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Generation of Mouse Induced Pluripotent Stem Cells with Plasmid Vectors

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ABSTRACT

Reprogramming of somatic cells into pluripotent stem cells has been reported by introducing several transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) in combination. The induced pluripotent stem (iPS) cells from patient's somatic cells would be useful source for drug discovery and cell transplantation therapies. However, most iPS cells were made by virus vectors, such as retrovirus and lentivirus. These vectors integrate host genome and increase the risk of tumor formation. Here we describe the detailed method to generate iPS cells by continual transfection of plasmid vectors from mouse embryonic fibroblasts (MEF). This protocol takes around 2 months from MEF isolation to iPS cell establishment. Still the reprogramming efficiency was low, the established iPS cells were most likely free from plasmid integration. This virus-free technique would advance the safety concern of iPS cell generation and usages, and provide you the cells for the investigation of mechanisms underlying reprogramming and pluripotency.

INTRODUCTION

Induced pluripotent stem (iPS) cells are able to be generated from somatic cells by several combination of transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) and chemical compounds in mouse and human[1,2,3,4]. They are similar to ES cells in morphology, proliferation and gene expression profile. Mouse iPS cells can differentiate into all three germ layers and contributed chimera mice when injected into blastocysts[5,6,7]. Human iPS cells can differentiate into neuron and cardiomyocyte in vitro[3]. These iPS cells would supply patient-specific pluripotent stem cells for elucidation of pathogenesis, drug discovery, toxicology study, and cell transplantation therapy. However, original method of iPS generation used retrovirus vectors, which integrate the reprogramming factors into host genome. One of the factors, c-Myc, is known as proto-oncogene, and its reactivation could give rise to transgene deriving-tumor formation[6]. To overcome the safety concern of iPS generation, several protocols were reported, such as omission of c-Myc from the reprogramming factors, removal of the integrated factors after iPS establishment, and transient expression of the factors using adenovirus vectors [1,8,9,10]. Here we describe the detailed protocol of iPS cell generation by transient expression of the reprogramming factors using plasmid vectors[11]. We designed two plasmids; one encoding Oct3/4, Klf4, and Sox2, and the other encoding c-Myc (Fig. 1a). The former 3 transcription factors are connected with 2A self-cleaving peptide of foot-and-mouth disease virus, which

enable polycistronic expression of these proteins from one coding region[12,13]. Repeated transfection of the plasmids can induce pluripotent stem cells from mouse embryonic fibroblasts (MEF; summarized in Fig. 1b). We provide protocols for MEF isolation, feeder preparation, iPS induction with the plasmids, and PCR screening to obtain iPS cells without any evidence of genomic integration. Although we could not rule out the integration of short fragment, at least they don't have functional transgene. The plasmid-iPS cells can develop teratomas when subcutaneously injected into nude mice and contribute to adult chimeras. With this protocol, you do not need infectious viral vectors to generate iPS cells. Since the estimated established rate of plasmid-iPS is much lower than that of viral induction, this method would be an important step to improve the safety concern of iPS cells for future medical application.

MATERIALS

REAGENTS

- pCX expression vectors[14] (see REAGENT SETUP)
- *Nanog*$^{\text{GFP-IRES-Puro}}$ mice[6] (see REAGENT SETUP)

  CAUTION Experiments involving live rodents must conform to International and Institutional regulations.

- SNL feeder cells[15] (see REAGENT SETUP)

- DMEM containing 4.5 g l^{-1} glucose (Nacalai tesque, cat. No. 14247-15)
- PBS without calcium and magnesium (Nacalai tesque, cat. no. 14249-95)
- $_{L}$-Gln (Invitrogen, cat. no. 25030-081)
- Nonessential amino acid solution (Invitrogen, cat. no. 11140-050)
- 2-Mercaptoethanol (Invitrogen, cat. no. 21985-023)
- Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)
- 0.25% (wt/vol) Trypsin/1 mM EDTA solution (Invitrogen, cat. no. 25200-056)
- 0.5% (wt/vol) Trypsin/5.3 mM EDTA solution (Invitrogen, cat. no. 25300-054) (see REAGENT SETUP)
- Gelatin (Sigma, cat. no. G1890) (see REAGENT SETUP)
- Puromycin (Sigma, cat. no. P7255) (see REAGENT SETUP)
- Opti-MEM I Reduced-Serum Medium (Invitrogen, cat. no. 31985-062)
- FuGENE 6 transfection reagent (Roche, cat. no. 11 814 443 001)
- Methanol (Nacalai tesque, cat. no. 12915-93)
- Crystal violet (Nacalai tesque, cat. no. 09804-52) (see REAGENT SETUP)
- ES medium (see REAGENT SETUP)
- SNL medium (see REAGENT SETUP)
- FP medium (see REAGENT SETUP)

- Proteinase K (Nacalai tesque, cat. no. 29442-14) (see REAGENT SETUP)
- TaKaRa Ex Taq (Takara bio, cat. no. RR001A)
- Cell lysis buffer (see REAGENT SETUP)

EQUIPMENT

- 100-mm tissue culture dish (Falcon, cat. no. 353003)
- 6-well tissue culture plate (Falcon, cat. no. 353046)
- 24-well tissue culture plate (Falcon, cat. no. 353047)
- 96-well tissue culture plate (Falcon, cat. no. 351172)
- 15-ml conical tube (Falcon, cat. no. 352196)
- 50-ml conical tube (Falcon, cat. no. 352070)
- 1-ml plastic disposable pipette (Falcon, cat. no. 357520)
- 5-ml plastic disposable pipette (Falcon, cat. no. 357543)
- 10-ml plastic disposable pipette (Falcon, cat. no. 357551)
- 25-ml plastic disposable pipette (Falcon, cat. no. 357525)
- Bottle-top filter (Techno Plastic Products, cat. no. 99500)
- 0.22-μm pore size filter (Millex GP; Millipore, cat. no. SLGP033RS)
- 10-ml disposable syringe (Terumo, cat. no. SS-10ESZ)

- Dissecting forceps *CAUTION* *Sterilize by autoclave*
- Dissecting scissors *CAUTION* *Sterilize by autoclave*
- Coulter counter (Z2; Beckman Coulter)
- CO_2 incubator
- Thermal cycler
- 0.2-ml PCR tube (Greiner bio-one, cat. no. 301301)
- Freezing container (Nalgene, cat. no. 5100-0001)

REAGENT SETUP

pCX expression vector containing cDNAs of *Oct3/4, Sox2, Klf4,* or *c-Myc*

Available from addgene (http://www.addgene.org/Shinya_Yamanaka). We use a pCX expression vector encoding the red fluorescence protein (DsRedExpress) to monitor transfection efficiency. It also serves as a negative control for iPS cell induction.

***Nanog*$^{\text{GFP-IRES-Puro}}$ mice**

Available from Riken Bioresource Center (http://www.brc.riken.jp/inf/en/index.shtml, BRC no. RBRC02290). The reporter cassette GFP-IRES-Puro^{R} was introduced into 5' untranslated region of the *Nanog* gene in a bacterial artificial chromosome (BAC). The modified BAC was

linearized and introduced into RF8 ES cells by electroporation. ES cells containing the modified BAC were injected into blastocysts to generate *Nanog* –GFP reporter mice.

SNL feeder cells

Available from Dr. Allan Bradley of the Sanger Institute (http://www.sanger.ac.uk/) or Health Protection Agency Culture Collections (http://www.hpacultures.org.uk/). SNL cells were clonally derived from a STO cell line and stably express a neomycin resistance cassette and a leukaemia inhibitory factor expression construct. SNLP 76/7-4 feeder cell line, which is puromycin-resistant derivative of SNL, is also available from Dr. Allan Bradley.

Gelatin-coated culture dishes

Prepare gelatin stock at 10 × concentration (1% wt/vol). Dissolve 1 g of gelatin powder in 100 ml of distilled water, autoclave and store at 4 ^{o}C. To prepare 0.1% (1 ×) gelatin solution, warm the 10 × gelatin stock in a water bath at 37 ^{o}C, add 50 ml of the stock into 450 ml of distilled water. Filter the solution with a bottle-top filter (0.22-μm) and store at 4 ^{o}C. To coat a culture dish, add enough volume of 0.1% gelatin solution to cover the entire area of the dish bottom. For example, 1, 3 or 5 ml of gelatin solution is used for a 35-, 60- or 100-mm dish, respectively. Incubate the dish for at least half an hour at 37 ^{o}C. Before using, aspirate excess gelatin solution.

0.5% Trypsin/5.3 mM EDTA solution

To prepare 0.05% trypsin/0.53 mM EDTA, mix 10 ml of 0.5% trypsin/5.3 mM EDTA solution with 90 ml of PBS. To prepare 0.1% trypsin/1 mM EDTA, add 20 ml of 0.5% trypsin/5.3 mM EDTA to 80 ml of PBS. Aliquot and store at -20 ^{o}C.

Puromycin

Dissolve in distilled water at 10 mg ml^{-1} and sterilize through a 0.22-μm filter. Aliquot and store at -20 ^{o}C.

Crystal violet

Working solution is 0.1% (wt/vol). Dissolve 0.1 g of crystal violet in 100 ml of methanol.

ES medium

DMEM containing 15% FBS (vol/vol), 2 mM $_{L}$-Gln, 1 × 10^{-4} M non-essential amino acids, 1 × 10^{-4} M 2-mercaptoethanol, and 50 U and 50 mg ml^{-1} penicillin and streptomycin. To prepare 500 ml of the medium, mix 75 ml of FBS, 5 ml of $_{L}$-Gln, 5 ml of nonessential amino acids, 1 ml of 2-mercaptoethanol and 2.5 ml of penicillin/streptomycin, and then fill to 500 ml with DMEM.

Store at 4 ^{o}C for a week.

SNL medium

DMEM containing 7% FBS, 2 mM $_{L}$-Gln, and 50 U and 50 mg ml^{-1} penicillin and streptomycin. To prepare 500 ml of the medium, mix 35 ml of FBS, 5 ml of $_{L}$-Gln and 2.5 ml of penicillin/streptomycin, and then fill to 500 ml with DMEM. Store at 4 ^{o}C for a week.

FP medium

DMEM containing 10% FBS, and 50 U and 50 mg ml^{-1} penicillin and streptomycin. To prepare 500 ml of FP medium, mix 50 ml of FBS and 2.5 ml of penicillin/streptomycin, and then fill to 500 ml with DMEM. Store at 4 ^{o}C for a week.

2 × Freezing medium

To make 10 ml of 2 × freezing medium, mix 2 ml of DMSO, 2 ml of FBS and 6 ml of DMEM, and sterilize through a 0.22 μm filter.

Proteinase K

Dissolve in distilled water at 10 mg ml^{-1}. Aliquot and store at -20 ^{o}C.

Cell lysis buffer

PCR buffer containing 150 μg ml^{-1} of Proteinase K. To prepare 100 μl of cell lysis buffer, mix 10 μl of 10 × Ex Taq buffer containing magnesium, 1.5 μl of 10 mg ml^{-1} Proteinase K and 88.5 μl of distilled water.

PROCEDURE

Preparation of fibroblasts from mouse embryos; TIMING 15 d

1. Euthanize 13.5-day pregnant female mice by cervical dislocation. Isolate uteri and wash with PBS briefly.
2. Separate embryos from their placenta and surrounding membranes with forceps. Remove the head, visceral tissues, and gonads from isolated embryos.
3. Wash embryos by transferring it to a 100-mm dish containing fresh PBS. Mince the bodies by using a pair of scissors, transfer into a 50-ml conical tube containing 0.1% trypsin/0.1 mM EDTA solution (3 ml per embryo), and incubate at 37 ^{o}C for 20 min.
4. Add additional 0.1% trypsin/0.1 mM EDTA solution (3 ml per embryo), and incubate the mixture at 37 ^{o}C for 20 min.
5. Add an equal amount of FP medium (6 ml per embryo), and pipette up and down a few

times to help with tissue dissociation.

6. Keep the tissue/medium mixture still for 5 min at room temperature (20~25 ^{o}C) to remove debris, and transfer the supernatant into a new 50-ml conical tube. Centrifuge at 200 *g* for 5 min, discard the supernatant, and resuspend the pellet in fresh medium.

7. Count the cell number and adjust the concentration to 1×10^6 cells ml^{-1} with FP medium. Generally, approximately 1×10^7 cells can be obtained from a single embryo. Transfer the cell suspension to 100-mm tissue culture dishes (1×10^7 cells per dish) and incubate at 37 ^{o}C with 5% CO_2 for 24 h (passage 1).

8. The next day, remove floating cells by washing with PBS, and add new FP medium..

9. When the cells become confluent (generally 2 or 3 d after the isolation), you can pass or store the cells. For passage, remove FP medium, wash once with PBS, and trypsinize with 1ml of 0.05% trypsin/0.53 mM EDTA for a 5 min. After detaching, add 9 ml of FP medium and suspend by pipetting. Passage to new 100-mm dishes at 1:4 dilution (passage 2). For the generation of iPS cells, we used MEFs within three passages to avoid replicative senescence.

Preparation of freeze stock of MEF; TIMING 1 h

10. Aspirate the medium, and wash the cells with 5 ml of PBS.

11. Remove PBS completely, add 1 ml of 0.05% trypsin/0.53mM EDTA and incubate at 37 ^{o}C for 5 min.

12. Add 9 ml of the FP medium and suspend the cells by pipetting up and down to single cell suspension.

13. Transfer the cell suspension to a 15-ml tube, count the cell number and spin the cells at 160 *g* for 5 min.

14. Discard the supernatant, and resuspend the cells with FP medium to the concentration at 1 × 10^7 cells per milliliter.

15. Prepare 2 × freezing medium and aliquot it at 0.5 ml per vial.

16. Transfer 0.5 ml of the cell suspension to freeze vials and mix gently.

17. Put the vials in a cell-freezing container and keep it at -80 ^{o}C overnight.

PAUSE POINT For long-term storage, keep frozen cells in the gas phase of a liquid nitrogen tank.

Thawing SNL cells; TIMING 0.5 h

18. Prepare 9 ml of SNL medium in a 15-ml tube.

19. Remove a vial of frozen SNL cells from the liquid nitrogen tank and put the vial into 37 ^{o}C water bath until most (but not all) cells are thawed.

20. Wipe the vial with ethanol, open the cap, and transfer the cell suspension to the tube prepared in step 18.

21. Centrifuge at 160 *g* for 5 min, and then discard the supernatant.

22. Resuspend the cells with 10 ml of SNL medium, and transfer to a gelatin-coated 100-mm dish. Incubate the cells in a 37 ^{o}C, 5% CO_2 incubator, until the cells become 80~90% confluent.

CRITICAL STEP Do not let the cells get over-confluent, or their ability as feeder cells may decrease.

Passage of SNL cells; TIMING 0.5 h

23. Discard the medium and wash the cells once with PBS.

24. Aspirate PBS, and add 0.5 ml per dish of 0.25% trypsin/1 mM EDTA, and incubate for 1 min at room temperature.

25. Add 4.5 ml of SNL medium, and break up the cells into a single cell suspension by pipetting up and down several times.

26. Adjust the cell suspension to 160 ml by addition of SNL medium, and transfer to gelatin-coated dishes (10 ml per 100-mm dish). This splits the cells 1:16. Incubate the cells at 37 ^{o}C, 5% CO_2 until the cells become 80-90% confluent. This should be happen 3-4 d

after passage.

Mitomycin C-inactivation of SNL cells; TIMING 3 h

27. Add 0.3 ml of 0.4 mg ml^{-1} mitomycin C solution directly to the culture medium of SNL dish, swirl it briefly, and incubate 2.25 h at 37 ^{o}C, 5% CO_2. The final concentration of mitomycin C will be 12 μg ml^{-1}.

28. After incubation, aspirate the mitomycin C-containing medium off the cells, and wash the cells twice with 10 ml of PBS.

29. Aspirate off PBS, add 0.5 ml of 0.25% trypsin/1 mM EDTA, swirl to cover the entire surface, and let sit for 1 min at room temperature.

30. Neutralize the trypsin by adding 5 ml of SNL medium, and break up the cells to a single cell suspension by pipetting up and down. Pour the cell suspension into a 50-ml tube and count the cell number. Seed the cells on gelatin-coated dishes (1×10^6 cells per 100-mm tissue culture dish, or 1.5×10^5 cells per well of 6-well plate).

31. Cells should be nicely spread with little gaps in between. They should become ready for usage by the next day.

PAUSE POINT The mitomycin C-treated SNL dishes can be left for up to a week before use.

Note: Mitomycin C-treated MEF could be used as feeders, but SNL seems to work slightly

better.

iPS induction with plasmid vector.

To use frozen stock of MEF, thaw the cells same as the protocols of thawing SNL cells (steps 18-22), except using FP medium instead of SNL medium.

Day 1: ***Preparation of fibroblasts***; TIMING 1 h

32. Culture MEF (passage < 3) to ~90% confluency in 100-mm dishes (~2×10^6 cells per dish).
33. Aspirate the culture medium and wash with 10 ml of PBS.
34. Discard PBS, add 1 ml per dish of 0.05% trypsin/0.53mM EDTA, and incubate at 37 ^{o}C for 5 min.
35. Add 9 ml of FP medium, suspend the cells to a single cell, and transfer to a 50-ml tube.
36. Count cell numbers, and adjust the concentration to 6.5×10^4 cells ml^{-1}. Transfer 2 ml of cell suspension (1.3×10^5 cells) to each well of 6-well plate. Incubate the dish overnight at 37 ^{o}C, 5% CO_2.

Day 2: ***Plasmid transfection***; TIMING 1 h

37. Aspirate the medium from a fibroblast dish, and add 2 ml of fresh FP medium.

38. Transfer 0.1 ml of Opti-MEM into a 1.5-ml tube.

39. Transfer 4.5 μl of FuGENE 6 transfection reagent directly into Opti-MEM prepared in step 38, mix gently by finger tapping, and incubate for 5 min at room temperature.

40. Mix 1.0 μg of pCX-OKS-2A and 0.5 μg of pCX-cMyc, add them into the FuGENE 6/Opti-MEM containing tube, mix gently by finger tapping, and incubate for 15 min.

41. Add the DNA/FuGENE 6 complex dropwise into the fibroblast dish, and incubate overnight at 37 ^{o}C, 5% CO_2.

CRITICAL STEP Also transfect with a suitable control; we use pCX vector encoding DsRedExpress to monitor transfection efficiency. We routinely obtain efficiency of more than 40% examined on day 10 (Fig. 2). High efficiency of transfection is crucial for iPS cell induction.

Day 3: ***Medium change***; TIMING 5 min

42. Aspirate the medium from a fibroblast dish, and add 2 ml of fresh FP medium.

Day 4: ***Plasmid transfection***; TIMING 1 h

43. Same as steps 37-41.

Day 5: ***Medium change***; TIMING 5 min

44. Aspirate the medium from a fibroblast dish, and add 2 ml of fresh ES medium.

Day 6: ***Plasmid transfection***; TIMING 1 h

45. Carry out same as steps 37-41 except that ES medium is used instead of FP medium.

Day 7: ***Medium change***; TIMING 5 min

46. Aspirate the medium from a fibroblast dish, and add 2 ml of fresh ES medium.

Day 8: ***Plasmid transfection***; TIMING 1 h

47. Carry out same as steps 37-41 except that ES medium is used instead of FP medium.

Day 9: ***Medium change***; TIMING 5 min

48. Aspirate the medium from a fibroblast dish, and add 2 ml of fresh ES medium.

Day 10: ***Passage of fibroblast***; TIMING 1 h

49. Discard the medium and wash the cells once with PBS.

50. Aspirate PBS, and add 0.5 ml per dish of 0.25% trypsin/1 mM EDTA, and incubate for 5

min at room temperature.

51. Add 4.5 ml of ES medium, and break up the cells into a single cell suspension by pipetting up and down several times.

52. Count the cell concentration, and adjust to 1×10^5 cells ml^{-1}. Transfer 10 ml of the suspension (1×10^6 cells) to 100-mm dish covered with mitomycin C-inactivated SNL cells. Gelatin-coated dishes are also available, but they would reduce induction efficiency. Incubate the cells at 37 ^oC, 5% CO_2.

Day 11~ : ***Medium change***; TIMING 5 min. each day

53. Change the medium every other day until the colonies become big enough to be picked up (Fig. 3). Colonies should first become visible approximately on day 20. They should become large enough to be picked up around day 30. If you use fibroblasts from *Nanog*$^{GFP\text{-}IRES\text{-}Puro}$ mice, the fluorescence will help you to choose colonies. We found that some GFP-negative or weak colonies become GFP-positive after picking up.

TROUBLESHOOTING

Picking up the iPS colonies; TIMING 1 h

54. Aliquot 20 µl of 0.25% trypsin/1 mM EDTA per well of 96-well plate.

55. Remove the medium from the dish, and add 10 ml of PBS.

56. Aspirate PBS, and add 5 ml of PBS.

57. Pick colonies from the dish using a Pipetman set at 2 μl, and transfer it into the 96-well trypsin plate. Pick up as many colonies as you can within 15 min. Incubate another 15 min in trypsin at 37 ^{o}C to dissociate cells in the colonies.

58. Add 180 μl of ES medium to each well, and pipette up and down to break up the colony to single cells.

59. Transfer cell suspension into the wells of 24-well plates with SNL feeder cells (use puromycin-resistant feeder cells for *Nanog*$^{GFP\text{-}IRES\text{-}Puro}$), add 300 μl ES medium, and incubate in 37 ^{o}C, 5% CO_2 incubator until the cells reach 50~60% confluency. Treatment of puromycin (1.5 μg ml^{-1}) support to select highly reprogrammed cells. At this point they could be screened by genomic PCR to exclude clones with apparent integrations (see steps 65~72).

Counting the colonies: staining with crystal violet; TIMING 1 d

60. After picking the colonies, aspirate PBS completely from the 100-mm dish and then add 5 ml of methanol to fix the remaining cells. Incubate for 1 min at room temperature.

61. Wash the dishes twice with water.

62. Add 5 ml of 0.1% crystal violet solution into the dish and incubate for 5 min at room temperature.

63. Wash the dishes with water.

64. Photograph the dishes and count the number of colonies.

Examination of genomic integration; TIMING 1 d

65. Aliquot 5 μl of cell lysis buffer per tube of 0.2-ml PCR tube.

66. Aspirate the medium, and wash the cells with 0.5 ml of PBS.

67. Remove PBS, and add 0.5 ml of PBS.

68. Pick a part of colonies (approximately 0.5 μl of volume) from the dish using a Pipetman set at 2 μl, and transfer it into the cell lysis buffer.

69. After picking the colonies, aspirate PBS completely from culture dishes and then add 0.5 ml of ES medium, and return them in 37 ^{o}C, 5% CO_2 incubator until the cells reach 80~90% confluency.

70. Incubate cell lysis buffer at 55 ^{o}C for 4-12 h in humidified chamber.

71. Add 10 μl of distilled water, and incubate at 95 ^{o}C for 3 min to inactivate proteinase.

72. Use 1 μl of the solution for PCR to detect genomic integration. Primer sequences and PCR conditions are listed in table 1. Select clones which do not show obvious integration for

further cultivation (Fig. 4).

TROUBLESHOOTING

Expansion of iPS cells; TIMING 1 h

73. Aspirate the medium, and wash the cells with 1 ml of PBS.

74. Remove PBS completely, add 0.1 ml of 0.25% trypsin/1 mM EDTA and incubate at 37 ^{o}C for 10 min.

75. Add 0.4 ml of the ES medium and suspend the cells by pipetting up and down to single cell suspension.

76. Transfer the cell suspension to a well of 6-well plate with SNL feeder cells (use puromycin-resistant feeder cells for *Nanog*$^{\text{GFP-IRES-Puro}}$), add 1.5 ml ES cell medium, and incubate in 37 ^{o}C, 5% CO_2 incubator. Addition of puromycin (1.5 μg ml^{-1}) support to select highly reprogrammed cells. When the cells reach 80~90% confluency, prepare frozen stock of the cells, as follows.

TROUBLESHOOTING

Preparation of freeze stock; TIMING 1 h

77. Aspirate the medium, and wash the cells with 2 ml of PBS.

78. Remove PBS completely, add 0.3 ml of 0.25% trypsin/1 mM EDTA and incubate at 37 ^{o}C for 10 min.

79. Add 2 ml of the ES medium and suspend the cells by pipetting up and down to single cell suspension.

80. Transfer the cell suspension to a 15-ml tube, count the cell number and spin the cells at 160 *g* for 5 min.

81. Discard the supernatant, and resuspend the cells with ES medium to the concentration at 2 × 10^6 cells per milliliter.

82. Prepare 2 × freezing medium and aliquot it at 0.5 ml per vial.

83. Transfer 0.5 ml of the cell suspension to freeze vials and mix gently.

84. Put the vials in a cell-freezing container and keep it at -80 ^{o}C overnight.

PAUSE POINT For long-term storage, keep frozen cells in the gas phase of a liquid nitrogen tank.

TROUBLESHOOTING

Troubleshooting

PROBLEM

No colonies appear after induction of the four factors.

SOLUTION

The efficiency of colony formation is low with the plasmid-based method. Approximately 10% of experiments can not obtain any colony (including non-ES like colony). Try another experiment. The transfection efficiency of plasmid is important, therefore use high quality of plasmid for the induction, and avoid repetitive freeze-and-thaw cycle to prevent DNA nicking and degradation. We use anion-exchange based column (QIAGEN plasmid kit; QIAGEN) to purify the plasmids, aliquot the plasmids, and store at -20 ^{o}C. Age of fibroblast (passage number) is also critical for iPS generation. We recommend using MEFs within passage 3 for iPS production. Also make sure that your ES medium can maintain mouse ES or iPS cells.

PROBLEM

Too many non-ES-like colonies appear after the induction.

SOLUTION

In some experiments (approximately 10%) more than 500 colonies appeared. It may because transfected plasmid(s) were integrated into fibroblast's genome and transformed them to non-ES-like cells in the early period of the induction. After several rounds of proliferation, they would spread out the 100-mm dish by the passage on day 10. In these cases, we could not obtain iPS clones without apparent integration. Try another experiment.

PROBLEM

Isolated clone is not pluripotent.

SOLUTION

Choose the superior clones by morphologies and marker gene expression. As iPS clones having genomic integration tend to become transformed, select the clones without apparent integration.

PROBLEM

The characters and potentials of iPS cell change during culture.

SOLUTION

iPS cells are less stable than ES cells. Make large amount of freeze stocks of iPS clones at early passages. These stocks should be stored in the vapor phase of liquid nitrogen. The recovery of iPS cells after freezing is approximately 50%. Continuous selection with antibiotics facilitates stability of iPS cells. In some cases, granulated (not ES-like) cells emerge in iPS cell cultures. The loosely attached GFP-negative cells can easily be washed out with PBS or can be removed by antibiotic selection.

ANTICIPATED RESULTS

During the series of plasmid transfection, some floating cells appear because of the cytotoxicity of repeated transfection. However, other cells grow robustly and become confluent around day 10.The transfection efficiency could be more than 40% if the procedure went well. The transfection efficiency can be evaluated by analyzing DsRedExpress-transfected cells with a flow cytometer (Fig. 2). The efficiency and mean expression level are lower than that of retroviral transduction.

We obtained iPS colonies without evidence of integration from 6 out of 10 experiments, but not from 4 experiments including 1 experiment showing no iPS colony formation. The number of colonies in a 100-mm dish is generally small (below 100). The estimated established rate of plasmid-iPS cells without evidence of integration is <0.0002%, which is at least 1000-fold lower than that of viral induction[11]. Although iPS colonies with genomic integration were frequently observed, genomic PCR screening (steps 65~72, Fig. 4) can distinguish them easily. We found that almost one-third of ES-like colonies did not show transgene-derived band in this screening. By using *Nanog*$^{\text{GFP-IRES-Puro}}$ MEF, GFP-positive cells became first apparent around day 19 after the induction (Fig. 3). Some clones did not show fluorescence at the time of colony picking up. However, they became GFP-positive during the culture in 24-well plate.

After picking up and PCR selection, most clones show ES-like proliferation and

morphology, including a round shape, large nucleoli, and scant cytoplasm, in ES cell culture condition. However, some clones show non-ES-like morphology or fail to proliferate. Most clones express ES cell marker gene, such as *Nanog*, *ERas*, *Zfp42*, and *Utf1*[16]. Teratoma developed by subcutaneous injection into nude mice can be used to demonstrate pluripotency of these cells (Fig. 5a-d). Most clones contribute to adult chimeric mice (Fig. 5e) and some of them transmitted through germline.

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Figure legends

Figure 1 Schematic diagram of iPS cell generation with plasmid vectors.

a. Expression vectors for iPS cell generation. pCX-OKS-2A (left) encodes three transcription factors (Oct3/4, Sox2, and Klf4) connected with 2A self-cleaving peptide (2A). The locations of CAG promoter (CAG), polyadenylation signal (pA), and ampicillin resistant gene (Amp^r) are also indicated. The c-Myc expression vector (pCX-cMyc) is on the right. b. Approximate time table of the iPS cell generation.

Figure 2 Transfection efficiency of repeated lipofection.

Expression plasmid encoding DsRedExpress was transfected 4 times with FuGENE6 reagent. Their fluorescence were photographed on day 10 of the iPS induction and examined by flow cytometory. MEF were also infected with pMXs-based retrovirus encoding DsRedExpress on day 2, and were analyzed. Bar indicates 500 μm.

Figure 3 Morphology of iPS colonies just before picking up.

Lower panels showed GFP fluorescence from *Nanog*$^{GFP\text{-}IRES\text{-}Puro}$ locus. Although the right colony barely showed the fluorescence, it became GFP positive after picking up. Bar indicates 500 μm.

Figure 4 PCR screening of genomic integration.

a. Schematic diagram of PCR primers. Open boxes indicate exons of Klf4 (left) and c-Myc (right), and arrows indicate primer sites. The primers of Klf4 are located on 2 exons flanking intron. Therefore they amplify 831 bp from endogenous locus, and 186 bp from transgene. The primers for c-Myc amplify 541 bp from endogenous locus and 237 bp from transgene. b. Detection of genomic integration by PCR. Open and black arrowheads indicate bands from endogenous alleles and transgenes, respectively. Retrovirus induced iPS cells (retro-iPS) were used as control. Some clones (#2, 3, and 8) do not seem to have integration.

Figure 5 Pluripotency of plasmid-iPS cells.

Plasmid-iPS cells developed teratoma when subcutaneously transplanted into nude mice (a-d). Shown are hematoxylin & eosin staining sections of neural tissue (a), gutlike epithelial tissue (b), epidermal tissue (c), and striated muscle (d). Bar indicates 50 μm. e. The iPS cells also contributed to chimeric mouse. iPS cells gave rise to gray hair.

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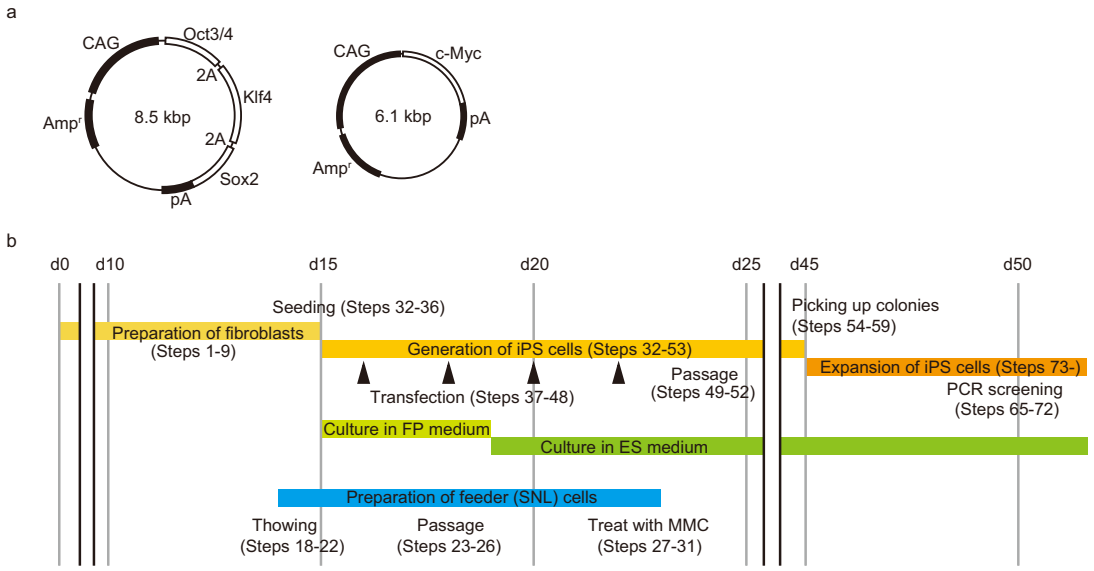
Table 1 PCR conditions

Gene	Primer set	Initial denaturing	Denature	Annealing	Extension	Final Extension
Klf4	GCG GGA AGG GAG AAG ACA CTG CGT C	92˚C	92˚C	64˚C	72˚C	92˚C
	TAG GAG GGC CGG GTT GTT ACT GCT	2 min	20 sec	20 sec	40 sec	3 min
c-Myc	ACA CTC CCC CAA CAC CAG GAC GTT T	92˚C	92˚C	64˚C	72˚C	92˚C
	GCT CGC CCA AAT CCT GTA CCT CGT CCG AT	2 min	20 sec	20 sec	40 sec	3 min
	GAG ATG AGC CCG ACT CCG ACC TCT T					

Fig. 1 Okita et al.



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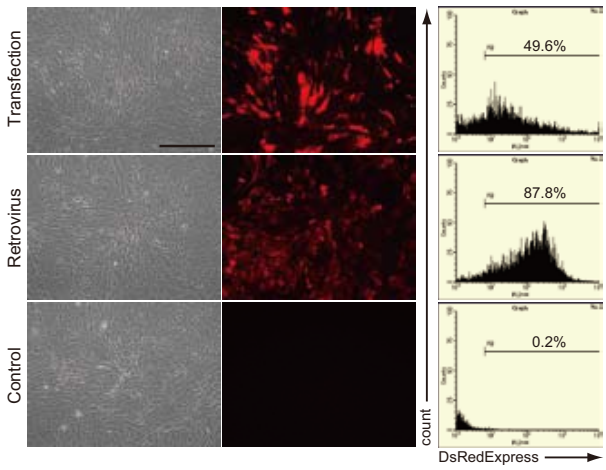

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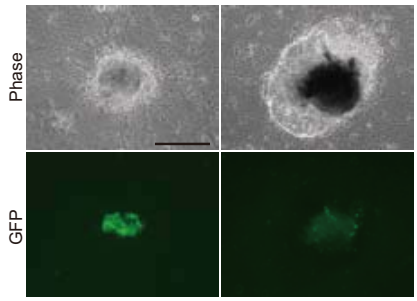

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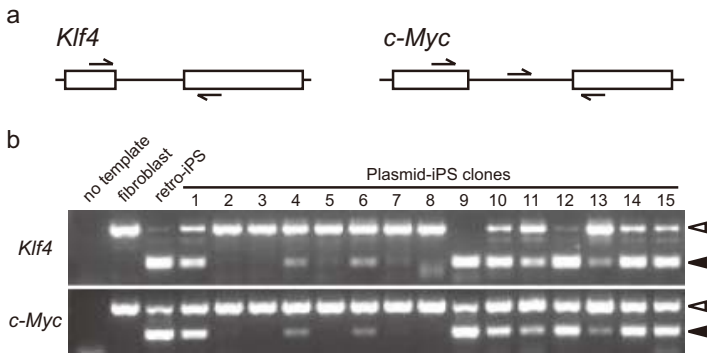

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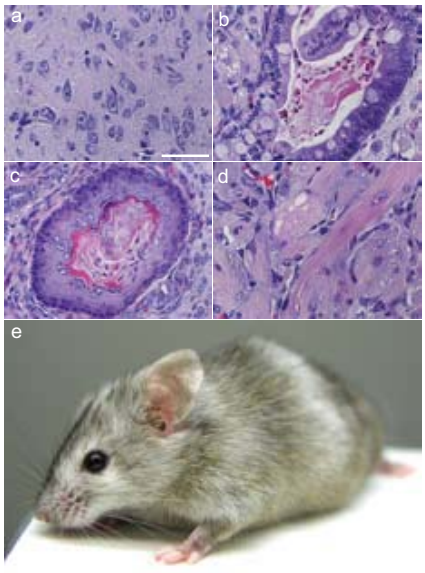
Fig. 5 okita et al.



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