

Chapter 2

Generation of a Three-Dimensional Retinal Tissue from Self-Organizing Human ESC Culture

Atsushi Kuwahara, Tokushige Nakano, and Mototsugu Eiraku

Abstract

A three-dimensional (3D) tissue generated in vitro is a promising source to study developmental biology and regenerative medicine. In the last decade, Yoshiki Sasai's group have developed a 3D stem cell culture technique known as SFEBq and demonstrated that embryonic stem cells (ESCs) have an ability to self-organize stratified neural tissue including 3D-retina. Furthermore, we have reported that ESC-derived retinal tissue can form an optic cup and a ciliary margin, which are unique structures in the developing retina. In this review, we focus on self-organizing culture technique to generate 3D-retina from human ESCs.

Key words SFEBq culture, Human ESCs, Retina, Neural retina, RPE, Optic cup, Ciliary margin

1 Introduction

The retina is the main visual sensory tissue in mammals. During retinal development, the optic cup derived from the rostral diencephalon is composed of the inner and outer walls that differentiate into neural retina (NR) and retinal pigment epithelium (RPE), respectively. Yoshiki Sasai's group pioneered methodology for inducing 3D neural tissues from embryonic stem cells (ESCs) (for review *see* [1, 2]). We have previously reported that embryonic stem cells (ESCs) have an ability to self-organize stratified 3D-retina by using a self-organizing stem cell culture technique known as SFEBq [3]. In this study, we also demonstrated the emergence of an optic cup, a unique structure in the developing retina. We then applied this mouse ESC culture technique to human ESCs and generated human 3D-retina and an optic cup [4]. We further developed a hESC-differentiation culture technique, named induction-reversal culture method, to generate human ciliary margin-like retinal stem cell niche [5]. Importantly, 3D-retina generated by these culture methods is now studying to apply in regenerative medicine field [6–8]. Since we have published a

detailed protocol for mESC differentiation [9], in this review we focus on our recent advances in the retinal differentiation culture method of hESCs.

2 Materials

1. Low-cell adhesion 96-well plates with V-bottomed conical wells (96-well V-bottomed plate).
2. 90-mm Petri Dishes for suspension cell culture (Floating culture dish).
3. Knockout Serum Replacement (KSR; *see Note 1*).
4. hESC maintenance medium: DMEM/F-12 (suitable for 2% CO₂ culture) supplemented with 20% (vol/vol) KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin. Filter the medium with a 0.2-µm filter bottle, store at 4 °C, and use within 2 weeks. Add 7.5 ng/mL bFGF freshly on the day of use.
5. Basic fibroblast growth factor (bFGF): To prepare a stock solution at 100 µg/mL, reconstitute 50 µg of bFGF in 500 µL of hESC maintenance medium. Store small aliquots at −20 °C for 3 months. To prepare the working solution (0.75 µg/mL), dilute in the hESC maintenance medium. Store the working solution at 4 °C for 3 weeks. Avoid freeze thaw cycle.
6. hESC dissociation solution: 0.25% (wt/vol) trypsin and 1 mg/mL collagenase IV in PBS containing 20% (vol/vol) KSR and 1 mM CaCl₂. Sterilize the solution by filtering through a 0.2-µm filter. Store small aliquots at −20 °C for several months.
7. GMEM differentiation medium: GMEM supplemented with 20% (vol/vol) KSR, 0.1 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin. Filter the solution with a 0.2-µm filter bottle, store at 4 °C, and use within 3 weeks.
8. gfCDM + KSR medium: growth-factor-free CDM (gfCDM) supplemented with 10% KSR medium, while gfCDM contains 45% Iscove's modified Dulbecco's medium (IMDM), 45% Ham's F12 (F12), Glutamax, 1% chemically defined lipid concentrate, monothioglycerol (450 µM), 100 U/mL penicillin, and 100 µg/mL streptomycin [10].
9. RPE-induction medium: DMEM/F-12-Glutamax medium supplemented with 1% (vol/vol) N2 supplement, 100 U/mL penicillin, and 100 µg/mL streptomycin. Filter the solution with a 0.2-µm filter bottle, store at 4 °C, and use within 2 weeks. Add CHIR99021 (3 µM) and SU5402 (5 µM) freshly on the day of use.

10. Retina maturation medium: DMEM/F-12-Glutamax medium supplemented with 1% (vol/vol) N2 supplement, 10% (vol/vol) FBS, 0.5 μ M retinoic acid, 0.1 mM taurine, 0.25 μ g/mL Fungizone, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Filter the solution with a 0.2- μ m filter bottle, store at 4 °C, and use within 2 weeks.
11. Gelatin solution: To prepare gelatin solution (0.1%, wt/vol), dissolve 0.5 g of gelatin in 500 mL of water by autoclaving. The solution can be stored at 4 °C for up to 3 months.
12. DNase I: To prepare a stock solution at 10 mg/mL, dissolve DNase I in PBS. Store small aliquots at –20 °C for several months.
13. Y-27632 (ROCK inhibitor) [11]: To prepare a stock solution at 10 mM, reconstitute Y-27632 in H₂O. Store small aliquots at –20 °C for several months.
14. Matrigel (growth factor-reduced): Thaw Matrigel overnight at 4 °C. Keep Matrigel on ice and make aliquots in 2 mL tubes using precool P1000 tips. Store small aliquots at –20 °C for several months (*see Note 2*).
15. IWR-1-endo (Wnt inhibitor): To prepare a stock solution at 10 mM, reconstitute IWR-1-endo in DMSO. Store small aliquots at –20 °C for several months.
16. Smoothed agonist (SAG): To prepare a stock solution at 10 mM, reconstitute SAG in DMSO. Store small aliquots at –20 °C for several months. To prepare the working solution (100 μ M), dilute the stock in PBS. Store the working solution at 4 °C for 1 month.
17. Recombinant human BMP4 (BMP4): To prepare a stock solution at 1 μ M, reconstitute 50 μ g of BMP4 in 1375 μ L of 0.1% BSA/PBS. Store small aliquots at –20 °C for 3 months. Store aliquots at 4 °C for 3 weeks. Avoid freeze thaw cycle.
18. CHIR99021 (GSK3 inhibitor; CHIR): To prepare a stock solution at 10 mM, reconstitute CHIR99021 in DMSO. Store small aliquots at –20 °C for several months.
19. SU5402 (FGFR inhibitor): To prepare a stock solution at 10 mM, reconstitute SU5402 in DMSO. Store small aliquots at –20 °C for several months.
20. All trans retinoic acid (RA): Prepare 100 mM stock solution in DMSO. Store small aliquots at –80 °C for several months. To prepare the working solution (3.3 mM), dilute the 100 mM stock in EtOH. Store the working solution at –20 °C for several months.
21. Taurine: Prepare 50 mM stock solution in PBS. Store small aliquots at –20 °C for several months.

3 Methods

3.1 Maintenance of Human ESCs

Undifferentiated hESCs are maintained on a feeder layer of mouse embryonic fibroblasts (MEF) inactivated by mitomycin C treatment in ESC maintenance medium under 2%-CO₂ conditions. For passaging, hESC colonies are detached with hESC dissociation solution and broken into smaller pieces by gentle pipetting. The passages are performed at a 1:3–1:5 split ratio every third or fourth days [5, 12].

3.1.1 Preparation of MEF Feeder-Layer Dish

1. Add 6 mL Gelatin solution (0.1%, wt/vol) in a tissue culture dish (100 mm).
2. Stand for 0.5–2 h at 37 °C.
3. Thaw the inactivated MEF stock and centrifuge it in a centrifuge tube.
4. Add 8 mL MEF medium and plate on gelatin-coated culture dish.
5. Incubate for 4–48 h at 37 °C.

3.1.2 Passage

1. Wash twice MEF feeder-layer dish with 10 mL PBS and incubate in hESC maintenance medium (w/o bFGF) at 37 °C.
2. Prepare 70% confluent hESCs cultured on a MEF feeder-layer dish (100 mm).
3. Aspirate hESC maintenance medium from 70% confluent hESCs, wash twice with 10 mL PBS, and then aspirate.
4. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
5. Add hESC maintenance medium (w/o bFGF) and detach en bloc from the feeder layer by pipetting.
6. Break hESC clumps into smaller pieces (several dozens of cells) by gentle pipetting.
7. Plate hESC clumps onto fresh feeder-layer dish (1:3–1:5 split ratio).
8. Culture in hESC maintenance medium supplemented with bFGF (7.5 ng/mL) at 37 °C under 2%-CO₂ conditions.

From the next day, change 10 mL hESC maintenance medium (+bFGF) every day and passage the cells every third or fourth days (60–70% confluent).

3.2 Generation of Retinal Progenitors from hESCs by Using “ECM-Addition Method” (Days 0–18)

Prepare hESCs on feeder layers grown to ~70% of confluency (Subheading 3.1, *see Note 3*). Undifferentiated hESCs can differentiate into retinal progenitors by using “extracellular matrix (ECM)-addition method” as described previously [4]. On culture day 18, aggregates contain retinal epithelium. The percentage of retinal progenitor marker Rx is typically around 60% as determined by FACS [4, 5].

Day 0: Plating

1. Prepare 70% confluent hESCs cultured on a MEF feeder-layer dish (100 mm).
2. Prepare gelatin-coated culture dish (100 mm).
3. Aspirate hESC maintenance medium from 70% confluent hESCs, wash twice with 10 mL PBS, and then aspirate.
4. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
5. Add hESC maintenance medium (w/o bFGF) and detach en bloc from the feeder layer by pipetting.
6. Plate hESC clumps in 6 mL hESC maintenance medium (w/o bFGF) supplemented with 10 μ M Y-27632 on a gelatin-coated dish.
7. Incubate at 37 °C for 1.0–1.5 h to adhere contaminated MEF cells to the dish bottom.
8. Collect the medium containing the floating ESC clumps from the dish and transfer into a 15 mL conical tube.
9. Centrifuge at $180 \times g$ for 3 min at 25 °C, remove the supernatant, and suspend with 10 mL of PBS.
10. Centrifuge at $180 \times g$ for 3 min at 25 °C and remove the supernatant.
11. Dissociate hESC clumps into single cells by using TrypLE Express supplemented with 20 μ M Y-27632 and 0.05 mg/mL DNase I.
12. Centrifuge at $180 \times g$ for 3 min at 25 °C and remove the supernatant.
13. Resuspend the cells in the GMEM differentiation medium.
14. Count the number of cells using a cell counter.
15. Adjust the concentration to 9.0×10^4 cells per mL in the GMEM differentiation medium supplemented with 20 μ M Y-27632 and 3 μ M IWR-1-endo.
16. Plate hESCs into a 96-well low-adhesion V-bottomed plate (9000 cells per 100 μ L per well) (*see Note 4*).
17. Culture at 37 °C under 5%-CO₂ conditions.

Define the day on which the SFEBq culture is started as day 0.

Day 2

18. On culture day 2, add 50 μ L GMEM differentiation medium supplemented with 3 μ M IWR-1-endo and 3% Matrigel to each well (3 μ M IWR-1-endo and 1% Matrigel at final concentration).

From days 6 to 12, change the medium containing 3 μ M IWR-1-endo and 1% Matrigel every 3–4 days.

Day 12

19. On culture day 12, transfer the floating aggregates to a 90-mm floating culture dish. Culture in suspension in the GMEM differentiation medium supplemented with 10% FBS, 100 nM SAG, and 1% Matrigel at 37 °C under 5%-CO₂ conditions.

From days 12 to 18, change the medium containing 10% FBS, 100 nM SAG, and 1% Matrigel every 3–4 days.

Day 18

20. On culture day 18, transfer the floating aggregates to a 90-mm floating culture dish and further culture in NR-selective culture, RPE-selective culture or induction-reversal culture (*see below*).

3.3 Generation of Optic Cups from hESCs by Using “ECM-Addition Method” (Days 12–30)

Prepare hESCs on feeder layers grown to ~70% of confluency (Subheading 3.1, *see Note 3*). Undifferentiated hESCs can differentiate into optic cups by using “ECM-addition method” as described previously [4]. Prepare the floating aggregates on culture day 12 as described (**steps 1–18** in Subheading 3.2).

Days 12–30

1. On culture day 12, transfer the floating aggregates to a 90-mm floating culture dish. Culture in suspension in GMEM differentiation medium supplemented with 10% FBS and 1% Matrigel at 37 °C under 5%-CO₂ conditions.
2. On culture day 15, culture the aggregates in the GMEM differentiation medium supplemented with 3 μ M CHIR, 100 nM SAG, 10% FBS, and 1% Matrigel at 37 °C under 5%-CO₂ conditions.
3. On culture day 18, culture the aggregates in the DMEM/F-12-Glutamax medium supplemented with 1% (vol/vol) N2 supplement, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37 °C under 5%-CO₂ conditions.

From days 12 to 30, change the medium every 3–4 days.

3.4 Generation of Retinal Progenitors from hESCs by “BMP Method” (Days 0–18)

Prepare hESCs on feeder layers grown to ~70% of confluency (Subheading 3.1, *see Note 3*). Undifferentiated hESCs can differentiate into retinal progenitors by using “BMP method” as described previously [5]. On culture day 18, aggregates contain retinal epithelium. The percentage of retinal progenitor marker Rx is typically around 80% as determined by FACS.

Day 0: Plating

1. Prepare 70% confluent hESCs cultured on a MEF feeder-layer dish (100 mm).
2. Prepare gelatin-coated culture dish (100 mm).
3. Aspirate hESC maintenance medium from 70% confluent hESCs, wash twice with 10 mL PBS, and then aspirate.
4. Add 1.5 mL ESC dissociation solution and incubate for 7–8 min at 37 °C.
5. Add hESC maintenance medium (w/o bFGF) and detach en bloc from the feeder layer by pipetting.
6. Plate hESC clumps in 6 mL hESC maintenance medium (w/o bFGF) supplemented with 10 μ M Y-27632 on a gelatin-coated dish.
7. Incubate at 37 °C for 1.0–1.5 h to adhere contaminated MEF cells to the dish bottom.
8. Collect the medium containing the floating ESC clumps from the dish and transfer into a 15 mL conical tube.
9. Centrifuge at $180 \times g$ for 3 min at 25 °C, remove the supernatant, and suspend with 10 mL of PBS.
10. Centrifuge at $180 \times g$ for 3 min at 25 °C and remove the supernatant.
11. Dissociate hESC clumps into single cells by using TrypLE Express supplemented with 20 μ M Y-27632 and 0.05 mg/mL DNase I.
12. Centrifuge at $180 \times g$ for 3 min at 25 °C and remove the supernatant.
13. Resuspend the cells in gfCDM + KSR medium.
14. Count the number of cells using a cell counter.
15. Adjust the concentration to 1.2×10^5 cells per mL in gfCDM + KSR medium supplemented with 20 μ M Y-27632. Concentration of Y-27632 is diluted into half by half medium change every 3–4 days.
16. Plate hESCs into a 96-well low-adhesion V-bottomed plate (12,000 cells per 100 μ L per well) (*see Note 4*).
17. Culture at 37 °C under 5%-CO₂ conditions.

Define the day on which the SFEBq culture is started as day 0. Add 50 μ L gfCDM + KSR medium on day 2 or 3 (150 μ L per well at final volume).

Day 6

18. On culture day 6, change medium with gfCDM + KSR medium supplemented with 3 nM BMP4 to each well (1.5 nM (55 ng/

mL) BMP4 at final concentration) (*see Note 5*). From days 6 to 18, change the medium with gfCDM + KSR medium every 3–4 days. Concentration of BMP4 is diluted into half by half medium change every 3–4 days.

Day 18

19. On culture day 18, transfer the floating aggregates to a 90-mm floating culture dish and further culture in NR-selective culture, RPE-selective culture, or induction-reversal culture (*see below*).

BMP addition promotes differentiation of hESCs to Rx⁺/Chx10⁺ retinal progenitors (Fig. 1).

3.5 Generation of Multilayered NR-Tissue from hESCs in NR-Selective Culture Condition (Days 18–60)

Prepare hESC-derived retinal progenitors by using ECM-addition method (Subheading 3.2) or BMP method (Subheading 3.4). On culture day 18, retinal progenitors form retinal epithelium and can differentiate into multilayered NR-tissue on day 35 by culturing in Retina maturation medium, which is DMEM/F-12-Glutamax medium supplemented with 1% N2 supplement, 10% FBS, 0.5 μ M retinoic acid, 0.1 mM taurine, 0.25 μ g/mL Fungizone, 100 U/mL penicillin, and 100 μ g/mL streptomycin [4, 5].

Day 18

1. On culture day 18, transfer the floating aggregates to 90-mm floating culture dish and further culture in Retina maturation medium under 40%-O₂/5%-CO₂ conditions.
From days 18 to 60, change the medium every 3–4 days.
2. (optional) On culture days 18–35, transfer the aggregates to Cell culture dish and dissect the NR-like tissue with fine forceps and scissors under a stereo microscope [4]. Return dissected aggregates to the 90-mm floating culture dish with fresh Retina maturation medium.

3.6 Generation of RPE-Like Tissue from hESCs in RPE-Selective Culture Condition (Days 18–35)

Prepare hESC-derived retinal progenitors by using the ECM-addition method (Subheading 3.2) or the BMP method (Subheading 3.4). On culture day 18, retinal progenitors form retinal epithelium and can differentiate into RPE-tissue on day 35 by culturing in the RPE-induction medium, which is DMEM/F-12-Glutamax medium supplemented with 1% N2 supplement, 100 U/mL penicillin, 100 μ g/mL streptomycin, 3 μ M CHIR99021, and 5 μ M SU5402 [4, 5].

Day 18

1. (optional) On culture day 18, transfer the aggregates to Cell culture dish and dissect the NR-like tissue with fine forceps and scissors under a stereo microscope. Return dissected aggregates to the 90-mm floating culture dish with fresh RPE-induction medium.

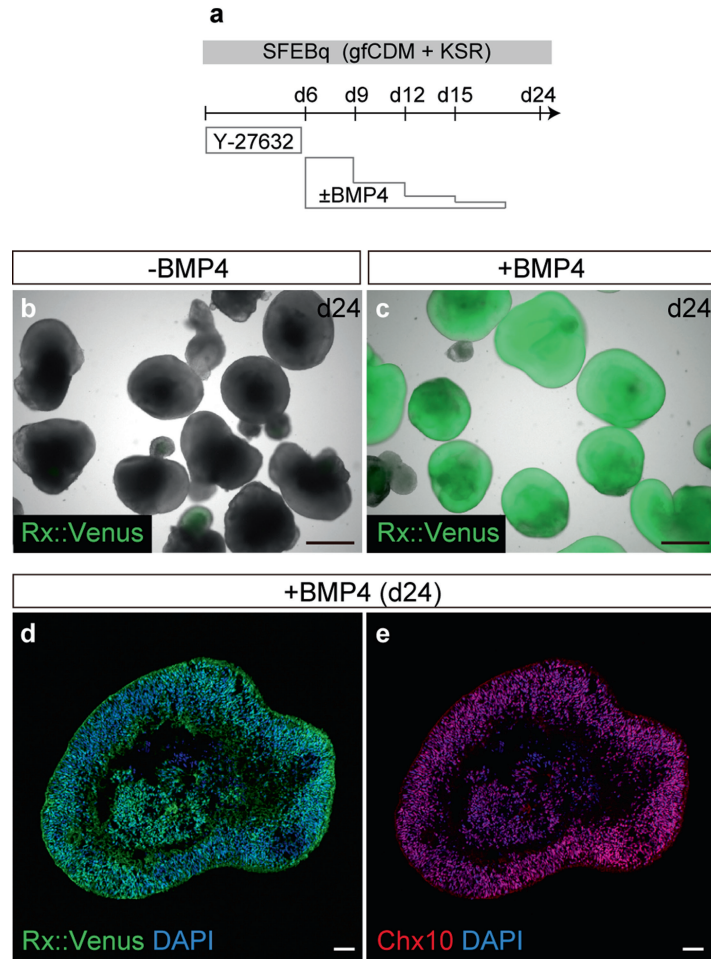


Fig. 1 Selective NR generation in self-organizing hESC culture by BMP method. **(a)** Timing of BMP4 treatment. BMP4 (1.5 nM) was added to medium on day 6, while its concentration was diluted into half by half medium change on days 9, 12, and 15. **(b, c)** Induction of Rx::Venus by transient BMP4 treatment (c; b, untreated control) in hESC aggregates. **(d, e)** Immunostaining of NR tissue (day 24) generated by BMP method with antibodies for Venus **(d)** and Chx10 **(e)**. Blue, nuclear staining with DAPI. Modified from Kuwahara et al. *Nat Commun* 2015

- On culture day 18, transfer the floating aggregates to 90-mm floating cell culture dish and further culture in RPE-induction medium under 5%-CO₂ conditions.

From days 18 to 24, change the medium every 3–4 days with RPE-induction medium.

Day 24

- On culture day 24, transfer the floating aggregates to 90-mm floating cell culture dish and further culture under 5%-CO₂ conditions in RPE-differentiation medium, which is DMEM/

F-12-Glutamax medium supplemented with 1% N2 supplement, 100 U/mL penicillin, 100 µg/mL streptomycin, 3 µM CHIR99021, and 1% FBS. From days 24 to 35, change the medium every 3–4 days with RPE-differentiation medium.

3.7 Generation of Ciliary Margin-Like Tissue from hESCs by “Induction-Reversal Culture Method” (Days 18–150)

Prepare hESC-derived retinal epithelium progenitors by using ECM-addition method (Subheading 3.2) or BMP method (Subheading 3.4). Culturing retinal epithelium in RPE-induction medium from days 18 to 24 induces transition from NR-fate into RPE-fate. Then, culturing in retina maturation medium from days 24 to 35 facilitates reversion of RPE-biased epithelium back to NR-fate. This step-wise “induction-reversal culture method” generates both RPE and NR in the same aggregate (turnip-shaped aggregate, Fig. 2d). Then, NR-RPE tissue boundary in turnip-shaped aggregate self-forms a ciliary margin-like tissue on culture day 63 [5].

Day 18

1. (optional) On culture day 18, transfer the aggregates to Cell culture dish and dissect the NR-like tissue with fine forceps and scissors under a stereo microscope.
2. For RPE-induction culture, transfer the floating aggregates (day 18) to a 90-mm floating culture dish and further culture in RPE-induction medium (Subheading 3.6) under 5%-CO₂ conditions (*see* **Note 6**).

From days 18 to 24, change the medium with RPE-induction medium every 3–4 days.

Day 24

3. For NR-reversal culture, transfer the floating aggregates (day 24) to a 90-mm floating culture dish and further culture in Retina maturation medium under 40%-O₂/5%-CO₂ conditions (Subheading 3.5).

From days 24 to 150, change the medium with Retina maturation medium every 3–4 days.

Multilayered stratified NR is often formed near the ciliary margin-like tissue (Fig. 2e).

3.8 Neurosphere Culture from hESC-Derived Ciliary Margin-Like Tissue (Day 60)

Prepare hESC-derived ciliary margin-like tissue by using the induction-reversal culture method (Subheading 3.7). Cells in ciliary margin-like tissue can form neurospheres after culturing in retinospere medium, which is DMEM/F12-Glutamax medium supplemented with 2% B27 supplement (without vitamin A), 20 ng/mL human bFGF, 20 ng/mL human EGF, 5 µg/mL Heparin, 0.25 µg/mL Fungizone, 100 U/mL penicillin, and 100 µg/mL streptomycin [5].

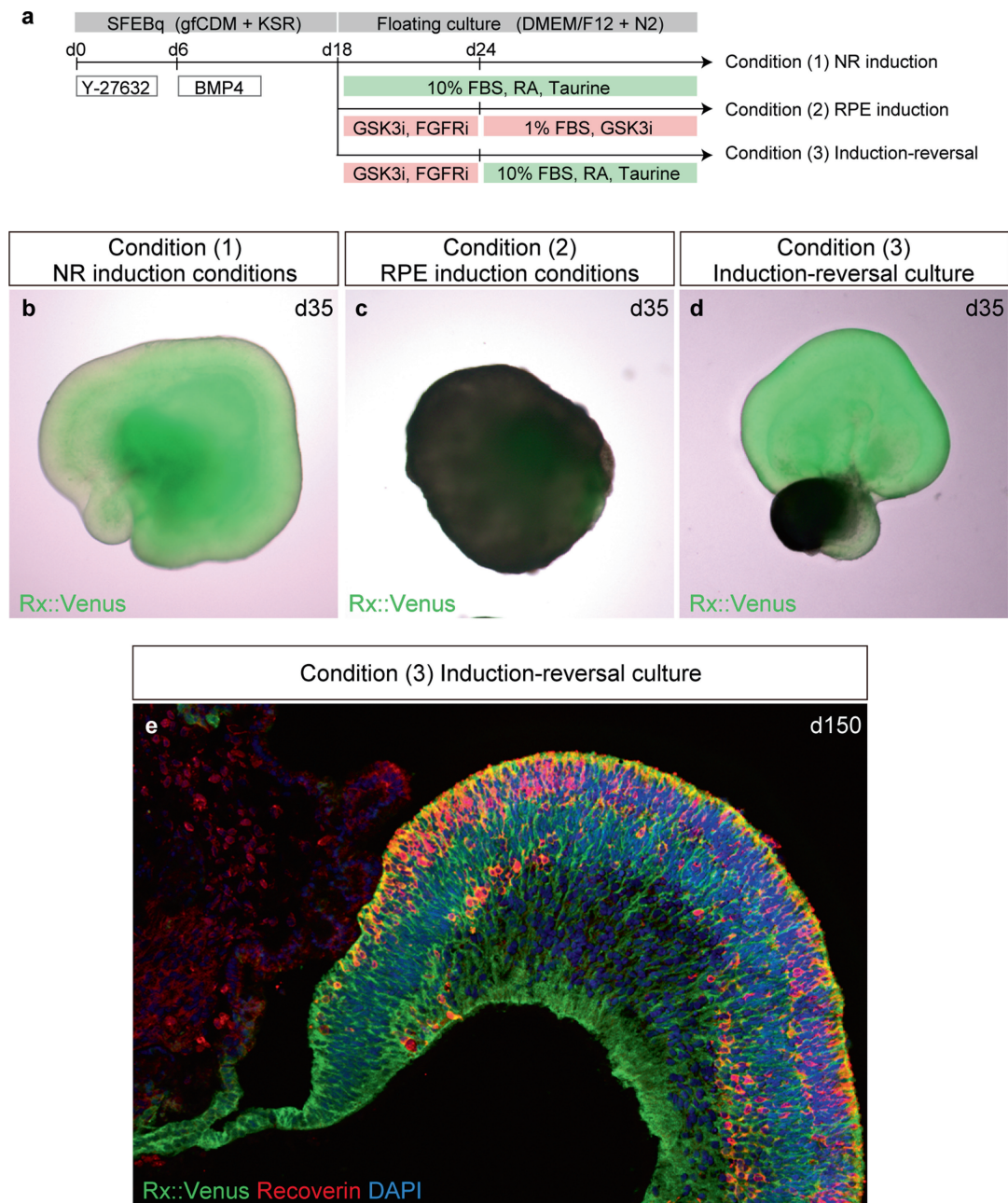


Fig. 2 Generation of turnip-shaped NR-RPE conjugated aggregates in induction-reversal culture. **(a)** Time table for three culture conditions. **(b–d)** External appearance of self-formed NR and RPE structures under different conditions on day 35. **(e)** Immunostaining of NR tissues in the turnip-shaped NR-RPE conjugated aggregates on day 150 sectioned along the central-peripheral axis. Modified from Kuwahara et al. *Nat Commun* 2015

1. (optional) Transfer the aggregates to Cell culture dish and dissect the ciliary margin-like tissue with fine forceps and scissors under a stereo microscope.

2. Digest cells at 37 °C for 30 min by using papain (Neural Tissue Dissociation Kit) and gently dissociate into single cells by pipetting.
3. Plate single cells on 96-well flat-bottom plates (MPC coated) at the density of 1000–3000 cells/well ($0.5\text{--}1.5 \times 10^4$ cells/mL) and culture for 7–14 days in retinosphere medium.
4. (optional) For secondary sphere formation assay, dissociate primary spheres by using papain (Neural Tissue Dissociation Kit) and culture in retinosphere medium.

3.9 Immunostaining of Aggregates

Prepare cell aggregates with retinal tissue. Cells are fixed in paraformaldehyde (PFA) and immunostained as described previously [5].

1. Transfer the SFEBq aggregates into 1.5 mL microtube or 15 mL conical tube and wash with PBS at 25 °C.
2. Fix aggregates with 4% (wt/vol) PFA at 4 °C for 15 min.
3. Wash with PBS.
4. Cryoprotect with 20% (wt/vol) sucrose in PBS at 4 °C for 12–72 h.
5. Transfer aggregates into a cryomold.
6. Embed aggregates with O.C.T. compound and freeze them.
7. Cut 10–15 μm thick frozen sections using a cryostat.
8. (optional) Treat frozen sections with heat-based antigen retrieval in Target Retrieval solution (15 min at 105 °C).

Both ECM-addition method and BMP method show a similar time course of differentiation: Brn3b⁺ cells (~d28), Crx⁺ cells (~d35), Recoverin⁺ (~d45), RXRG⁺ cells (~d60), NRL⁺ cells (~d100), S-opsin⁺ cells (~d130), and Rhodopsin⁺ cells (~d130). When we applied the “induction-reversal” methods, differentiation of these markers tended to be delayed by several days (corresponding to the time for RPE induction phase).

4 Notes

1. It is important to choose KSR lot suitable for retinal differentiation.
2. It is important to choose Matrigel lot suitable for ECM-addition method.
3. The quality of human ESCs is critical for retinal differentiation.
4. The number of plating hESCs should be optimized for each hESC/iPSC line, because it affects retinal differentiation efficacy. We typically seed 6000–15,000 cells per well in a 96-well V-bottomed plate.

5. BMP4 was added to culture to final 1.5 nM on day 6, and its concentration was diluted into half by half medium change every third day. The addition of BMP4, started on day 3 and day 6, induced Rx⁺ NR epithelium at day 18 with similar efficiency.
6. The addition of 5 μ M SU5402 treatment to RPE-induction medium increased efficiency and reproducibility of RPE induction and formation of ciliary margin-like tissues after the reversal culture, while CHIR99021 treatment without SU5402 tended to give higher variations in the level of RPE induction.

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Competing financial interests

A.K. is employed by Sumitomo Dainippon Pharma Co., Ltd. T.N. is employed by Sumitomo Chemical Co., Ltd. The authors are inventors on patent applications.

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