

Directed differentiation of human pluripotent stem cells into intermediate mesoderm

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Abstract

The induction of kidney lineage cells from human pluripotent stem cells (hPSCs) has been previously described in some reports, but there have been no protocols so far for the robust induction of intermediate mesoderm (IM), an early embryonic germ layer that gives rise to kidneys. In the present study, we have established a robust induction protocol using combinational treatment of growth factors, which produces up to 90% IM cells expressing the IM-specific marker OSR1. This novel method thus supplies an unprecedented system to elucidate the mechanisms of IM development and potentially provide a cell source for kidney regeneration.

Introduction

The kidneys have highly differentiated and complicated structures, and play critical roles in many physiological processes. Once damaged, kidneys rarely recover their functions. To overcome this problem, kidney regeneration using embryonic stem cells (ESCs)¹⁻³ or induced pluripotent stem cells (iPSCs)⁴⁻⁶, which have an unlimited self-renewal capability and the potential to differentiate into any cell type in the body, is a valuable alternative. Kidneys are derived from IM, and Odd-skipped related 1 (Osr1) is one of the earliest markers specific for IM, although the expression also extends into the lateral plate mesoderm at early stages in mouse, chick and fish embryos⁷⁻⁹. The differentiation of PSCs into OSR1-expressing IM cells is the first step toward the induction of renal lineage cells¹⁰. In this protocol, we describe how to efficiently induce OSR1⁺ IM cells from hPSCs.

Reagents

- SNL feeder cells (Available from Dr. Allan Bradley of the Sanger Institute or European Collection of Cell Cultures)
- hPSCs
- Primate ES medium (ReproCELL, cat. no. RCHEMD001)
- DMEM (Nacalai Tesque, cat. no. 08459-35)
- DMEM/F12+Glutamax (Invitrogen, cat. no. 10565-018)
- FBS (HyClone, cat. no. SH30070.03)
- Knockout serum replacement (KSR, Invitrogen, cat. no. 10828)
- Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)

- Non-essential amino acids (Invitrogen, cat. no. 11140-050)
- 2-mercaptoethanol (Invitrogen, cat. no. 21985-023)
- 0.25% trypsin (Invitrogen, cat. no. 25200-056)
- 0.1% collagenase IV (Invitrogen, cat. no. 17104-019)
- CaCl₂ (Nacalai Tesque, cat. no. 08894-25)
- Accutase (Innovative Cell Technologies, Inc., cat. no. AT104)
- PBS (-) (Nacalai Tesque, cat. no. 14249-95)
- Recombinant human basic fibroblast growth factor (bFGF, Wako, cat. no. 064-04541)
- Y27,632 (Wako, cat. no. 253-00513)
- Recombinant human/mouse/rat activin A (R&D Systems, cat. no. 338-AC)
- Recombinant mouse Wnt3a (R&D Systems, cat. no. 1324-WN)
- CHIR99021 (Wako, cat. no. 039-20831)
- Recombinant human BMP7 (R&D Systems, cat. no. 354-BP)
- Matrigel (BD, cat. no. 354230)
- Human Collagen Type I (BD, cat. no. 354265)
- Gelatin, from porcine skin (SIGMA, cat. no. G1890)

All media should be sterilized by 0.22 µm filtration.

- hPSC medium for maintenance culture

Primate ES medium supplemented with 500 U/ml penicillin/streptomycin and 4 ng/ml recombinant human bFGF

- Stage 1 medium

DMEM/F12+Glutamax supplemented with 500 U/ml penicillin/streptomycin and 2% FBS

- Stage 2 medium

DMEM/F12+Glutamax supplemented with 0.1 mM non-essential amino acids, 500 U/ml penicillin/streptomycin, 0.55 mM 2-mercaptoethanol and 10 % KSR

- CTK dissociation solution

PBS (-) supplemented with 0.25% trypsin, 0.1% collagenase IV, 20% KSR and 1 mM CaCl₂

Equipment

- CO2 incubator with controlling and monitoring system for CO2, humidity and temperature
- Biosafety cabinet for cell culture
- Cell culture centrifuge
- Cell culture disposables: Petri dishes, multi-well plates, centrifuge tubes, pipettes, pipette tips, and cell scrapers

Procedure

Overview

Steps 1 – 9: Maintenance of hPSCs

Steps 10 – 22: Induction of IM cells by embryoid body (EB) formation (EB method)

Steps 23 – 29: Induction of IM cells by colony formation (Colony method)

Steps 30 – 40: Induction of IM cells with single cell dissociation (Single cell method)

Maintenance of hPSCs

Preparation of SNL feeder cells

1. Partially thaw the frozen vial of mitotically inactivated SNL feeder cells in 37 °C water bath, resuspend the 1 ml cell solution in 10 ml DMEM/10 % FBS medium, and spin down at approximately 160 g for 5 min.
2. Remove the supernatant and resuspend the pellet in 10 ml DMEM/10 % FBS medium.
3. Plate the cell suspension onto gelatin-coated plates at a density of approximately 50,000 cells/cm² and incubate overnight. Passage of hPSCs
4. Remove differentiated hPSCs using aseptic technique.
5. Aspirate old hPSC medium from plate, wash with 5 ml PBS (-), and add warm CTK dissociation solution (1 ml/10cm plate).
6. Incubate at 37 °C for 3 min.
7. Wash out SNLs twice with 5 ml PBS (-).
8. Harvest the hPSC colonies in 5 ml fresh hPSC medium by using a cell scraper.

9. Pipette and triturate the hPSC colonies in hPSC medium using P1000 pipetman and split onto new feeder cells at a ratio between 1:3 and 1:6.

Induction of IM cells by embryoid body (EB) formation (EB method)

10. Repeat steps 4 - 7.
11. Add 5 ml hPSC medium and scrape hPSCs off the surface with a cell scraper, then pipet the cells up and down 5-10 times using P1000 pipetman.
12. Plate the suspension onto gelatin-coated plate and incubate at 37 °C for 30 min to remove SNLs.
13. Transfer the hPSC cell solution to 15ml tube and allow cells to settle via gravity.
14. Remove the supernatant and resuspend with 10 ml Stage 1 medium, then allow cells to settle via gravity.
15. Remove the supernatant and resuspend with 8 ml Stage 1 medium containing 100 ng/ml recombinant human/mouse/rat activin A and 100 ng/ml recombinant mouse Wnt3a or 3 µM CHIR99021.
16. Distribute the cell suspension into a low attachment 6-well plate (2 ml/well) to form EB. Culture at 37 °C for 2 days.
17. Transfer the EB solution to a 15ml tube and allow EBs to settle via gravity.
18. Remove the supernatant and resuspend with 10 ml Stage 2 medium, then allow cells to settle via gravity.
19. Remove the supernatant and resuspend with 8 ml Stage 2 medium containing 100 ng/ml recombinant human BMP7 and 100 ng/ml recombinant mouse Wnt3a or 3 µM CHIR99021.
20. Transfer the cell solution onto a gelatin-coated 6-well plate (2 ml/well).
21. Change medium with BMP7 and Wnt3a or CHIR99021 every 2 - 3 days and culture at 37 °C for 4 - 20 days.

Induction of IM cells by colony formation (Colony method)

22. Repeat steps 10 - 14.
23. Remove the supernatant, resuspend with 12 ml SNL-conditioned hPSC medium containing 10 ng/ml recombinant human bFGF and seed on Matrigel-coated 24-well plate (500 μ l/well).
24. Grow the cells until hPSC colonies reach approximately 70% confluency.
25. Aspirate the medium and wash twice with PBS (-).
26. Add Stage 1 medium containing 100 ng/ml recombinant human/mouse/rat activin A and 100 ng/ml recombinant mouse Wnt3a or 3 μ M CHIR99021 (500 μ l/well) and culture at 37 °C for 2 days.
27. Aspirate the medium and wash with Stage 2 medium.
28. Add Stage 2 medium containing 100 ng/ml recombinant human BMP7 and 100 ng/ml recombinant mouse Wnt3a or 3 μ M CHIR99021 (500 μ l/well).
29. Change medium with BMP7 and Wnt3a or CHIR99021 every 2 - 3 days and culture for 8 days.

Induction of IM cells with single cell dissociation (Single cell method)

30. Repeat steps 4 - 7.
31. Add 2 ml warm Accutase.
32. Incubate at 37 °C for 6 min.
33. Pipet and dissociate the hPSC colonies into single cells using P1000 pipetman.
34. Add 8 ml Stage 1 medium and pipet the cells up and down using P1000 pipetman.
35. Count the cell number.
36. Transfer the hPSC cell solution to 15ml tube and spin down at 160 g for 5 min.
37. Remove the supernatant and resuspend with Stage 1 medium containing 100 ng/ml recombinant human/mouse/rat activin A, 100 ng/ml recombinant mouse Wnt3a or 3 μ M CHIR99021, and 10 μ M Y27,632 at a density of 6.0 X 10⁵ cells/ml.

38. Distribute into Human Collagen Type I-coated 24-well plates at a density of 1.5×10^5 cells/cm² (500 μ l/well) and culture at 37 °C for 2 days.
39. Aspirate the medium and wash with warm Stage 2 medium.
40. Add Stage 2 medium containing 100 ng/ml recombinant human BMP7 and 100 ng/ml recombinant mouse Wnt3a or 3 μ M CHIR99021 (500 μ l/well).
41. Change medium with BMP7 and Wnt3a or CHIR99021 every 2 - 3 days and culture at 37 °C for 8 days.

Troubleshooting

(a) No formation of EBs

Check the size and the density of hPSC colonies

Do not form smaller EBs

Check the concentration of activin A and Wnt3a or CHIR99021

Check for contamination of Mycoplasma species in hPSCs or SNLs

Change the batch of hPSCs or SNLs

(b) Low attachment of EBs

Check the gelatin-coated plate

Matrigel-coated plate is better for attachment

Check the concentration of BMP7 and Wnt3a or CHIR99021

(c) Low survival rate of the cells in Colony method

Check the size and the density of hPSC colonies

Check the concentration of activin A, BMP7 and Wnt3a or CHIR99021

Check for contamination of Mycoplasma species in hPSCs or SNLs

Change the batch of hPSCs or SNLs

(d) Low survival rate of the differentiated cells in Single cell method

Check the plating density of hPSCs

Do not incubate in Accutase for an excessive amount of time during the single cell dissociation step

Check the activity of Y27,632

Check the concentration of activin A, BMP7 and Wnt3a or CHIR99021

Check for contamination of Mycoplasma species in hPSCs or SNLs

Change the batch of hPSCs or SNLs

Anticipated Results

The temporal expression pattern of OSR1 will show a gradual increase, with a peak at culture day 19 with the EB method and at day 11 with the Single cell method (Figure 1a, b). The Single cell method produces up to 90% OSR1+ cells (Figure 1c). These human intermediate mesoderm (IM) cells can differentiate into multiple cell types constituting IM-derived organs, such as kidney, adrenal cortex and

gonad, in vitro (Figure 2).

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Figures

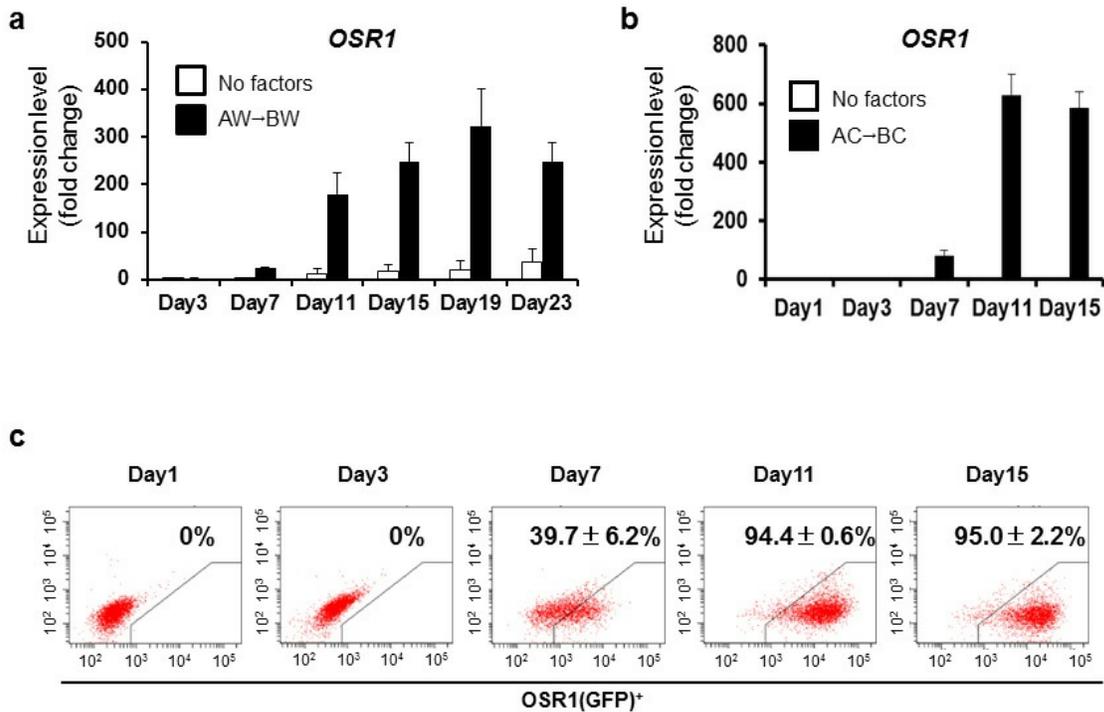


Figure 1

Differentiation of hiPSCs into the intermediate mesoderm (IM). (a, b) The time course analysis of OSR1 expression in the differentiation culture with EB method (a) and Single cell method (b). Each value was normalized to the samples on day 1 before treatments. Note that the OSR1 expression of samples on culture day 3 in a and on culture days 1 and 3 and without factors in b is so low that the histogram bars are at the baseline. White bars: no factors; black bars (AW→BW): Stage 1, 100 ng/ml activin A + 100 ng/ml Wnt3a, Stage 2, 100 ng/ml BMP7 + 100 ng/ml Wnt3a. (AC→BC): Stage 1, 100 ng/ml activin A + 3 μ M CHIR99021, Stage 2, 100 ng/ml BMP7 + 3 μ M CHIR99021. (c) The temporal differentiation pattern of OSR1+ cells using the Single cell method. The data from three independent experiments are presented as the means \pm SD (n=3) in a-c.

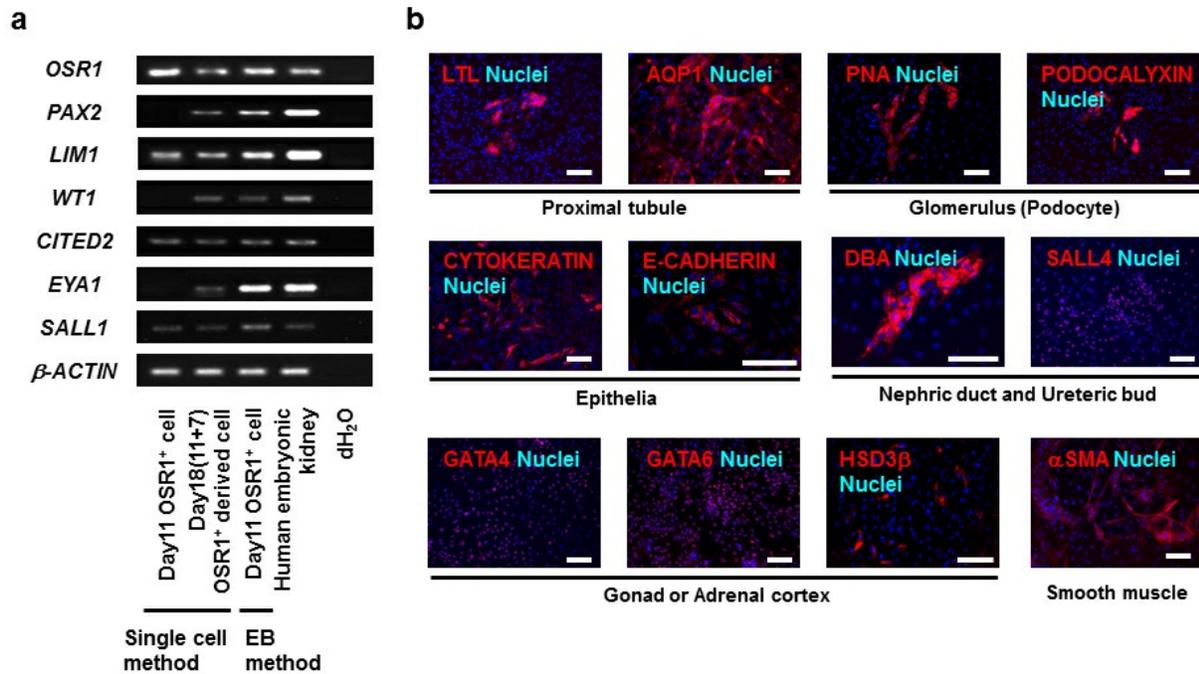


Figure 2

In vitro differentiation of OSR1+ cells into IM derivatives. (a) RT-PCR analyses of marker gene expression for IM or kidney lineage in isolated OSR1+ cells on culture day 11 using two induction protocols (EB and Single cell methods), and the differentiated cells on day 18 (after an additional 7-day culture of the day 11 isolated OSR1+ cells generated with the single cell method). (b) The differentiated cells on day 18 were stained with antibodies or lectins against markers for IM derivatives: Lotus Tetragonobulus lectin (LTL) and Aquaporin (AQP1) for proximal renal tubule, Peanut agglutinin (PNA) and PODOCALYXIN for glomerular podocytes, CYTOKERATIN and E-CADHERIN for renal epithelia, Dolichos biflorus agglutinin (DBA) and SALL4 for nephric duct and ureteric bud, GATA4, GATA6 and HSD3 β for the gonads or adrenal cortex, and α SMA for smooth muscle. Scale bars, 100 μ m.

Monitoring and robust induction of nephrogenic intermediate

mesoderm from human pluripotent stem cells

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