometer and count the number of alive (unstained) and dead (stained blue) cells.
3. Calculate the average number of unstained cells in at least three quadrants and multiply by 10^4 to find the number of cells per ml. The percentage of viable cells is the number of viable cells divided by the number of total cells.

The following steps are used to count the CD133+ selected cells or Phoenix Amphotropic 293 cells in 10 μ l of cell suspension:

1. Place 10 μ l of cell suspension in an appropriate tube and add 10 μ l of Trypan Blue and mix gently.
2. Place 10 μ l of stained cells in a hemocytometer and count the number of alive (unstained) and dead (stained blue) cells.
3. Calculate the average number of unstained cells in at least three quadrants and multiply by 10^3 to find the number of cells per ml.

9| Centrifuge the sample for 5 min at 200g at room temperature to eliminate platelets.

▲ **CRITICAL STEP** Activation of platelets may disturb further isolation.

? TROUBLESHOOTING

10| Pipette off the supernatant and resuspend cell pellet in a final volume of 300 μ l of PBS–EDTA per 10^8 total cells.

▲ **CRITICAL STEP** For less than 10^8 total cells, use 300 μ l of PBS–EDTA.

Isolation of CD133+ cells ● **TIMING** ~1 h

11| Add 100 μ l FcR blocking reagent to 10^8 total cells resuspended in 300 μ l of PBS–EDTA.

12| Add 100 μ l CD133 microbeads to 10^8 total cells.

▲ **CRITICAL STEP** Work fast and keep the cells cold using cold solutions to avoid non-specific cell labeling.

▲ **CRITICAL STEP** When working with smaller cell numbers, use 100 μ l of CD133 microbeads and FcR blocking. When working with larger cell numbers, scale up all reagent volumes (e.g., for 2×10^8 total cells use twice the volume of all indicated reagents).

13| Mix well and incubate cells for 30 min at 4–8 °C.

▲ **CRITICAL STEP** During incubation, gently shake the tube every few minutes to avoid clumping and cellular adherence.

14| Wash cells by adding up to 50 ml of PBS–EDTA.

15| Centrifuge for 5 min at 300g at room temperature.

16| Pipette off the supernatant.

17| Resuspend cell pellet in 500 μ l of PBS–EDTA per 10^8 total cells and proceed to magnetic separation.

18| Use one MS column up to 10^7 magnetically labeled cells or up to 2×10^8 total cells.

▲ **CRITICAL STEP** When working with larger cell numbers, divide the cell suspensions using two MS columns.

19| Place the column in the magnetic field of the MACS Separator and place a 15-ml tube under the column. Rinse the column with 500 μ l of PBS–EDTA.

20| Apply 500 μ l cell suspension onto the column and allow the negative cells to pass through.

▲ **CRITICAL STEP** Avoid forming bubbles/foam that can disturb migration of cells through the column.

21| Wash the column twice with 500 μ l PBS–EDTA.

22| Remove the column from separator and place the column on a new collection 15-ml tube.

23| Pipette 1 ml of PBS–EDTA onto the column and firmly flash out the cell fraction magnetically labeled using the plunger.

24| Repeat magnetic separation Steps 19–23. Apply the eluted cells to a new pre-filled column.

BOX 2 | FLOW CYTOMETRY ANALYSIS

Flow cytometry analysis provides information about the purity of isolated CD133+ cells.

1. To prepare cells for flow analysis, add 10 μ l of CD133-PE and 20 μ l of CD45-APC in cell suspension (50,000 in 500 μ l of PBS), mix well and incubate for 15 min in the dark at room temperature.
2. Wash the cells with 2 ml of PBS with 2% human serum albumin (HSA) and centrifuge at 600g for 5 min at room temperature.
3. Resuspend the cell pellet in 500 μ l of PBS with 2% HSA containing propidium iodide (final concentration 10 μ g ml⁻¹) to detect dead cells.
4. Analyses: all our analyses were carried out on Moflo Cell Sorter (DakoCytomation) applying Summit software.

- 25| Count the cells using hemacytometer and assess their viability using Trypan Blue exclusion method (as described in Box 1).
- 26| Take 5×10^4 cells and carry out the flow cytometry (FACS) analysis to evaluate the efficiency of the magnetic separation (as described in Box 2).
- 27| Plate the CD133+ cells (50,000 ml⁻¹) in complete DMEM supplemented with SCF (50 ng ml⁻¹), Flt-3 ligand (50 ng ml⁻¹), IL-6 (10 ng ml⁻¹) and TPO (10 ng ml⁻¹) in a 6-well plate. Incubate at 37 °C, 5% CO₂ for 24 h.
- ▲ **CRITICAL STEP** As the integration and expression of retroviral constructs require mitotic division of the target cells, it is important to carry out a pre-stimulation step of 24 h to induce the quiescent CD133+ cells to enter in a proliferative status.

Retrovirus production ● TIMING ~1 week

28| Defrost a vial of Phoenix Amphotropic 293 cells (for details see Box 3). If using other virus or production approaches, jump to Step 42.

! **CAUTION** Use Category 2 (or higher) tissue culture hoods and exercise due caution in the production, storage and use of recombinant retroviral particles.

- 29| Plate $\sim 2 \times 10^6$ cells in complete DMEM medium in 100-mm tissue culture dish and incubate at 37 °C, 5% CO₂ for 2 d.
- 30| When the cells reach 80% confluence (1–2 d), aspirate medium, wash gently with PBS, aspirate and add 3 ml 0.05% Trypsin–EDTA. Incubate for 1 min at 37 °C. Gently tap the tissue culture plate ensuring all cells are in suspension.
- 31| Add 10 ml complete DMEM medium to the plate, collect cell suspension and transfer to a 50-ml tube.
- 32| Centrifuge at 200g for 5 min at room temperature.
- 33| Resuspend pellet in 10 ml complete DMEM medium and count cells (as described in Box 1).
- 34| Plate 4×10^6 cells in 10 ml final volume in 100-mm culture dishes and place in a 37 °C, 5% CO₂ incubator.
- 35| The next day, co-transfect Phoenix Amphotropic 293 cells with FuGENE–DNA complex according to the manufacturer's instructions. Briefly, place 0.873 ml of OPTIMEM into separate 1.5-ml tube (one for each of the plasmids to be transfected) and add 27 μ l of FuGENE 6 transfection reagent, gently tapping the tube to mix. Next incubate at

BOX 3 | THAWING PHOENIX AMPHOTROPIC 293 CELLS

To thaw Phoenix Amphotropic 293 cells, prepare and warm 10 ml of complete DMEM in a 37 °C water bath and proceed as follows:

1. Remove a vial of Phoenix Amphotropic 293 from liquid nitrogen tank and place immediately in a 37 °C water bath.
2. When the cell suspension is almost fully thawed, remove the vial and spray with ethanol.
3. Open the vial and dropwise add 500 μ l of complete DMEM medium.
4. Using a 5 ml pipette containing 3 ml of complete DMEM medium, aspirate the cell suspension and transfer the cells into a 15-ml tube containing 7 ml of complete DMEM medium.
5. Centrifuge at 200g for 5 min at room temperature.
6. Pipette off the supernatant and resuspend the cell pellet in 8 ml of complete DMEM medium.
7. Plate the cell suspension in a 100-mm tissue culture dish and place in a 37 °C, 5% CO₂ incubator.

Figure 3 | Phoenix Amphotropic 293 cells post transfection.

(a) Representative image of Phoenix Amphotropic 293 cells 48 h after transfection (phase contrast image; scale bar, 100 μ m). (b) The transfection efficiency of Phoenix Amphotropic 293 cells after 48 h should be close to 100%. Scale bar, 100 μ m.

room temperature for 5 min. Add 9 μ g of pMSCV DNA plasmids dropwise into separate solutions of FuGENE–OPTIMEM and mix by tapping with a finger. Incubate at room temperature for 15 min.

36 | Add the FuGENE–DNA solution dropwise onto plate and return to a 37 °C, 5% CO₂ incubator overnight.

▲ **CRITICAL STEP** Gently add FuGENE–DNA complex solution dropwise. Phoenix Amphotropic 293 cells get detached easily from the plate if L-polylysine coating is not carried out.

37 | Next day, change the media gently (10 ml per plate) and incubate overnight in a 32 °C, 5% CO₂ incubator. If desired check transfection efficiency (see **Fig. 3**).

▲ **CRITICAL STEP** Virus is more stable at 32 °C, resulting in higher infectivity, although 37 °C is acceptable.

▲ **CRITICAL STEP** At this time point, near 100% confluence of cells should be transfected. Monitor transfection efficiency by using preferred GFP reporter plasmid (**Fig. 3a,b**).

38 | Collect 5 ml viral supernatant from every plate 48 h after transfection using plastic pipettes and filter the supernatant through a 0.45- μ m PVDF filter to remove any residual cells.

▲ **CRITICAL STEP** Use low protein-binding filters to avoid trapping the virus and reducing the titer.

■ **PAUSE POINT** Virus can be snap-frozen in cryovials using liquid nitrogen and stored at –80 °C or in liquid nitrogen for several months. We have observed some loss in infectivity upon freezing.

39 | Add fresh 5 ml of complete DMEM to every plate and place in a 37 °C, 5% CO₂ incubator.

▲ **CRITICAL STEP** Take care to avoid cells detaching from the tissue culture plates.

40 | Add 1 μ l (10 mg ml^{–1}) of polybrene for each ml viral supernatant needed.

41 | Every 12 h repeat Steps 38–40 to collect more viral supernatant. Collect twice more. Continue with Step 42 while carrying out these collections.

Retroviral transduction of CD133+ cells ● TIMING ~2d

42 | Infection day 1: dispense an appropriate volume of retronectin solution (15 μ g cm^{–2}) into each well of a 24-well plate and incubate for 2 h at room temperature.

▲ **CRITICAL STEP** We recommend diluting the retronectin solution in PBS and dispensing 500 μ l of diluted retronectin into each well of a 24-well plate.

43 | Remove retronectin solution and add 500 μ l of PBS containing 2% HSA (vol/vol) into each well for blocking.

44 | Incubate the plate for 30 min at room temperature.

45 | Remove the PBS–HSA solution and wash once with PBS.

46 | Pre-load the viral supernatant derived from Phoenix Amphotropic 293 cells that have been filtered onto retronectin-coated plates. Use equal amounts of each transcription factor to reach a total volume of 1 ml (500 μ l for OCT4 and 500 μ l for SOX2).

47| Set the plate into a centrifuge pre-warmed to 32 °C and centrifuge for 1 h at 2,000g at 32 °C.

▲ **CRITICAL STEP** If the titer of viral vector is high enough, then incubate for 4–6 h in a 32 °C, 5% CO₂ incubator. Centrifugation increases infectivity when the viral titer is low.

48| During centrifugation collect the CD133+ cells from Step 27 in a 15-ml tube and count the number of living cells (as describe **Box 1**).

49| Centrifuge for 5 min at 200g at room temperature.

50| Suspend the pellet cells in complete DMEM medium supplemented with cytokines (SCF, Flt- 3, IL-6 and TPO) at a concentration of 8×10^4 cells per ml.

51| Pipette off the viral supernatant from each well, taking care that the virus bound to the retronectin does not dry out, and wash each well with 1 ml of PBS.

52| Pipette off PBS and immediately add 1 ml of cell suspension into each well.

53| Incubate for 12 h in a 37 °C, 5% CO₂ incubator.

54| Infection day 2: repeat Steps 38–40.

55| Remove 500 µl from each well of the 24-well plates containing CD133+ cells and add 500 µl of fresh viral supernatant to infect the cells a second time.

▲ **CRITICAL STEP** It is important to add the cytokine cocktail described in Step 27 (half the amount) to the fresh viral supernatant to keep the cells alive and proliferating (**Fig. 4a**).

56| Incubate for 12 h in a 37 °C, 5% CO₂ incubator.

57| Repeat Steps 55 and 56 to infect the cells a third time and incubate for 24 h in a 37 °C, 5% CO₂ incubator.

▲ **CRITICAL STEP** We have noticed that infection efficiency, monitored by a constitutive GFP reporter retrovirus, can vary (10–40%).

? TROUBLESHOOTING

Culturing transduced CD133+ cells ● **TIMING** ~2 weeks

58| Plate the HFF in a gelatin-treated 6-well plate.

59| Collect the cells in a 15-ml tube 1 d after the last infection.

60| Centrifuge for 5 min at 200g at room temperature.

61| Resuspend in 1 ml hES medium and plate the infected CD133+ cells onto a 6-well plate containing HFF feeder and 1 ml of hES medium.

62| After 2 d change the medium daily and maintain in culture until the iPS cell colonies emerge.

▲ **CRITICAL STEP** Because the first week of culture CD133+ cells are still growing in suspension, it is important to

Figure 4 | Morphology of cord blood (CB) CD133+ cells after infection. (a) CB CD133+ cells growing attached on retronectin during the viral infection could change morphology, losing the round shape. (b) During the first week of reprogramming, CB CD133+ cells grow in suspension and only few of them start to attach to the feeder layer. Scale bar, 100 µm.

PROTOCOL

Figure 5 | Induced pluripotent stem cells (iPSC) from cord blood CD133+ cells. (a) Morphology of cord blood iPS (CBiPS) cell colonies obtained after the introduction of two transcription factors (OCT4 and SOX2). (b) CBiPS colonies grow as compact and domed colonies that express alkaline phosphatase (AP). Scale bar, 100 μ m.

aspirate the medium gently using a 1-ml pipette and to add fresh medium dropwise to prevent the few cells attached to the feeder from coming off (**Fig. 4b**).

63 | After 9 d small colonies start to appear. At 15 d of culture the colonies exhibit typical hESC morphology (**Fig. 5a,b**).

Picking and expanding iPS cell colonies ● **TIMING** ~9 d

64 | Day 1: identify colonies of iPS-like morphology and mark them on the bottom of the dish under the inverted microscope.

65 | Prepare the required number of 6-well plates with irradiated HFF feeder layers, calculating 1-well per colony to be picked.

66 | Day 2: aspirate the medium and add 2 ml of fresh hES medium in each well of the reprogramming dishes and 6-well feeder plates.

67 | Place the plate in a 37 °C, 5% CO₂ incubator for 20 min.

68 | Working on the stereomicroscope placed inside a tissue culture hood, manually pick the single colonies from each well using a stripper micropipette. Transfer colony fragments into a well with an HFF feeder. Incubate in a 37 °C, 5% CO₂ incubator.

▲ **CRITICAL STEP** To avoid cross-contamination with colonies originated in the same well, pick each colony with a new micropipette.

69 | Day 4: change the medium in each well. Many colony fragments should be attached to the feeder.

70 | Days 5–9: change the medium daily. Attached colony fragments will form iPS-like colonies, ready to be passaged by days 8–9.

Freezing of iPS cells ● **TIMING** ~1 h

71 | Aspirate the medium and add 2 ml of fresh hES medium in each well.

72 | Pick approximately 20 colonies from each well and transfer the cell suspension in a 15-ml tube containing 2 ml pre-warmed hES medium. Centrifuge for 1 min at 200g at room temperature and aspirate the supernatant.

73 | Drop-by-drop, add 1 ml of freezing medium and transfer the cells into freezing vial.

74 | Keep the vials in a cell-freezing container overnight at –80 °C and then transfer them into a liquid nitrogen tank the next day.

! **CAUTION** Liquid nitrogen is dangerous; use special protective clothing.

● **TIMING**

Step 1, Umbilical CB collection: ~5 min

Steps 2–10, Isolation of umbilical CB mononuclear cells using Lympholyte-H density gradient: ~2 h

Steps 11–27, Isolation of CD133+ cells: ~1 h

Steps 28–41, Retrovirus production: ~1 week
 Steps 42–57, Retroviral transduction of CD133+ cells: ~2 d
 Steps 58–63, Culturing transduced CD133+ cells: ~2 weeks
 Steps 64–70, Picking and expanding iPS cell colonies: ~9 d
 Steps 71–74, Freezing of iPSC: ~1 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
9	White fiber formation during the process	Contamination of cells suspension with platelets	Centrifuge cells slowly 200 × g/5 min; discard the supernatant with platelets
57	Low infection efficiency	Viral titer is low	Ensure that Phoenix Amphotropic cells are healthy and the transfection efficiency is close to 100%. Use low passage Phoenix cells
		Infectivity of other cell lines is high	Ensure to work with a purity of CD133+ cells more than 90%. Extend the centrifugation to promote viral vectors binding to Retronectin to 2 h

ANTICIPATED RESULTS

Cord blood CD133+ cells represent a small fraction of total nucleated cells (0.1–0.5%) in one CB unit. Depending on the quality of the CB unit (total volume of CB, total number of nucleated cells, the percentage of CD133+ cells and the age of the CB unit), the recovery and the purity of CD133+ cells after immunoselection can vary (from 400,000 to 1.2×10^6 CD133+ cells, with a purity range of 90–94%) (**Fig. 6a**).

On average, 8×10^4 CD133+ cells seeded over retro-nectin-coated plates previously pre-absorbed with viral particles (**Fig. 4**) and infected with OS give rise to five iPS colonies in 12–15 d. CBiPS colonies exhibited typical hESC morphology with sharp borders and small, tightly packed cell population with large nuclei (**Fig. 5**). CBiPS cells show a differential potential similar to hESC *in vitro*, and when injected into immunocompromised SCID mice, CBiPS cells generate complex intratesticular teratomas, comprising structures and tissue derived from the three embryonic germ layers (**Fig. 6b,c**). In addition, CBiPS cells show a genome-wide transcriptional profile similar to hESC and other iPS cell lines (**Fig. 6d**)¹².

Figure 6 | Characterization of CD133+ and cord blood-induced pluripotent stem (CBiPS) cells. **(a)** Representative dot-plot for CD133 cells after standard immunoselection. **(b)** Hematoxylin and eosin staining of teratomas derived from SCID Beige mice injected with CBiPS2F cells show tissues representative of all three embryonic germ layers, including cartilage and smooth muscle (mesoderm), gut epithelium (endoderm) and immature neural tissue (ectoderm). **(c)** Fluorescent microscope staining of teratoma sections reveal the presence of derivatives of the three embryonic germ layers (Tuj1 (green)/GFAP (red)-positive ectoderm, AFP (green)/FoxA2 (red)-positive endoderm and ASM (green)/ASA (red)-positive mesoderm. Scale bar, 25–50 μ m. Experiments using laboratory animals complied with the institutional and national guidelines. **(d)** Principal component analysis shows that CBiPS2F have a similar gene expression profile to human ES cell (hES) and other iPS cell lines derived in our laboratory.

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