Rapid Generation of Induced Pluripotent Stem Cells from Mouse Pre-B Cells

Bruno Di Stefano (bruno.distefano@crg.es)
Graf's Lab, Centre for Genomic Regulation
Thomas Graf (thomas.graf@crg.es)
Graf's Lab, Centre for Genomic Regulation

Method Article

Keywords: Induced pluripotent stem cells, Cell reprogramming, B lymphocytes, Transcription factor C/EBPα, Deterministic process, Embryonic stem cells, Oct4, Sox2, Klf4, c-Myc

Posted Date: February 4th, 2016

DOI: https://doi.org/10.1038/protex.2016.001

Abstract

One of the major obstacles in generating induced pluripotent stem \(iPS) cells suitable for therapeutic application is the low efficiency of the process and the long time required, with many iPS lines acquiring genomic aberrations. In this protocol we describe a highly efficient iPS reprogramming system based on the transient expression in pre-B cells of the transcription factor C/EBPα, followed by the induction of the four Yamanaka factors \(OSKM) in a serum-free medium.

Introduction

Resetting the somatic epigenome to a pluripotent state has been achieved by several approaches, including somatic cell nuclear transfer, cell fusion, and overexpression of defined transcription factors such as Oct4, Sox2, Klf4, and c-Myc \(OSKM, also known as the Yamanaka factors) \(1). The induced pluripotent stem cells \(iPS cells) derived by overexpression of the OSKM factors have opened up new opportunities for disease modeling and cell therapy applications \(2,3). However, the low efficiency of iPS cell derivation \(0.1–1 %) \(3, 4) has hindered these opportunities \(5). Therefore, creating an efficient and rapid system to convert somatic cells into pluripotent cells would be beneficial for the generation of high-quality iPS cells suitable for cell therapy and also for the full understanding of the molecular mechanism underlying the reprogramming process. Here we report a method with which primary pre-B cell precursors derived from the bone marrow of the reprogrammable mouse \(6) can be converted rapidly and efficiently into iPS cells. The method consists in the transient expression of the transcription factor C/EBPα followed by OSKM induction in a serum-free medium. This system allows the derivation of iPS cells within 1 week at very high efficiency. Pluripotency genes are activated as early as 1 day after OSKM induction and transgene- independent colonies are generated within 4 days. The method described here should help to fully understand the reprogramming mechanism of somatic cells into pluripotent cells and could be beneficial for the generation of high-quality iPS cells suitable for disease modeling and cell therapy.

Reagents

1. 8-16 weeks old, male and female mice obtained from the cross between ‘reprogrammable mice’ containing a doxycycline-inducible OSKM cassette and tetracycline transactivator, and the Oct4-GFP reporter mice. 2. S17 stromal cells. 3. Mouse embryonic fibroblasts \(MEFs). 4. Retroviral vector carrying C/EBPα-ER-hCD4. 5. RPMI with L-glutamine \(Life Technologies 11875-093). 6. Interleukin 7 \(Peprotech, 217-17). 7. Interleukin 4 \(Peprotech 214-14). 8. Interleukin 15 \(Peprotech 210-15). 9. D-PBS without Ca2+ and Mg2+. 10. TrypLE select cell dissociation reagent \(Life Technologies, 12605036). 11. Fetal bovine serum \(FBS), E.U. Approved \(Life Technologies 10437-028). 12. ESGRO Leukemia inhibitor factor \(LIF) \(Millipore, ESG1107). 13. β-Mercaptoethanol, penicillin-streptomycin, sodium pyruvate, NE amino acids \(Life Technologies). 14. Gelatin 0.1 % \(Millipore, ES-006-B). 15. β-estradiol \(Calbiochem, CAS 50-28-2). 16. Doxycycline \(Stemcell technologies, 72742). 17. Deionized water \(B. Braun Medical SA). 18. D-MEM Life Technologies, A14431-01). 19. DMEM/F12 \(Life Technologies, 10565-018). 20.

Neurobasal medium \(Life Technologies, 10888-022). 21. N2 Supplement \(Life Technologies, 17502-048). 22. B27 supplement \(Life Technologies, 12587-010). 23. PD0325901 \(Tocris, 4192). 24. CHIR99021 \(Tocris, 4423). 25. Monoclonal antibody to CD19 \(BD Pharmingen, 553784). 26. MACS microbeads beads \(Miltenyi Biotech, 130-048-101). 27. Knock-out serum replacement \(Life Technologies, 10828-028). 28. LS columns \(Miltenyi Biotech, 130-042-401). 29. PlatE cells \(Cell Biolabs, RV-101). 30. Monoclonal antibody against hCD4 \(eBioscience, 12-0049-42). 31. FC block \(BD Pharmingen, 553141)

Equipment

1. Cell culture hood \(i.e., biosafety cabinet). 2. Inverted microscope. 3. Incubator at 37C. 4. Water bath at 37C. 5. Centrifuge. 6. Micropipettes. 7. Pipettor. 8. Liquid nitrogen tank. 9. Cryovial storage rack. 10. Freezers. 11. Sterile plastic pipettes \(5-mL, 10-mL). 12. 15 and 50 ml conical tubes. 13. 100-mm tissue culture-treated dishes. 14. 96-, 24-, 12- and 6-well tissue culture-treated plates. 15. Filter pipette tips \(0.5-10, 2-20, 20-200, 200-1000 µl). 16. 0.22-µm vacuum filtration \(500 ml). 17. 0.45-µm pore size filter. 18. Glass Pasteur pipettes, 9 inches, sterilized by autoclave. 19. Sterile syringes \(1, 5, 20, 50 ml). 20. Cryovials. 21. 40µm cell strainers.

Procedure

Solutions _B Cell Medium_ To prepare 100 ml of B cell medium mix the following: RPMI with L-Glutamine 1X 88.9ml Fetal bovine serum 10% 10ml Penicillin-Streptomycin 100X 1ml β-Mercaptoethanol 1000X 100µl Interleukin 7 1000X 100µl The medium can be stored at 4ºC for up to two weeks. _Cell Reprogramming Medium_ To prepare 500 ml of N2B27 medium mix the following*: DMEM/F12 231ml Neurobasal medium 231ml Penicillin-Streptomycin 100X 5ml β-Mercaptoethanol 1000X 500µl Sodium pyruvate 100X 5ml NE-aminoacids 100X 5ml Leukemia inhibitor factor 10000X 50µl N2 supplement 100X 5ml B27 supplement 50X 10ml Interleukin 7 1000X 500µl Interleukin 4 500X 1000µl Interleukin 15 5000X 100µl Doxycycline 500X**** 1000µl * From day 2 onwards supplement the N2B27medium with 20% KSR, 3uM CHIR99021 and 1uM PD0325901. ****Prepare the reprogramming medium without inducer and supplement Doxycycline when needed. _S17/MEF Medium_ To prepare 100 ml of OP9/S17 cell medium mix the following: DMEM 89ml Fetal bovine serum 20% 20ml Penicillin-Streptomycin 100X 1ml β-Mercaptoethanol 1000X 100µl _Interleukin 7, 4 and 15 Stock Solutions_ To prepare 1ml of a 10µg/ml interleukin solution, mix the following: Interleukin 10µg Deionized water 1ml _Estradiol Stock Solution_ To prepare 1mM b-Estradiol solution \(10.000X), mix the following: b-Estradiol 272ug EtOH 1ml _Doxycycline Stock Solution_ To prepare 1ml of a 50mg/ml Doxycycline solution, mix the following: Doxycycline 50mg Deionized water 1ml _Labeling Buffer_ To prepare 250ml of labeling buffer, mix the following: PBS 1X 240ml FBS 10ml EDTA 0.5M 1ml **B cell Isolation** One week before performing the reprogramming experiment purify ‘B cells’ from the bone marrow of two reprogrammable mice by sorting CD19 surface antigen positive cells, which consist of a mixture of pre-B and pro-B cells. 1. Sacrifice two 8-16 week old reprogrammable mice/Oct4GFP mice by ventilating them with CO^2. 2. Aseptically remove

femurs and tibiae. 3. Use scissors to remove all tissues from the bones. 4. Crush the bones in a mortar in 10 ml of labeling buffer. 5. Thoroughly resuspend the cells by pipetting them up and down. 6. Filter the cells with a 40µm cell strainer in a 50 ml conical tube. 7. Centrifuge the cells for 5 minutes at 300xg, resuspend them in fresh labeling buffer and count them in a hemocytometer. 8. Resuspend the cells in labeling buffer to a final concentration of $10X10^6$ cells/ml. 9. Add Fc-block to the cell suspension at a concentration of $0.1µg/10^6$ cells and incubate for 10 minutes on ice. 10. Add CD19-biotin conjugated antibody \(0.2ug/10^6 cells) and incubate for 20 minutes on ice. 11. Wash the cells with 10ml of labeling buffer, centrifuge for 5 minutes at 300xg. Resuspend them in 90µl of labeling buffer and 10µl of Streptavidin beads per 10 $x10^6$ cells and incubate for 20 minutes on ice. 12. Wash the cells with 10ml of labeling buffer and proceed with purification using the MACS LS column. 13. Place the LS column in the MACS separator. 14. Wash the column with labeling buffer. 15. Pipette 500µl of cells in the LS column allowing the suspension to run through and collect the effluent as negative fraction \(this fraction is enriched for myeloid progenitors). 16. Wash the column with 3ml of labeling buffer. 17 Repeat STEP 17 three times. 18. Remove the column from the MACS separator and place it in a new 15ml collection tube. 19. Apply 5ml of labeling buffer and firmly flush out the cells using the plunger provided. This yields the CD19 positive B cell fraction. **Reprogramming of Bells** _Retrovirus Production and B Cell Infection_ 1. Two days before viral transduction seed $9x10^6$ PlatE cells \(Cell Biolabs) into two 10cm dishes. To detach PlatE cells wash the cells once with PBS and then add 1-3ml of TrypLE select cell dissociation reagent. After 3-5 minutes at 37°C add PlatE medium and count the cells in the hemocytometer. 2. The following morning transfect PlatE cells with 20µg of C/EBP-ER-hCD4 plasmid using the calcium phosphate protocol. 3. Replace the transfection medium after 8 hours with 6 ml of fresh B cell medium per 10cm plate. Culture the cells at 37°C, 5% CO2 incubator. 4. After 18-20 hours of culture at 37°C collect the supernatant, filter it using a 0.45µm sterile filter, add 10ng/ml of IL-7 and use the filtered medium to infect the B cells. Use 2ml of supernatant to infect $2X10^6$ B cells per well of a 6-weel plate. Add 6 ml of fresh B cell medium to the PlatE cells. Place the cells in a 37°C, 5% CO2 incubator. 5. 24 hours later collect the supernatant of the PlatE cells and use it to infect the B cells a second time. _Plating of B cells on S17 Stromal Cells_ We use S17 stromal cells for the first days of B cell expansion before the reprogramming is initiated. 1. The day of the viral transduction experiment prepare gelatin-coated dishes. Then plate Mitomycin C inactivated S17 cells at a density of $3X10^4$ cells/cm^2 onto 10cm plates. To detach S17 cells, wash the cells with PBS and add the adequate amount of TrypLE select cell dissociation reagent to the cells. After 3-5 minutes, add S17 medium and count the cells in the hemocytometer. 2. Collect the infected B cells from the 6 well plate and centrifuge them at 300xg for 5 min. Then aspirate the supernatant, and resuspend the cells in 10 ml of B cell medium. Plate the B cells on the inactivated S17 cells. 3. Grow the cells 4 days or until they reach confluence \(note that besides B cells that remain non-adherent, many of them move under the feeders, forming clusters of flattened cells with smooth edges that can easily be distinguished from the feeder cells). 4. Collect the B cells, stain them with human CD4 antibody and sort $hCD4^+$ transgene containing cells using a MACS column or a fluorescence activated cell sorter. 5. The day before the sorting prepare gelatin-coated 12 well dishes. Plate Mitomycin C inactivated MEF cells at a density of $3X10^4$ cells/cm^2. To detach MEF cells add the adequate amount of TrypLE select cell

dissociation reagent. After 3-5 minutes, add S17/MEF medium and count the cells in the hemocytometer. 6. After the sorting of the hCD4^{+} B cells, count the cells using the desired method and seed the cells on MEF feeders at a density of 25.000 cells/cm^{2}. Let the cells grow for two days. 7. To activate C/EBPa add β-estradiol at a concentration of 100nM to the B cell medium. 8. After 18h collect the supernatant and carefully wash the cultures twice with 500µl of PBS. Then centrifuge the supernatant/PBS at 300xg for 5 min. 9. Resuspend the pellet in fresh reprogramming medium containing 2ug/ml of doxycycline and re-seed the cells into the original wells. 10. Gently replace the medium every two days. 11. Starting at day 1 Oct4GFP positive cells can be observed under a fluorescence microscope or recorded by FACS. 12. After 2 days supplement the reprogramming medium with 20%KSR, 3uM CHIR99021 and 1uM PD0325901. 13. Starting at day 4 Oct4GFP positive colonies with ES-like morphology appear. 13. At day 8 change the medium to reprogramming medium without doxycycline to select transgene-independent iPS cell colonies.

Timing

The total procedure can be completed in 3 weeks.

Troubleshooting

1. Sterilize the abdomen and hind legs with 70% ethanol. Make an incision in the midline of the abdomen. Clip outward to expose the hind legs. 2. Wash the bones carefully with PBS to avoid possible contamination with cells outside the bones. 3. We suggest to use Türk's solution to count the cells at this step as the erythrocytes are hemolyzed by the acetic acid and the leukocytes are stained by the dye the solution contains. 4. The use of FC-block is suggested to avoid unspecific staining with the CD19 primary antibody. 5. We add 30µM Cloroquine to the medium used to transfect the PlatE cells to increase transfection efficiency. 6. We suggest infecting the B cells immediately after the CD19 purification. 7. We observed increased viability if 1 ml of fresh medium is added to the cells after infection. We do not recommend "freeze-thawing" the virus samples since the titers decrease upon thawing. 8. We initially culture the B cells on S17 stromal cells to increase the number of cells as the proliferation is greatly enhanced in this condition compared to the MEF feeder cells. 9. The hCD4 cell sorting is performed as for the CD19 biotin. 10. Maintaining the precise β-estradiol exposure time of 18 hours is crucial to obtain an optimal reprogramming efficiency. 11. We have observed variability in the outcome of our reprogramming depending on the batch number of KSR, N2 and B27 supplements.

Anticipated Results

Under the conditions described, iPS colonies are obtained from B cells pulsed with C/EBPa in less than 1 week. Oct4GFP expression becomes detectable after 1 day of OSKM induction, and small colonies with an ES-like morphology appear already after 4 days.

References

1. Jaenisch R, Young R \(2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132\(4):567–582. doi:10.1016/j.cell.2008.01.015. 2. Robinton DA, Daley GQ \(2012) The promise of induced pluripotent stem cells in research and therapy. Nature 481\(7381):295–305. doi:10.1038/nature10761 3. Takahashi K, Yamanaka S \(2013) Induced pluripotent stem cells in medicine and biology. Development 140\(12):2457–2461. doi:10. 1242/dev.092551 4. Takahashi K, Yamanaka S \(2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126\(4):663–676. doi:10.1016/j.cell. 2006.07.024. 5. Buganim Y, Faddah DA, Jaenisch R \(2013) Mechanisms and models of somatic cell reprogramming. Nat Rev Genet 14\(6):427–439. doi:10.1038/nrg3473. 6. Carey BW, Markoulaki S, Beard C, Hanna J, Jaenisch R \(2010) Single-gene transgenic mouse strains for reprogramming adult somatic cells. Nat Methods 7\(1):56–59. doi:10.1038/nmeth.1410.

Acknowledgements

We thank all the member of the Graf lab for helpful discussions. This work was supported by Ministerio de Educacion y Ciencia, SAF.2012- 37167, Fundacio La Marató TV3 120410, AGAUR SGR 1136 and European Research Council Synergy Grant \(4D-Genome).