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Fabrication of three-dimensional lacrimal gland-like tissue organoids from human pluripotent stem cells

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Abstract

We have established a method for producing three-dimensional lacrimal gland-like tissue organoids from human pluripotent stem cells (hPSCs). These resemble native lacrimal glands on the basis of their morphology, immuno-labelling characteristics and gene expression patterns. When transplanted into the connective tissue above the eyes of recipient rats the organoids undergo functional maturation, producing tear film proteins.

Introduction

The generation of *in vitro* tissue organoids of exocrine glands from human pluripotent stem cells will facilitate research to provide a deeper understanding of the biological mechanisms that underpin gland development. The organoids also represent a valuable resource in the field of drug discovery and have potential in the field of regenerative medicine for use as transplantable material. The tear-producing lacrimal glands are the main exocrine glands of the eyes. Several methods for inducing lacrimal gland-like organoids from mouse and human lacrimal gland cells have been reported[1-8], however, human lacrimal gland cells and tissues are difficult to source, thus hampering research efforts. Accordingly, the generation of lacrimal gland-like tissue organoids from hPSCs would represent a significant advance for researchers in the field. Here, we report a protocol whereby cells with characteristics of lacrimal gland primordia emerge in two-dimensional cellular multi-zones, cultivated from hPSCs,[9,10] which partially recapture the developmental events in ocular morphogenesis. When isolated by cell sorting and grown under defined conditions, the cells form a three-dimensional lacrimal gland-like tissue organoid with ducts and acini, enabled by budding and branching.

Reagents

Human induced pluripotent stem cell (hiPSC) line 201B7 (RIKEN Bio Resource Center, #HPS0063).

hiPSC line YZWJs524 (Kyoto University's Center for iPS Cell Research and Application).

Human embryonic stem cells (hESC) line KhES-1 (RIKEN Bio Resource Center, #HES0001).

Laminin LN511E8 fragment (iMatrix-511; Nippi, #892011).

StemFit medium (Ajinomoto, #AK02N or #AK03N).

GMEM (Thermo Fisher Scientific, #11710-035).

Knockout serum replacement (KSR) (Thermo Fisher Scientific, #10828-028).

Sodium pyruvate (Thermo Fisher Scientific, #11360-070).

Non-essential amino acids (Thermo Fisher Scientific, #11140-050).

L-glutamine (Thermo Fisher Scientific, #25030-081).

Penicillin potassium and streptomycin sulfate (Meiji Seika Pharma).

Monothioglycerol (StemSure 50 mmol/L Monothioglycerol Solution (x100)) (Wako, #195-15791).

CnT-PR w/o; EGF and FGF2 (CELLnTEC Advanced Cell Systems, #CnT-PR-EF).

KGF (Wako, #119-00661).

Y-27632 (CultureSure Y-27632; Wako, #034-24024).

DMEM/F12 (Thermo Fisher Scientific, #11320-033).

B-27 supplement (Thermo Fisher Scientific, #17504-044).

Accutase (Thermo Fisher Scientific, #A11105-01).

Alexa Fluor 647 (AF647)-conjugated anti-ITGB4 (450-9D, BD Biosciences, #555721).

PE-conjugated anti-SSEA-4 (MC813-70, BioLegend, #330406).

PE-Cy7-conjugated anti-CD200 antibodies (OX-104, BD Biosciences, #562125).

Matrigel (growth factor reduced, Corning, #354230).

EGF (Wako, #059-07873).

FGF10 (Wako, #558-61851).

BMP7 (Wako, #026-19171).

PBS (Wako, #166-23555).

TrypLE^{TM} select (Thermo Fisher Scientific, #12563-029).

0.5 mM EDTA solution (Nacalai tesque, #13567-84).

DMEM without glutamine (Thermo Fisher Scientific, #10313-021).

Nutrient Mixture F-12 Ham (Thermo Fisher Scientific, #11765-054).

Hydrocortisone succinate (Wako, #080-05581).

3,3',5-Triiodo-L-thyronine sodium salt (MP Biomedicals, #194585).

Cholera toxin (List Biological Laboratory, #100B).

Bovine transferrin, holo form (Thermo Fisher Scientific, #11107-018).

Insulin transferrin selenium solution (Thermo Fisher Scientific, #41400-045).

FBS (Japan Bio Serum, #15-001-JBS or Life Thermo Fisher Scientific, #12483-020).

Equipment

SH800 cell sorter (Sony Biotechnology Inc.).

FACSAriaII cell sorter (BD Biosciences).

CO_2 Incubator (Panasonic, #MCO-19AICUVH-PJ).

Bio-clean bench (Panasonic, #MCV-B131F-PJ).

Microscope (Axiovert D1, Carl Zeiss).

Centrifuge (TOMY, #LC-230).

Culture plates; 6-well and 12-well (Corning, #353046, #353043).

Culture inserts; 12-well (FALCON, #353180).

Non-adhesive, round-bottomed 96-well plate (Sumitomo Bakelite Co., Ltd., #MS-9096U).

STEMFULLTM tubes (Sumitomo Bakelite, #MS-90150).

Cell Strainer (40 µm) (Corning, #352340).

Countess (Thermo Fisher Scientific).

Cell Scraper (Sumitomo Bakelite, #MS-93100).

Procedure

Culture Media

Differentiation medium (DM): GMEM supplemented with 10% knockout serum replacement (KSR), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 unit/mL penicillin potassium, and 100 µg/mL streptomycin sulfate and 55 µM monothioglycerol.

Epithelial differentiation medium (EDM): DM and CnT-PR w/o; EGF and FGF2 (1:1) containing 20 ng/mL KGF and 10 µM Y-27632, 100 unit/mL penicillin potassium and 100 µg/mL streptomycin sulfate.

Ocular surface epithelial differentiation medium (OSEM): DMEM/F12 containing 2% B-27 supplement, 20 ng/mL KGF, 10 μM Y-27632, 100 unit/mL penicillin potassium and 100 μg/mL streptomycin sulfate.

Lacrimal gland culture medium (LGM): DMEM/F12 containing 2% B-27 supplement, 10 ng/mL EGF, 10 μM Y-27632, 100 unit/mL penicillin potassium and 100 μg/mL streptomycin sulfate.

KCM medium: DMEM without glutamine and Nutrient Mixture F-12 Ham (3:1) supplemented with 5% FBS, 0.4 μg/ml hydrocortisone succinate, 2 nM 3,3',5-Triiodo-L-thyronine sodium salt, 1 nM cholera toxin, 2.25 μg /ml bovine transferrin HOLO form, 2 mM L-glutamine, 0.5% insulin transferrin selenium solution, and 100 unit/mL penicillin potassium and 100 μg/mL streptomycin sulfate.

Procedure

The use of an appropriate hPSC clone is important to generate lacrimal gland-like tissue organoids. In our hands hiPSC lines 201B7 and YZWJs524, and hESC line KhES-1 all were able to generate lacrimal gland-like organoids. Initially, hPSCs are cultured in StemFit^{TM} medium on LN511E8-coated 6-well culture plates.

Plate coating

1. Coat new 6-well culture plates with LN511E8 at 0.5 μg/cm^2 in 1.5 ml PBS by incubation at 37°C for at least 60 min.

2. Add 0.7 mL StemFit^{TM} medium to the culture plates.

3. Remove the mixed solution.

4. Immediately add 1.5 mL StemFit^{TM} medium containing 10 μM Y-27632 and incubate at 37°C in 5% CO_2.

Cell passaging

1. Remove the medium.

2. Wash hPSCs once with 1 mL PBS.

3. Add 300 µL dissociation solution (50% TrypLE select and 50% 0.5 mM EDTA solution) and incubate for 4-5 min at 37°C.

4. Remove the dissociation solution carefully (hPSCs are still attached to the culture plates at this point).

5. Wash the hPSCs once with 2 mL PBS, and then add 1 mL StemFit™ medium containing 10 µM Y-27632.

6. Harvest the hPSCs with a Cell Scraper.

7. Pipette the hPSCs about 10 times with a 200 or 1000µL micropipette, then collect the dissociated hPSCs in a 1.5 mL tube.

8. Count the collected hPSCs using a Countess® Machine after staining with Trypan blue (settings for Countess®; Sensitivity: 5, Minimium size: 8, Maximum size: 30, Circularity: 75).

9. Seed hPSCs on LN511E8 coated 6-well culture plates at 13,000 cells/well.

10. Incubate the plates at 37°C in 5% CO_2.

11. The next day, change the medium to fresh StemFit^{TM} medium without Y-27632 and incubate at 37°C in 5% CO_2 up to day seven. The medium should be changed every two days.

Critical point: If hPSCs are maintained under other conditions (e.g. co-cultivation with MEF feeder layers) after removal of the feeder cells, the dissociated hPSCs must be seeded in the StemFit^{TM}/LN511E8 culture system at 13,000 cells/well and be passaged at least five times to adapt to the conditions.

Differentiation culture

Preparing hPSCs for differentiation (Day -10)

1. Coat a 6-well or 12-well culture plate with LN511E8 at 0.5 µg/cm^2 as previously indicated.

2. Harvest hPSCs using the same method (above) as that employed for hPSC passaging.

3. Seed dissociated hPSCs on 6-well plates at 3000-6000 cells/well, or on 12-well plates at 1000-2000 cells/well in 1.5 mL (6-well plate) or 0.6 mL (12-well plate) of StemFit^{TM} medium containing 10 µM Y-27632.

4. Incubate the plates at 37°C in 5% CO_2.

5. The next day, change the medium to fresh StemFit™ medium without Y-27632.

6. Incubate the plates at 37°C in 5% CO_2 for 10 days in total. The medium should be changed every two days before day seven, and every day thereafter.

Ocular surface epithelial cell differentiation culture (Day 0-)

7. After 10 days culture in StemFit™ medium, change the medium to 2 mL (6-well plate) of DM (Day 0).

8. Incubate the plates at 37°C in 5% CO_2 for four weeks, changing the medium every 2-3 days (i.e. three times per week). At around three weeks, hPSC colonies should appear as multi-cellular colonies formed of 3-4 concentric zones (this is called a "SEAM"; Self-formed Ectodermal Autonomous Multi-zone)[9,10]. Cells in the third zone from the centre of the colony are ocular surface ectodermal cells, which we have shown can differentiate into corneal and conjunctival epithelium.[9-12]

EDM culture (Day 28-)

9. After four weeks of incubation in DM, change the medium to 2 mL (6-well plate) of EDM.

10. Incubate the plates for an additional four weeks at 37°C in 5% CO_2 (i.e. eight weeks in total). The medium should be changed every 2-3 days (three times per week). Optional: To promote retinal cell differentiation, the medium can be changed to OSEM at day 28. After incubation in OSEM for an additional 2-3 weeks (i.e. 6-7 weeks in total), cells with characteristics of pigmented retinal pigment epithelial (RPE) cells are frequently observed.

Manual pipetting to remove non-epithelial cells (Day 49-)

During cultivation in EDM, manual pipetting should be performed to remove non-epithelial cells (e.g. neuronal cells, retinal cells and/or lens cells) from the SEAMs. We have found that this significantly enhances the yield of corneal epithelial cells on subsequent cell sorting compared to the yield without manual pipetting.

11. At around three weeks of incubation in EDM (i.e. after seven weeks in total), perform manual pipetting using a PIPETMANTM (1000 or 200 µL) on a clean bench.

12. After pipetting, change the medium (which will contain the detached cells) to fresh EDM.

13. Incubate the plates for an additional one week in EDM (i.e. eight weeks in total).

OSEM culture (Day 56-)

14. After EDM culture for four weeks (i.e. eight weeks overall) change the medium to 2 mL (6-well plate) of OSEM.

15. Incubate the plates for either two weeks (i.e. 10 weeks in total) or four weeks as a maximum (i.e. 12 weeks in total) at 37°C in 5% CO_2. The medium should be changed every 2-3 days (three times per week).

FACS for isolating ocular surface epithelial stem cells (Day 70-)

16. After OSEM culture, wash the hPSCs once with PBS.

17. Add Accutase™ to the differentiated hPSCs and incubate for 45 min at 37°C.

18. Perform pipetting several times and again incubate for 15 min at 37°C.

19. Collect and re-suspend the dissociated cells in ice-cold KCM medium in a STEMFULL™ 15 mL tube.

20. Filter the cells using a Cell Strainer (40 μm) and count the cell number.

21. Centrifuge the cells at 1200 rpm for 8 min.

22. Aspirate the supernatant and re-suspend the cells in ice-cold KCM containing FITC-conjugated SSEA-4 (MC813-70), PE-conjugated CD104 (ITGB4; 58BX4) and PE-Cy7-conjugated anti-CD200 (OX-104) antibodies.

23. Incubate the cells for 1 hr at 4°C (agitate the cells every 20 min).

24. Wash the cells twice with PBS.

25. Resuspend the cells in PBS by filtration using a Cell strainer (40 μm). Cells should be stained with non-specific isotype IgG (for SSEA-4, CD104 and CD200) as controls. For each fluorescent probe, single-colour stained cells should be prepared for colour compensation during flow cytometry.

26. Set up the FACSAriaII or SH800 cell sorter.

27. Subject stained cells to sorting in the FACSAriaII or SH800.

28. Perform compensation between the detectors for FITC, PE, and PE-Cy7 using each of the single stained cells.

29. Analyze the triple colour-stained cells.

30. Sort the SSEA-4+, CD104+, and CD200- population (i.e. the ocular surface epithelial stem cell fraction) to 8 mL of KCM in a STEMFULL^{TM} 15 ml tube. Typically, in CD200 negative cells, around 30-50% of the cells are detected as the ocular surface epithelial stem cell fraction.

31. Collect the ocular surface epithelial stem cells by centrifugation at 1200 rpm for 8 min.

32. Re-suspend the cells in LGM on ice.

Formation of lacrimal gland organoid (Day 70-)

33. Cultivate the sorted ocular surface epithelial stem cells (i.e. CD200−, SSEA-4+, ITGB4+) in OSEM in the wells of a non-adhesive, round-bottomed 96-well plate for one day at 1×10^5 cells per well. This results in the formation of a spheroid of lacrimal gland-like progenitors.

34. Embed spheroids in 50% (vol/vol) of Matrigel (growth factor reduced) diluted in LGM, and culture at 37°C in 5% in CO_2 for approximately 20 days. The medium should be changed every 2-3 days (three times per week).

Troubleshooting

If no lacrimal gland-like organoid formation is observed from the ocular surface epithelial stem cells, it might be better to shorten the culture period in OSEM (e.g. reduce from 2 weeks to 1 week or even several days) and adjust the concentration of EGF in LGM (e.g. increase from 10 ng/mL to 50 ng/mL or more). If the organoids still do not form, it might be worth trying a different hPSC clone.

Time Taken

The entire protocol from the seeding of hPSCs to the formation of lacrimal gland-like organoids takes a total of 84 days minimum.

Anticipated Results

SEAM multi-zonal colony formation

In the initial stages of this protocol, SEAM colonies with concentric multi-cellular zones (typically 3-4 zones) should have spontaneously formed by four weeks in culture.[9,10] For the validation of ocular surface epithelial induction, PAX6 and p63-double staining is useful. When double positive cells are detected in SEAM zone-3 at 3-4 weeks of differentiation and more widely in the SEAM at 10 weeks, the method is deemed to have worked successfully.

Formation of lacrimal gland-like organoids

Bright field or phase-contrast microscopy can be used to visualize a branched morphology starting to emerge in spheroids within a few days of three-dimensional culture in LGM. This usually continues for another four weeks as the organoid forms.

References

1. Hirayama, M. et al. Functional lacrimal gland regeneration by transplantation of a bioengineered organ germ. *Nat. Commun.* **4**, 2497 (2013).

2. Ackermann, P. et al. Isolation and investigation of presumptive murine lacrimal gland stem cells. *Invest. Opthalmol. Vis. Sci.* **56**, 4350 (2015).

3. Ueda, Y. et al. Purification and characterization of mouse lacrimal gland epithelial cells and reconstruction of an acinar like structure in three-dimensional culture. *Invest. Ophthalmol. Vis. Sci.* **50**, 1978–1987 (2009).

4. Gromova, A. et al. Lacrimal gland repair using progenitor cells. *Stem Cells Transl. Med.* 6, 88-98 (2017).

5. Tiwari, S. et al. Establishing human lacrimal gland cultures with secretory function. *PLoS One* **7**, e29458 (2012).

6. Hirayama, M. et al. Identification of transcription factors that promote the differentiation of human pluripotent stem cells into lacrimal gland epithelium-like cells. *NPJ Aging Mech. Dis.* **3**, 1 (2017).

7. Bannier-Hélaouët, M. et al. Exploring the human lacrimal gland using organoids and single-cell sequencing. *Cell Stem Cell* **28,** 1221-1232 (2021).

8. Jeong, S. Y. et al. Establishment of functional epithelial organoids from human lacrimal glands. *Stem Cell Res. Ther.* **12**, 247 (2021).

9. Hayashi, R. et al. Co-ordinated ocular development from human iPS cells and recovery of corneal function. *Nature* **531**, 376-380 (2016).

10. Hayashi, R. et al. Coordinated generation of multiple ocular-like cell lineages and fabrication of functional corneal epithelial cell sheets from human iPS cells. *Nat. Protoc.* **12**, 683-696 (2017).

11. Watanabe, S. et al. Human iPS cells engender corneal epithelial stem cells with holoclone-forming capabilities. *iScience* **24**, 102688 (2021).

12. Nomi, K. et al. Generation of functional conjunctival epithelium, including goblet cells, from human iPSCs. *Cell Rep.* **34**, 108715 (2021).

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