Editorial Summary: This protocol describes how to grow a functional and transplantable corneal epithelium and how to generate ocular-like cell lineages resembling neuroectoderm, neural crest, ocular surface ectoderm or surface ectoderm derived from human iPS cells.

Co-ordinated generation of multiple ocular-like cell lineages and fabrication of functional corneal epithelial cell sheets from human iPS cells

Ryuhei Hayashi[1, 2*], Yuki Ishikawa[2], Ryousuke Katori[2], Yuzuru Sasamoto[2], Yuki Taniwaki[2], Hiroshi Takayanagi[2], Motokazu Tsujikawa[2], Kiyotoshi Sekiguchi[3], Andrew J. Quantock[4], & Kohji Nishida[2*]

[1] Department of Stem Cells and Applied Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, 565-0871, Japan. [2] Department of Ophthalmology, Osaka University Graduate School of Medicine, Suita, Osaka, 565-0871, Japan. [3] Division of Matrixome Research and Application, Institutes for Protein Research, Osaka University, Suita, Osaka, 565-0871, Japan. [4] Structural Biophysics Group, School of Optometry and Vision Sciences, College of Biomedical and Life Sciences, Cardiff University, Cardiff, CF24 4HQ, Wales, UK.

*Co-corresponding authors: Ryuhei Hayashi (ryuhei.hayashi@ophthal.med.osaka-u.ac.jp) and Kohji Nishida (knishida@ophthal.med.osaka-u.ac.jp)

ABSTRACT

We describe a protocol for the generation of a functional and transplantable corneal epithelium derived from human induced pluripotent stem (iPS) cells. In our experiments a proportion of iPS cells spontaneously form circular colonies, each of which is comprised of four concentric zones. Cells in these zones have different morphologies and immunostaining characteristics, resembling neuroectoderm, neural crest, ocular surface ectoderm or surface ectoderm. We term this two-dimensional (2D) colony a SEAM (a self-formed, ectodermal, autonomous, multi-zone), and propose that cells within it have the potential to give rise to anlages of different ocular lineages including retinal cells, lens cells, and ocular surface ectoderm. To investigate the translational potential of the SEAM, cells within it that resembled ocular surface epithelia were isolated by pipetting and sorted by FACS into a population of corneal epithelial-like progenitor cells. These were then expanded and differentiated to form an epithelial layer, which expressed K12 and PAX6 and which was able to recover function in an animal model of corneal epithelial dysfunction after surgical transplantation. The whole protocol, encompassing human iPS cell preparation, autonomous differentiation, purification and subsequent differentiation takes between 100 and 120 days, and is of potential use to researchers with an interest in eye development and/or ocular surface regeneration. We suggest that experience with human iPS cell culture and sorting via FACS will be of benefit for researchers performing this protocol.

INTRODUCTION

Induced pluripotent stem (iPS) cells are highly influential in life sciences research, and the fields of fundamental and translational eye research are no exceptions[1,2]. Noteworthy in this regard are studies that have used embryonic stem (ES) cells and iPS cells to advance our knowledge of ocular morphogenesis, and the potential of stem cell-derived tissue for surgical use[3-7]. The front of the eye is formed by the cornea, a curved and clear collagen-rich tissue that is overlaid by a specialised multi-layered epithelium that supports the tear-film. Research into the corneal epithelium is active owing to the prevalence of blinding and difficult-to-treat ocular surface injuries or diseases. At present, convincing methods for the production of corneal epithelial stem cells and progenitor cells by direct differentiation from iPS cells, and the subsequent expansion of these cells into functional epithelial tissues, are lacking. To try to address this and to investigate the possible generation of multiple ocular cell lineages, we recently reported the fabrication of a self-formed, ectodermal, autonomous, multi-zone (SEAM) of ocular cells from human iPS cells[8]. The SEAM contains four concentric zones of cells, each of which has the characteristics of a particular ocular cell lineage or lineages. From the cell zone of the SEAM that most closely resembles the ocular surface epithelium we isolated and sorted functional corneal epithelial stem/progenitor cells[8]. Here, we report a detailed protocol for generating a SEAM from human iPS cells and the subsequent fabrication of functional corneal

epithelial cell sheets.

Development of the Protocol

Recent research with pluripotent stem cells in culture has identified a previously underappreciated level of intrinsic cellular self-organization, and has provided evidence of cupping of the neural retina when a three-dimensional (3D) culture system was used[4]. However, this work provided no signs of ocular surface development. We have now developed a culture technique that can partially mimic whole eye development encompassing all key ocular component tissues. To achieve this, we adopt a 2D culture system and use laminin 511 E8-fragment as a substrate for ocular cell growth. Various types of serum-free differentiation medium were used to promote spontaneous eye-like differentiation of human iPS cells.

Comparison with other methods

As mentioned, research using 3D culture techniques has successfully demonstrated spontaneous retinal tissue formation from human iPS cells[4]. However, this approach has not led to the generation of corneal or other anterior eye tissues. It has also been shown, by us and others, that the use of a 2D-culture system with feeder cells or their conditioned medium (e.g. corneal limbal fibroblasts or PA6 mouse feeder cells) is able to induce corneal epithelial-like cells from iPS cells, but the purification of these cells was ultimately not achievable[9,10]. In essence, these

cells appeared to be “corneal epithelial-like cells” and, indeed, they expressed corneal epithelial markers. But, ultimately, they did not undergo subsequent physiological development and differentiation[9-11]. The protocol we describe here is distinct because it generates a range of cellular anlages that have characteristics of several quite separate tissues in the eye. The culture techniques do not require the use of feeder cells or FBS, and the iPS cell-derived corneal epithelial stem and progenitor cells undergo purification by FACS using a combination of antibodies. This approach confers a crucial advantage for future potential clinical applications because the final product (i.e. the corneal epithelial cell sheets) will be devoid of non-corneal iPS-derived epithelial cells and of cellular impurities that might originate from feeder cells.

Limitations

In this series of experiments, it became apparent that different human iPS cell clones had different propensities for differentiation. Indeed, some clones only infrequently formed a SEAM or resulted in the successful generation of a FACS-sorted corneal epithelial cell layer. Commercially available 201B7 and 454E2 iPS cell clones, along with clone 1383D2, provided by the Center for iPS Cell Research and Applications (CiRA) at Kyoto University, displayed a good differentiation potential. Clone 253G1, on the other hand, was found to be poor in terms of its differentiation into corneal epithelium[8]. This differential differentiation propensity of

different human iPS cell clones might be a consequence of dissimilarities in epigenetic status[11-13], but at this stage of our research it is too early to definitively identify a particular human iPS cell clone(s) as best-suited for use in SEAM-formation and subsequent corneal differentiation. Of course, this question could be resolved by optimizing the cultivation protocol for each human iPS cell clone, but we have not undertaken this task. As such, and based on our experience to date, it would seem sensible to recommend the use of clone 201B7, a frequently used clone in the field of human iPS cell research[14-16], for the experiments we describe. Finally, we note that the protocol described here, especially in its latter parts, is targeted at inducing corneal epithelial cells from the human iPS cell-derived SEAM. Effectively obtaining other cell types such as neural crest cells or presumptive lens cells will likely require modifications to the protocol.

Applications

SEAMs generated by this protocol have potential use as *in vitro* cellular models for human eye development. This will likely be of significant interest for research into human eye development, particularly in the early stages, because most current information is based on mouse or avian eye development. Moreover, because human iPS cells can be infinitely amplified in culture a SEAM has the potential to act as a significant research resource for

human ocular cells, such as retinal cells or corneal epithelial cells, which are difficult to obtain otherwise.

Experimental Design

This protocol is described in two parts: the first covers the human iPS cell culture and SEAM formation (Steps 1–34; PART I); the second, the isolation of corneal epithelial cells from the SEAM and their fabrication into functional and transplantable cell sheets (Steps 35–59; PART II) (Fig.1).

PART I: Human iPS cell culture and SEAM formation (Steps 1–34) Human iPS cells to be used for SEAM-formation and subsequent corneal epithelial differentiation were maintained on Laminin-511 E8 fragment (LN511E8)-coated culture dishes in serum-free medium (StemFit™ medium) for a minimum of three to four cell passages before initiating differentiation[15,17] (Fig. 2). The initial seeding density -- typically, we use 1500-4500 cells/6-well plate -- is an important factor for the differentiation propensity. For example, at higher cell densities (e.g. 4500-6000 cells/6-well plate) the likelihood of corneal epithelial differentiation is enhanced. Seeding at lower cell densities, (e.g. 1000-2000 cells/6-well plate), on the other hand, has the effect of encouraging neural crest differentiation.

After the initial cell passaging, cultivation was continued in specific growth factor-free medium (Differentiation medium; DM), whilst still on LN511E8, until the formation of a SEAM (Fig. 3A). As we reported recently[8], a typical SEAM contains neuronal cells in the innermost zone (zone 1), retina-like and neural crest-like cells in the adjacent radial zone (zone 2), ocular surface ectoderm-like cells in the next zone outwardly (zone 3), lens-like cells in zones 2 and 3, and non-ocular surface epithelial-like cells in the outermost zone (zone 4) (Fig. 3B).

After approximately four weeks of differentiation culture, we examined each zonal cell population in the SEAM via immunofluorescence staining (Box 1). To verify the existence of ocular cell lineages in each zone, the following antibody combinations were used and are recommended: a PAX6/p63-double-positive phenotype for ocular surface ectoderm (an anlage of the corneal epithelium)[8,18,19]; TUBB3 and p63 for neuronal/epithelial cells[20-22]; CHX10 and MITF for neural-retina/retinal pigment epithelium (RPE)[8,23]; SOX10 and p75 for neural crest cells[8,24]; and PAX6/α-crystallin for lens cells[25,26] (Fig. 3B, Table 1).

At the four-week timepoint we further studied cells in zone 3, i.e. those with characteristics of the ocular surface ectoderm, anlages of corneal epithelial stem and progenitor cells. To do this the SEAM was further cultivated in corneal differentiation medium (CDM) containing growth

factors and the ROCK inhibitor, Y-27632, for an additional four weeks. Three weeks into this CDM culture period, (i.e. after seven weeks total culture time) we remove zone 1 and 2 cells from the SEAM by manual pipetting, as is described later. During this second culture period, immature ocular surface ectodermal cells proliferate and gradually differentiate into what will later become functional corneal epithelial stem/progenitor cells. (As an alternative option for encouraging retinal cell differentiation, we suggest a direct switch to corneal epithelial medium (CEM), rather than CDM after the initial four week differentiation in DM).

PART II: Isolation of corneal epithelial cells from the SEAM and their fabrication into functional and transplantable cell sheets (Steps 35–59). As mentioned, corneal epithelial-like stem/progenitor cells form zone 3 of the SEAM. Immunolabelling shows that cells with characteristics of other ocular cell lineages (i.e. retinal cells, lens cells, or neuronal cells) are generated in other zones of the same SEAM in the same culture dish (Fig. 1, 3B). To enrich and purify corneal epithelial-like cells, we remove these non-epithelial cellular zones from the SEAM by manual pipetting (Fig. 4A). This can be carried out at around seven weeks of differentiation, i.e. three weeks into the four-week CDM culture period. As described later, the pipetting can selectively remove neuronal-like cells from zones 1 and 2 of the SEAM without removing the cells in zones 3 and 4 because these adhere more tightly to the culture dish. After

pipetting away zone 1-2 cells, those remaining are further differentiated for an additional week in CDM, so that a total of four weeks in CDM is achieved. Following this, i.e. at the eight-week timepoint, the culture medium is switched to CEM (Fig. 4B) and cultivation is continued for a further four weeks. Thus, the recommended culture time is 12 weeks in total, although this can be reduced to 10-weeks by performing an abridged two-week CEM cultivation. This, however, carries the risk of lessening the efficiency of corneal differentiation, which becomes more stable after 12 weeks. (The maximum recommended culture time, depending on the logistics of the experiments, is 16 weeks, which can be achieved by extending the final CEM cultivation period to eight weeks). At the recommended 12-week timepoint FACS was performed using anti-ITGB4, SSEA-4, and TRA-1-60 antibodies to isolate corneal epithelial stem/progenitor cells (Fig. 4C). TRA-1-60, a pluripotent stem cell marker, was used to remove undifferentiated iPS cells[14,27], ITGB4 was used to select basal cells of the stratified epithelium, rich in epithelial stem/progenitor cells[8,28,29], whilst SSEA-4 was employed as it is expressed in corneal epithelial cells as well as in a significant proportion of stem and progenitor cells in the corneal epithelial stem cell niche at the edge of the cornea at the limbus of the eye[8,30]. This approach enabled the high-purity isolation of corneal epithelial stem/progenitor cells consisting of TRA-1-60^{-}/ITGB4^{+}/SSEA-4^{+} cells.

After sorting in this way, the FACS-isolated corneal stem/epithelial progenitor cells were allowed to proliferate and form epithelial colonies on LN511E8-coated dishes in a feeder-less culture system. After reaching confluence, cells became stratified and had formed a multi-layered structure. To conduct cell-sheet transplantation in an animal model of corneal epithelial injury, we harvested cell sheets at this juncture (i.e. after two-to-three weeks in culture). Sheets were recoverable as intact cellular constructs, similar to those we have used in previous corneal epithelial transplantation investigations[31]. Additionally, longer-term cultivation (up to 10 weeks) of the sorted cells was conducted to promote cellular stratification and maturation, after which we performed immunofluorescence microscopy to examine how patterns of protein expression compared to those of normal cultivated human corneal limbal epithelium (Box 2). We recommend immunofluorescence staining using the corneal epithelial-specific markers, K3, K12, PAX6, p63, MUC1 and MUC16 (Fig. 5).

MATERIALS

Human iPS cell (201B7; RIKEN Bio Resource Center, #HPS0063)

Caution: All work with human iPS cells must be carried out in accordance with all relevant institutional and governmental regulations, applicable in the investigator's home laboratory and country. In our case, the experiments were approved by the Recombinant DNA Committees of

Osaka University and were performed according to our institutional guidelines.

Critical: Human iPS cells should be routinely verified by microscopic observation. They should exist as circular colonies composed of uniformly packed cells that exhibit immunostaining for pluripotent stem cell markers, TRA-1-60 and SSEA-4.

Caution: The 201B7 human iPS cell line was tested as *Mycoplasma* negative by the provider and by us. Human iPS cells should be regularly tested for *Mycoplasma* contamination by an appropriate method (e.g., we used a PCR-based method employing the e-Myco™ Mycoplasma PCR Detection Kit; iNtRON Biotechnology, # 25235).

PE-conjugated CD104 (ITGB4) (58BX4; Bio Legend, #327808)

PE-conjugated isotype control IgG (MOPC-173; BioLegend, #400214)

FITC-conjugated SSEA-4 (MC-813-70; BioLegend, #330410)

FITC-conjugated isotype control IgG (MG3-35; BioLegend, #401317)

Alexa647-conjugated TRA-1-60 (TRA-1-60-R; BioLegend, #330606)

Alexa647-conjugated isotype control IgM (MM-30; BioLegend, #401618)

Anti-PAX6 antibody (PRB-278P; BioLegend, #901301)

Anti-PAX6 antibody (AD2.35; Santa Cruz Biotechnology, #sc-53108)

Anti-p63 antibody (4A4; Santa Cruz Biotechnology, #sc-8431)

Anti-CHX10 antibody (N-18; Santa Cruz Biotechnology, #sc-21690)

Anti-MITF antibody (C5; Exalpha Biologicals, #X1405M)

Anti-TUBB3 antibody (T2200; Sigma-Aldrich, #T2200)

Anti-SOX10 antibody (N-20; Santa Cruz Biotechnology, #sc-17342)

Anti-p75 antibody (ME20.4; Advanced Targeting Systems, #AB-N07)

Anti-α-Crystallin antibody (SPA-224; Stressgen Biotechnologies, #SPA-224)

Anti-K12 antibody (N-16; Santa Cruz Biotechnology, #sc-17098)

Anti-K3/76 antibody (AE5; PROGEN Biotechnik, #61807)

Anti-MUC1 antibody (M4H2; Abcam, #ab10120)

Anti-MUC16 antibody (OV185:1; Abcam, #ab697)

Alexa Fluor 488 donkey anti-Rabbit IgG (H+L) (Life Technologies, #A21206)

Alexa Fluor 594 donkey anti-Rabbit IgG (H+L) (Life Technologies, #A21207)

Alexa Fluor 568 donkey anti-Goat IgG (H+L) (Life Technologies, #A11057)

Alexa Fluor 488 donkey anti-Mouse IgG (H+L) (Life Technologies, #A21202)

Alexa Fluor 568 donkey anti-Mouse IgG (H+L) (Life Technologies, #A10037)

4% (wt/vol) Paraformaldehyde phosphate buffer solution (PFA) (Wako, #163-20145)

Cellstain® Hoechst 33342 Solution (Wako, #346-07951)

Triton X-100 (SIGMA, #T8787-100ML)

Normal donkey serum (Jackson ImmunoResearch, #017-000-121)

TBS powder (TakaraBio, #T903)

StemFit™ medium (Ajinomoto, #AK02 or AK03)

Accutase™ (Life Technologies, #A11105-01)

TrypLE™ Select (Life Technologies, #12563-029)

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

Trypan Blue Stain 0.4% (Thermo Fisher Scientific, #T10282)

KGF (R&D, #251-KG or Wako, #112-00813)

Y-27632 (Wako, #034-24024)

B-27 supplement (Life Technologies, #17504-044)

LN511E8 fragment (i-Matrix-511; Nippi, #892011)

GMEM (Life Technologies, #11710-035)

Penicillin-Streptomycin solution (Life Technologies, #15140-122)

Non-essential amino acids (Life Technologies, #11140-050)

Sodium pyruvate (Life Technologies, #11360-070)

Knockout serum replacement (KSR) (Life Technologies, #10828-028)

2-mercaptoethanol (2-ME) (Life Technologies, #21985-023)

Caution: 2-ME is toxic and flammable. Wear gloves and handled it carefully.

Monothioglycerol (replacement for 2-ME) (Wako, #195-15791)

CnT-PR [w/o; EGF and FGF2] (CELLnTEC, #CnT-PR-EF)

DMEM/F-12 [1:1] (Life Technologies, #11320-033)

DMEM without glutamine and Nutrient Mixture F-12 Ham (3:1) (Life Technologies, #10313-021, #11765-054)

Hydrocortisone succinate (Wako, #080-05581)

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

Cholera toxin (List Biological Laboratory, #100B)

Caution: Cholera toxin is potentially harmful. Wear gloves and handle it carefully in a biosafety cabinet.

Bovine transferrin HOLO form (Life Technologies, #11107-018)

L-glutamine (Life Technologies, #25030-081)

Insulin transferrin selenium solution (Life Technologies, #41400-045)

FBS (Japan Bio Serum, #15-001-JBS or Life Technologies, #12483-020)

STEM-CELLBANKER (TakaraBio, #CB046)

DPBS, no calcium, no magnesium (Life Technologies, #14190-144)

Equipment

Cell sorter (BD Biosciences, FACSAriaII)

1 CO_2 Incubator (Panasonic, #MCO-20AIC)

2 Bio-clean bench (Panasonic, #MCV-B131F)

3 Bio-safety cabinet (ESCO, #JA2-4A7)

4 Ultra-low freezer (Panasonic, #MDF-U33V)

5 Ultra-low freezer (Panasonic, #MDF-C2156VAN)

6 Fluorescent microscope (Carl Zeiss, Axio Observer. D1)

7 Fluorescent microscope (Thermo Fisher Scientific, EVOS®)

8 Stereomicroscope (Olympus, #SZ61)

9 Centrifuge machine (TOMY, #LC-230)

10 Culture plate 6-well, 12-well (Corning, #353046, #353043)

11 Culture insert 12-well (FALCON, #353180)

12 UpCell® 6-well plate (CellSeed, #CS3004)

13 STEMFULL™ tube (Sumitomo Bakelite, #MS-90150)

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

15 Automated cell counter (Thermo Fisher Scientific, Countess®)

16 PVDF membrane filter (Millipore, #SVWG04700)

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

18 Falcon 50-mL polypropylene conical tube (FALCON, #352070)

Falcon 15-mL polypropylene conical tube (FACLON, #352096)

PermaFluor™ Aqueous Mounting Medium (Thermo Fisher Scientific, # TA-030-FM).

REAGENT SET-UP

Differentiation medium (DM) GMEM supplemented with 10% (vol/vol) knockout serum replacement (KSR), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1% (vol/vol) penicillin-streptomycin solution, and 55 μM 2-mercaptoethanol (2-ME) or monothioglycerol (a replacement for 2-ME) [32].

The medium should be stored at 4 °C and used within two weeks.

Caution: 2-ME is toxic and flammable. Wear gloves and handle it carefully.

KGF frozen stock Dissolve 1.0 mg of KGF in 50 mL of PBS containing 0.1% (wt/vol) human serum albumin and store at 50-100 μL/tube (20 μg/mL) at -80°C or -30°C for up to three months.

Y-27632 frozen stock Dissolve 25 mg of Y-27632 in 7.4 mL of PBS and store at 50 μL/tube (10 mM) at -30°C or -80°C for up to six months.

Corneal differentiation medium (CDM) Prepare DM and CnT-PR [w/o EGF and FGF2] (1:1) containing 10-20 ng/mL KGF, 10 μM Y-27632, and 1% (vol/vol) penicillin-streptomycin solution. Add KGF and Y-27632 to the basal medium of the CDM (w/o KGF and Y-27632)

before use. The basal medium should be stored at 4 °C and used within two weeks.

Corneal epithelium maintenance medium (CEM) Prepare DMEM/F-12 [1:1] containing 2% (vol/vol) B-27 supplement, 10-20 ng/mL KGF, 10 μM Y-27632, and 1% (vol/vol) penicillin-streptomycin solution. Add B-27 supplement, KGF, and Y-27632 to the basal medium of CEM (w/o B-27, KGF, and Y-27632) before use. The basal medium should be stored at 4°C and used within two weeks.

KCM medium Prepare DMEM without glutamine and Nutrient Mixture F-12 Ham (3:1) supplemented with 5% (vol/vol) 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% (vol/vol) insulin transferrin selenium solution, and 1% (vol/vol) penicillin-streptomycin solution. The medium should be stored at 4 °C and used within three weeks.

Corneal epithelium-maturation medium (CMM) Prepare KCM medium containing 10-20 ng/mL KGF and 10 μM Y-27632. Add KGF and Y-27632 to the KCM before use. The basal medium (KCM medium) should be stored at 4°C and used within three weeks.

PROCEDURE

Human iPS cell maintenance and subculture: *Plate coating* TIMING ~3 h

CRITICAL The use of an appropriate human iPS cell clone is important to achieve successful

ocular surface epithelial cell differentiation. As discussed earlier, we found clone 201B7 to be suitable for the protocol described here, however other clones conceivably could be used with appropriate modifications to the protocol.

1. Coat new 6-well culture plates with LN511E8 (i-Matrix) at 0.5 µg/cm^2 in 1.5 mL of PBS by incubation at 37°C for 2 hr.

2. Add 0.7 mL of StemFit™ medium to the culture plates.

3. Remove the mixed solution and immediately add 1.5 mL of StemFit™ medium containing 10 µM Y-27632.

Critical step: Do not allow the surface of the culture plates to dry. Add StemFit™ medium immediately after the removal of the coating solution.

4. Incubate plates at 37°C in 5% CO_2 for around 15 min whilst preparing human iPS cells, as described in steps 5-12.

Human iPS cell maintenance and subculture: ***Human iPS cell seeding from frozen cell stock***

TIMING 7 d

5. Add 5 mL of StemFit™ medium to a 15-mL conical tube.

6. Take a frozen stock of 2 × 10^5 of human iPS cells, preserved in 0.2 mL STEM-CELLBANKER from a liquid nitrogen tank or an ultra-deep freezer (-150°C) and thaw in a water bath at 37°C.

Critical step: Do not leave the frozen cells in the water bath for too long, as this could cause cell death.

7. Immediately after thawing, transfer the cell suspension into 5 mL of StemFit™ medium in the 15-mL conical tube.

Critical step: Do not perform manual pipetting more than twice, to reduce the risk of cell damage.

8. Centrifuge the cells at 160 × *g* for 5 min at room temperature (20-28°C).

9. Remove the supernatant.

10. Add 0.5 mL of StemFit™ medium and pipette the cells.

11. Count the collected cells using a Countess® Machine immediately after staining with Trypan blue (settings for Countess®: sensitivity = 5, minimum size = 8, maximum size = 30, circularity = 75).

12. Seed cells on the LN511E8 coated 6-well culture plates (from Step 4) at 65,000 cells/well.

Critical step: Spread the cells evenly after seeding.

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

14. The next day, replace the medium with fresh StemFit™ medium without Y-27632 and incubate at 37°C in 5% CO_2 up to day 7. The medium should be changed every two days.

TROUBLESHOOTING

Human iPS cell maintenance and subculture: *Cell passaging* **TIMING at least 7-21 d**

15. Prepare LN511E8-coated 6-well culture plates as described in Steps 1-4.

16. Remove the culture medium from human iPS cells.

17. Wash the cells once with 1 mL of PBS.

18. Add 300 µL of dissociation solution (50% (vol/vol) TrypLE Select and 50% (vol/vol) 0.5 mM EDTA solution) and incubate for 4-5 min at 37°C.

19. Remove the dissociation solution.

Critical step: Perform this step carefully because the human iPS cells are still attached to the culture plates at this point.

20. Wash the cells once with 2 mL of PBS and then add 1 mL of StemFit™ medium containing 10 µM Y-27632.

21. Detach the cells from the culture plates mechanically using a Cell Scraper.

22. Pipette the cells about 10 times with a 200- or 1000-µL micropipette and transfer the dissociated ones to a 1.5-mL tube.

23. Count the collected human iPS cells using a Countess® Machine immediately after staining with Trypan blue (settings for Countess®: sensitivity = 5, minimum size = 8, maximum size = 30, circularity = 75).

24. Seed the cells on LN511E8 coated 6-well culture plates (from Step 15) at 13,000 cells/well.

Critical step: Spread out the cells evenly after seeding.

25. Incubate overnight at 37°C in 5% CO_2.

26. The next day, replace the medium with fresh StemFit™ 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 STEP : In modified protocols iPS cells might have been maintained in conditions other than those described above (for example, using co-culture with MEF feeder layers or in other feeder-less culture systems). If this is the case, after the removal of the feeder cells the dissociated iPS cells should be seeded in the StemFit™/LN511E8 culture system at 13,000 cells/well and passaged at least three to four times to adapt to the changed conditions.

CRITICAL STEP: We used human iPS cells before passage 40 for our differentiation experiments, but we have no definitive data regarding the influence of cell passage number on iPS cells and their differentiation.

Differentiation culture: ***Preparing human iPS cells for differentiation culture*** **TIMING 10 d**

27. Coat a 6- or 12-well culture plate with LN511E8 at 0.5 μg/cm^2 as described in Steps 1-4.

28. Harvest human iPS cells by the same method used for their passaging in steps 15-26.

29. Seed the dissociated cells on 6-well plates at 1500-6000 cells/well, or on 12-well plates at 500-2000 cells/well in 1.5 mL (6-well plate) or 0.6-mL (12-well plate) of StemFit™ medium containing 10 μM Y-27632.

Critical step: The initial seeding density is important. To generate SEAMs, a relatively low cell density (e.g. 1500-4500 cells/6-well plate) is recommended. This will help to avoid the fusion of colonies. We found that to successfully generate corneal epithelial progenitor cells a relatively high cell density (e.g. 4500-6000 cells/6-well plate) is required. The high seeding density, however, is accompanied by the associated risk that colonies might fuse, which makes it difficult to obtain regular concentric multiple cellular zones within all SEAMs. For neural crest induction in SEAM zone-2, a lower cell density (e.g. 1000-2000 cells/6-well plate) is suitable.

30. Incubate overnight at 37°C in 5 % CO_2.

31. The next day, 24 hrs after seeding, replace the medium with fresh StemFit™ medium without Y-27632.

32. Incubate the plates at 37°C in 5% CO_2 (for 10 days in total). Change the medium every two days before day 7 and every day thereafter.

Differentiation culture: ***Ocular cell differentiation culture (Day 0-)*** **TIMING 4 w**

33. After 10 days in culture in StemFit™ medium, replace it with DM and use volumes of 2 mL (in a 6-well plate) or 1 mL (in a 12-well plate) (Day 0).

34. Incubate at 37°C in 5% CO_2 for 4 weeks, changing the medium every 2-3 days to achieve three changes per week.

At 3-4 weeks, approximately 60-70% of human iPS cell colonies should appear as circular

colonies comprised of 3-4 concentric zones[8]. As mentioned previously, this type of colony has been termed a "SEAM" -- a self-formed ectodermal autonomous multi-zone[8]. To verify the existence of the ocular cell lineages in each zone, immunostaining for ocular cell makers is useful (BOX 1). Within a SEAM cells in the third zone from the center have characteristics of ocular surface ectodermal cells (PAX6/p63-double-positive phenotype) and the ability to differentiate into functional corneal epithelial cells.

TROUBLESHOOTING

Differentiation culture: *CDM culture (Day 28-)* TIMING 4 w

35. After 4 weeks of incubation in DM, replace the medium with either 2 mL (in a 6-well plate) or 1 mL (in a 12-well plate) of CDM.

36. Incubate the plates for an additional 4 weeks at 37°C in 5% CO_2 (8 weeks culture in total). The medium should be changed every 2-3 days (around 3 times per week).

Optional: To encourage retinal cell differentiation, change the medium to CEM, rather than CDM, at day 28. After incubation in CEM for an additional 2 to 3 weeks (i.e. 6 to 7 weeks of culture in total) pigmented retinal pigment epithelium (RPE)-type cells can be observed in zone 2 of the SEAM.

Differentiation culture: *Manual pipetting to remove non-epithelial cells (Day 49)* TIMING 1d

37. At around three weeks of incubation in CDM (i.e. after seven weeks of culture in total) perform manual pipetting using a PIPETMAN™ (1000 µL or 200 µL) on a clean bench to remove non-epithelial like cells (e.g. neuronal-type cells, retinal-type cells, and/or lens-type cells) from zones 1 and 2 of the SEAM. Cells in zones 1 and 2 of the SEAM adhere to the culture plates less strongly than cells in zones 3 and 4. Because of this, manual pipetting can be used to rinse away zone 1-2 cells from the SEAM, whilst leaving zone 3-4 cells intact. To do this, simply aspirate some CDM medium from the culture dish and flush it over the SEAM. This can either be carried out using 150-200 µl of medium and a 200 µl PIPETMANTM or 500-800 µl of medium and a 1000 µl PIPETMANTM. Typically, one wash is sufficient using the larger pipette, whereas two or three repeats tend to be needed using the smaller one. In both cases, the pipette tip is larger than the SEAM. Depending on seeding density and closeness of the SEAMs, the larger pipette can often treat several SEAMs in one action. The procedure seems to work best if the pipette is angled away from 90º to the surface of the SEAM.

CRITICAL STEP: Pipetting causes the yield of corneal epithelial-type cells for subsequent cell sorting to be higher than it would have been had manual pipetting not been carried out.

CRITICAL STEP: Caution: Some practice is required here. Do not perform the pipetting too vigorously; otherwise, it will detach not only zone 1 and 2 cells, but those in zones 3 and 4 as well. Also, take care that the pipette tip does not actually touch the SEAM.

CRITICAL STEP: A stereomicroscope is useful, but not essential, for performing this step.

38. After pipetting, discard the medium containing the detached cells, and add fresh CDM to the culture plate.

39. Incubate the plates for one additional week in CDM for eight weeks of culture in total.

Differentiation culture: *CEM culture (Day 56-)* TIMING at least 2-4 w

40. After CDM culture for four weeks (i.e. eight weeks overall), change the medium to 2 mL (in a 6-well plate) or 1 mL (in a 12-well plate) of CEM.

41. Incubate the plates for between two weeks (i.e. 10 weeks in total) and eight weeks (i.e. 16 weeks in total) at 37°C in 5 % CO_2. As before, the medium should be changed every 2-3 days (i.e. three times per week). In most case, 12 weeks is suitable for subsequent cell sorting.

FACS for isolating corneal epithelial stem/progenitor cells (Day 70-): *Cell staining with antibodies.* TIMING at least 3 h

42. After CEM culture, wash the differentiated human iPS cells once with PBS.

43. Add 1mL (in a 12-well plate) or 2 mL (in a 6-well plate) of Accutase™ and incubate for 45 min at 37°C.

44. Perform pipetting several times using a PIPETMAN™ (1000 μL) to dissociate the cells and incubate, once more, for 15 min at 37°C.

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

46. Filter the cells using a Cell Strainer (40 μm) and count the cells.

47. Centrifuge at 280 × *g* for 8 min at room temperature.

48. 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 Alexa647-conjugated TRA-1-60 (TRA-1-60R) antibodies. For isotype control, stain cells with FITC-, PE-, or Alexa647-conjugated non-specific isotype antibodies (for SSEA-4, ITGB4 and TRA-1-60, respectively). For colour compensation on flow cytometry, to be performed according to manufacturer's protocols, stain cells with each single antibody against SSEA-4, ITGB4 and TRA-1-60. The cell density should be less than 1×10^7 cells/mL.

49. Incubate the cells for 1 hr at 4°C, agitating every 20 min.

50. Wash the cells twice with PBS.

51. Re-suspend the cells in PBS by filtration using a Cell Strainer (40 μm).

TROUBLESHOOTING

FACS for isolating corneal epithelial stem/progenitor cells: *Cell sorting* TIMING at least 2.5 h

52. Set up the FACSAriaII machine according to manufacturer's protocol.

53. Subject isotype antibody stained samples and single colour-stained samples sequentially to the FACSAriaII and acquire the data.

54. Perform compensation between the detectors for FITC, PE, and Alexa647 using each of the single-stained cells according to manufacturer's protocols applicable to FACSAriaII.

CRTICAL STEP: A positive region for each antibody staining needs to be defined, and this is defined as a region that contains almost no cells (e.g. less than 0.3%) stained with the corresponding isotype control antibody.

55. Analyze the triple colour-stained cells according to manufacturer's protocols of FACSAriaII. Briefly, a Alexa647 negative region (TRA-1-60 negative cell population) is selected, after which the population is analyzed by FITC (SSEA-4) and PE (ITGB4) using dot plot.

TROUBLESHOOTING

56. Sort the SSEA-4^{+}/ITGB4^{+}/TRA-1-60^{-} population (i.e. the human iPS cell-derived corneal epithelial cell (human iCEC) fraction) to 8 mL of KCM in a STEMFULL™ 15-mL tube. Typically, in TRA-1-60 negative cells, 5-20% of the cells are identified as the human iCEC fraction.

TROUBLESHOOTING

57. Collect human iCECs by centrifugation at 280 × *g* for 8 min at room temperature.

58. Re-suspend the cells in CEM on ice.

TROUBLESHOOTING

Fabrication of human iCEC sheet (Day 70)

59. At this stage, two options exist for the fabrication of human induced corneal epithelial cell (iCEC) sheets. Follow Option A for cultivation on a temperature-responsive culture plate or Option B for longer-term culture on a cell culture insert.

Option A. Cultivation on a temperature-responsive culture plate and cell harvest. **TIMING 10-22 d**

i. Coat a 6-well UpCell® plate with LN511E8 at 0.25-0.5 μg/cm^2 and incubate at 37°C for 60 min.

ii. Seed FACS-sorted SSEA-4$^+$/ITGB4$^+$/TRA-1-60$^-$ cells (i.e. human iCECs) on the 6-well UpCell® plate at 1.5-6.0 ×10^5 cells/well in CEM.

iii. Incubate the plates at 37°C in 5% CO_2 for 5-12 days until cells reach 100% confluence. Change the medium every two days.

iv. After cells reach confluence, replace the medium with CMM.

v. Incubate the cells in CMM for a further 3-10 days at 37°C in 5% CO_2 to allow stratification. We find that the procedure works as long as the CMM incubation is within the 3-10 day window.

Change the medium every two days.

vi. Incubate the UpCell® plate at 20°C for 30 min.

vii. Place a PVDF membrane with a diameter of 25-mm and a 15-mm hole in the centre on the cell sheet to act as a carrier as described in detail in reference 33.

viii. Harvest the cell sheet using fine surgical tweezers under a stereomicroscope. Specifically, detach the peripheral part of the cell sheet from the dish and place it on the membrane. Subsequent to this, it is straightforward to harvest the cell sheet by simply lifting the membrane with the cell sheet using surgical tweezers.

TROUBLESHOOTING

Option B. Insert culture (for longer-term culture or for promoting maturation) **TIMING 10-80 d**

i. Coat a 12-well culture insert with LN511E8 at 0.25-0.5 μg/cm^2 and incubate at 37°C for 60 min.

ii. Seed FACS-sorted SSEA-4$^+$/ITGB4$^+$/TRA-1-60$^-$ cells (i.e. human iCECs) on the 12-well culture inserts at 0.4-1.5 × 10^5 cells/well in CEM.

iii. Incubate the cell culture inserts at 37°C in 5% CO_2 for 5-12 days until cells reach 100% confluence. The medium should be changed every two-to-three days. For longer-term culture, maintain human iCECs on cell culture inserts up to 10 weeks in CEM changing the medium every two-to-three days.

v. To promote the maturation of the confluent cells, change the medium to CMM approximately one week before the culture is completed. Although CMM culture promotes maturation, it is not essential for stratification.

Timing

The entire protocol from the initial preparation of human iPS cells to the fabrication of corneal epithelial cell sheets can take a total of 102 days minimum, but most often, we find that 120+ days is required.

Steps 1-4, Human iPS cell maintenance and subculture: Plate coating: 3 h

Steps 5-14, Human iPS cell maintenance and subculture: Human iPS cell seeding from frozen cell stock: 7 d

Steps 15-26, Human iPS cell maintenance and subculture: Cell passaging: at least 7-21 d

Steps 27-32, Differentiation culture: Preparing human iPS cells for differentiation culture: 10 d

Steps 33-34, Differentiation culture: Ocular cell differentiation culture (Day 0-): 4 w

Steps 35-36, Differentiation culture: CDM culture (Day 28-): 4 w

Steps 37-39, Differentiation culture: Manual pipetting to remove non-epithelial cells (Day 49): 1 d

Steps 40-41, Differentiation culture: CEM culture (Day 56-): at least 2-8 w

Steps 42-51, FACS for isolating corneal epithelial stem/progenitor cells (Day 70-): Cell staining with antibodies: at least 3 h

Steps 52-58, FACS for isolating corneal epithelial stem/progenitor cells: Cell sorting: at least 2.5 h

Step 59 Option A, Fabrication of human iCEC sheet: Cultivation on a temperature-responsive culture plate and cell harvest: 10-22 d

Step 59 Option B, Fabrication of human iCEC sheet: Insert culture (for longer-term culture or for promoting maturation): 10-80 d

BOX1, Recommended method for the characterization of cells in a SEAM: 2-7 d

BOX2, Recommended method for validation of human iPS cell-derived corneal epithelial cell sheets: 2-6 d

See Table 2 for Troubleshooting guidance.

Anticipated Results

Multi-zonal colony (SEAM) formation By four weeks of differentiation, colonies with concentric multi-cellular zones (typically 3-4 zones as shown in Fig. 3A) will have generated spontaneously. For the validation of ocular surface epithelial induction (in zone 3) PAX6 and p63 double-staining is useful (Fig. 3B). When double-positive cells are detected at three-to-four

weeks in the peripheral region of the colony and at 10 weeks overall, the method is deemed to have worked successfully.

Fabrication of corneal epithelial cell sheets. After the removal of non-epithelial cells from the SEAM by pipetting, those remaining at 12 weeks of differentiation are subjected to FACS (Fig. 4A, B). Flow cytometric analysis at this time typically shows that 5-20% of TRA-1-60 negative cells are human iCECs (i.e. SSEA-4^{+}/ITGB4^{+} cells) (Fig. 4C). The FACS-sorted human iCECs typically exhibit a small cobblestone-like morphology by phase contrast microscopy and when observed by immunostaining after 2 to 3 weeks of culture in CEM and CMM have become stratified into two to four layers. The stratified cell sheets express K12 and PAX6 throughout all cell layers, as well as MUC16 (on the sheet's surface) and p63 (strongly in the basal region) (Fig. 5).

BOX1

Recommended method for the characterization of cells in a SEAM Timing: 2-7 d

1. Fix differentiated human iPS cells after four weeks (or two weeks for neural crest staining) of differentiation culture in 4% (wt/vol) PFA at room temperature. Fix for 20 min. For CHX10 and MITF staining, fix cells that had been differentiated by the retinal differentiation protocol (shown in Step 36).
2. Wash the cells with Tris-buffered saline (TBS) three times for 10 min.

3. Incubate the cells in TBS containing 5% (vol/vol) normal donkey serum and 0.3% (wt/vol) Triton X-100 (blocking buffer) for 1 hr at room temperature for blocking of non-specific binding and cell permeabilization. For the mouse monoclonal PAX6 antibody (AD2.35), cell permeabilization should be performed at 4°C for three days.
4. Incubate the cells with each of following primary antibodies; PAX6 (PRB-278P, BioLegend, 1:300), p63 (4A4, Santa Cruz Biotechnology, 1:200), α-Crystallin (SPA-224, Stressgen Biotechnologies, 1:200), TUBB3 (T2200, Sigma-Aldrich, 1:100), SOX10 (N20, Santa Cruz Biotechnology, 1:100), p75 (ME20.4, Advanced Targeting Systems, 1:100), CHX10 (N-18, Santa Cruz Biotechnology, 1:500) and MITF (C5, Exalpha Biologicals, 1:2000) at 4°C overnight or at room temperature for 3 hrs. For the mouse monoclonal PAX6 antibody (AD2.35, Santa Cruz Biotechnology, 1:60), incubate cells at 4°C for 3 days. Dilute antibodies with TBS containing 1% (vol/vol) normal donkey serum and 0.3% (wt/vol) Triton X-100 (dilution buffer).
5. Wash the cells 3 times with TBS for 5 min.
6. Incubate cells with the secondary antibody, which corresponds to the primary antibody. Use a 1:200 diluted with dilution buffer for 1 hr at room temperature in the dark.
7. Wash the cells 3 times with TBS for 5 min.
8. Repeat Steps 3-7 for double immunostaining.

9. Stain cells (for nuclear staining) with Hoechst 33342 for 10 min at room temperature under protection from light.
10. Wash the cells 3 times with TBS for 5 min.
11. Mount the cells with PermaFluor™ and observe by fluorescence microscopy.

BOX 2

Recommended method for validation of human iPS cell-derived corneal epithelial cell sheets Timing: 2-6 d

1. Embed human iCEC sheets in OCT compound and store at -80°C.
2. Cut 10 μm-thick cryostat sections.
3. Dry the frozen sections for at least 2 hrs at room temperature.
4. Wash the slides once with TBS. (For p63 staining, the cells should be fixed with cold methanol for 1 hr before washing with TBS).
5. Incubate the slides in blocking buffer for 1 hr at room temperature.
6. Incubate the slide with each of following antibodies; PAX6 (PRB-278P, BioLegend, 1:300), p63 (4A4, Santa Cruz Biotechnology, 1:200), K12 (N16, Santa Cruz Biotechnology, 1:200), K3 (AE5, PROGEN Biotechnik, 1:200), MUC1 (M4H2, Abcam, 1:200) and MUC16 (OV185:1, Abcam, 1:200) at 4°C overnight or at room temperature for 3 hrs. Antibodies should be diluted with dilution buffer.

7. Wash the slides 3 times with TBS for 5 min.

8. Incubate the slides with the secondary antibody, which corresponds to the primary antibody (1:200 diluted with dilution buffer) for 1 hr at room temperature under protection from light.

9. Wash the cells with TBS 3 times at room temperature.

10. Stain with Hoechst 33342 for 10 min at room temperature under protection from light.

11. Wash the cells three times with TBS for 5 min.

12. Mount the cells with PermaFluor™ and observe by fluorescence microscopy.

Table 1: Antibodies to verify the existence of ocular cell lineages in each zone

Zone	Cell type	Markers	References
1	Neuronal cell	TUBB3 (T2200, Sigma-Aldrich, 1:100)	8), 20)
2 (early period)	Neural crest	p75 (ME20.4, Advanced Targeting Systems, 1:100)/ SOX10 (N20, Santa Cruz Biotechnology, 1:100)	8), 24)
2 (inner side)	Neuro retinal cell	CHX10 (N-18, Santa Cruz Biotechnology, 1:500)	8), 23)
2 (outer side)	Retinal pigment epithelial cell	MITF (C5, Exalpha Biologicals, 1:2000)	8), 23)
2-3	Lens cell	PAX6 (AD2.35, Santa Cruz Biotechnology, 1:60)/ α–Χρψσιταλλιν (SPA-224, Stressgen Biotechnologies, 1:200)	25), 26)
3	Ocular surface epithelial cell	PAX6 (PRB-278P, BioLegend, 1:300)/ p63 (4A4, Santa Cruz Biotechnology, 1:200)	8), 18), 19)
4	Epithelial cell	p63 (4A4, Santa Cruz Biotechnology, 1:200)	21), 22)

Table 2. Troubleshooting Table

Step	Problem	Possible reason	Solution
Step 14 and 26	Human iPS cells are not in a good condition or in an unstable condition.	1) Cells are differentiated	To verify whether or not human iPS cells have maintained an undifferentiated status, examine the expression of pluripotent stem cell markers, TRA-1-60 and SSEA-4 by immunostaining. If cells have differentiated, restarting the iPS cell culture is recommended.
		2) The duration of Y-27632 treatment is too short or too long.	Incubate human iPS cells in Y-27632-containing StemFit™ medium for 24 to 30 hr after cell seeding. An appropriate period of Y-27632 treatment is essential for the survival of human iPS cells during cell passages.
Step 26	The colony forming efficiency (CFE) of human iPS cells at cell passaging is too low or too high. (N.B. The CFE of the 201B7 human iPS cell line is around 6-14% in our experiments). If a satisfactory outcome is not achieved, it is likely to reflect changes in cell characteristics during passaging. If subsequent SEAM formation and corneal epithelial cell expansion is not achieved, restarting the iPS cell culture is recommended.	1) LN511E8-coating on culture plates is insufficient	Do not allow the surface of the culture plates to dry at steps 3 and 15. Add StemFit™ medium immediately after the removal of the coating solution.
		2) Human iPS cells are damaged by enzymatic treatment or mechanical harvesting at cell passaging	Keep the treatment time with the dissociation solution brief at around 4-5 min, and gently harvest human iPS cells using the Cell Scraper at cell passaging.
		3) The duration of Y-27632 treatment is too short or too long.	Incubate human iPS cells in Y-27632-containing StemFit™ medium for 24 hr to 30 hr after cell seeding. An appropriate treatment with Y-27632 is essential to ensure survival of human iPS cells during passaging.

Step 34	Cells do not generate zone 3, nor a typical SEAM structure by 4 weeks of differentiation culture.	1) The cell density is not appropriate.	Seed human iPS cells at several cell densities ranging from 1500 to 6000 cells/well (6-well plate) or 500 to 2000 cells/well (12-well plate) because the seeding density has a significant influence over the formation of a SEAM and the induction of zone 3. When the seeding density is high, immunostaining for PAX6 and p63 is useful to detect zone 3 because it can become difficult to identify by microscopy alone.
		2) The human iPS cell clone might not be appropriate for conditions in the protocol we describe here.	The best results were achieved with clone 201B7, and the protocol might need optimization for other clones.
Step 51	The yield of cells after antibody staining is low.	Cells might have been lost in collection tubes during antibody staining.	Use low absorbing tubes (StemFull™) and serum containing medium, CMM. This is recommended to prevent cell absorption to tubes during incubation with antibodies.
Step 55	The ratio of the corneal epithelial cell fraction is low (i.e. around 5% or lower) based on FACS analysis. In this case, restarting the differentiation culture is strongly recommended, although possible explanations and solutions are indicated here.	1) The culture period is too short.	Prolong the culture period to 12 weeks. In some cases, even 10 weeks is too brief to obtain a sufficient amount of human iCECs.
		2) Manual pipetting of cells outside SEAM zone 3 is not sufficient.	Perform manual pipetting carefully to remove the non-epithelial like cells that reside in zone 1 and 2 of the SEAM. Even though manual pipetting is not essential to obtain human iCECs, it greatly improves the efficiency of the final corneal epithelial-like cell yield.
Step 56	The yield of sorted human iCECs is low after FACS.	Sorted human iCECs might have been lost in collection tubes.	Use low absorbing tubes (StemFull™) and serum containing medium, CMM. This is recommended to prevent cell absorption to tubes.

Step 59	Human iCECs do not form epithelial cell sheets.	1) There is a low cell density at human iCEC seeding.	Seed human iCECs at an appropriate cell density as described in step 59. A low seeding density tends to promote EMT (epithelial-to-mesenchymal transition) or the proliferation of cells without corneal epithelial characteristics. When sufficient numbers of human iCECs, as shown in step 59, are not collected after FACS, we recommend redoing the FACS.
		2) The human iCEC shrinks after reaching confluence in CEM.	Change CEM to CMM before reaching 70-80% confluence.
		3) The culture period is too short.	To ensure that human iCECs become fully stratified they should be cultivated, first in CEM and then CMM, for at least 14 days combined time.

Figure legends

Figure 1

Schematic illustration outlining the reconstruction of corneal epithelial tissue from human iPS cells via SEAM formation

PART I: Induction of a SEAM containing various ocular cell anlages (Steps 1-34), indicating human iPS cell maintenance culture, preparation for differentiation culture, and the induction of multiple ocular cell lineages that form the SEAM.

PART II: Isolation of corneal epithelial stem/progenitor cells from a SEAM and the steps required to reconstruct corneal epithelial tissue *in vitro* (Steps 35-59). This includes corneal epithelial cell differentiation and isolation, and the generation of corneal epithelial cell sheets using SEAM-derived corneal epithelial stem/progenitor cells.

Figure 2

The protocol for SEAM formation and fabrication of corneal epithelial cell sheets.

Human iPS cells should be passaged three to four times in StemFit™ medium on an LN511E8-coated dish. Differentiation culture for inducing SEAM formation should then be initiated by changing the medium to DM. Corneal epithelial cells can be further induced by changing the medium to CDM (at four weeks) and then to CEM (at eight weeks); these contain KGF and Y-

27632 for promoting corneal differentiation. After CEM culture at around 12 weeks of differentiation, the corneal epithelial cells, including stem/progenitor cells, can be isolated by FACS and cultivated again in CEM and then CMM to develop corneal epithelial tissue. DM: Differentiation medium, CDM: Corneal differentiation medium, CEM: Corneal epithelial maintenance medium, CMM: Corneal epithelium maturation medium.

Figure 3

Characterization of cells in a SEAM

(A) Phase-contrast images for undifferentiated human iPS cell colonies and a SEAM after four and six weeks of differentiation culture. Scale bars, 200 μm. The macro photo (scale bar, 2 mm) shows several SEAMs, variously sized.

(B) Characterization of cells in a SEAM by immunostaining for ocular cell-related markers: PAX6 (PRB-278P, BioLegend, 1:300, green) and p63 (4A4, Santa Cruz Biotechnology, 1:200, red) indicate corneal epithelial cells at four weeks of differentiation; PAX6 (AD2.35, Santa Cruz Biotechnology, 1:60, green) and α-crystallin (SPA-224, Stressgen Biotechnologies, 1:200, red) indicate lens cells at four weeks of differentiation); TUBB3 (T2200, Sigma-Aldrich, 1:100, green) and p63 (4A4, Santa Cruz Biotechnology, 1:200, red) indicate neuronal/epithelial cells at four weeks of differentiation); p75 (ME20.4, Advanced Targeting Systems, 1:100, green) and

SOX10 (N20, Santa Cruz Biotechnology, 1:100, red) indicate neural crest cells at two weeks of differentiation); MITF (C5, Exalpha Biologicals, 1:2000, green) and CHX10 (N-18, Santa Cruz Biotechnology, 1:500, red) indicate retinal cells at seven weeks of differentiation using the protocol for retinal differentiation. CRYA: α-crystallin, Nuclei, Blue. Scale bars, 100 µm.

Figure 4

Isolation of corneal epithelial cells from a SEAM

(A) Enrichment of corneal epithelial-type cells from zone 3 of the SEAM by manual pipetting at seven weeks of differentiation. Scale bars, 200 µm.

(B) A 12-week differentiated SEAM-derived cells that underwent pipetting. Scale bars, 200 µm.

(C) Isolation of corneal epithelial cells by FACS using anti-TRA-1-60, SSEA-4 and ITGB4 antibodies from 14-week differentiated human iPS cells (upper graphs). In the TRA-1-60 negative cell population, 12% of the cells are corneal epithelial cells defined as SSEA-4^+/ITGB4^+ cells (range typically 5-20%). The results for the cells stained with isotype control antibodies are shown in lower graphs indicating almost no cells (<0.1%) in the SSEA-4^+/ITGB4^+ region.

Figure 5

Fabrication of a corneal epithelial cell sheet

Immunostaining of human SEAM-derived cell sheets (upper panels; 23 days: lower panels; 76 days of cultivation on cell culture inserts after FACS) for corneal epithelial-related markers, PAX6 (PRB-278P, BioLegend, 1:300), p63 (4A4, Santa Cruz Biotechnology, 1:200), K12 (N16, Santa Cruz Biotechnology, 1:200), K3 (AE5, PROGEN Biotechnik, 1:200), MUC1 (M4H2, Abcam, 1:200) and MUC16 (OV185:1, Abcam, 1:200). Nuclei stain red. Scale bars, 50 μm.

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Author contributions

R.H., and K.N. designed the research; R.H., Y.I., R.K., and H.T. performed the experiments on human iPS cells differentiation culture and acquired the data; Y.T. performed the experiments on human iPS cell maintenance in culture and acquired the data; Y.I and R.K performed the cell sorting experiments and acquired the data; R.H., Y.I., R.K., Y.T. and H.T. analyzed the data and provided technical assistance to the protocol; Y.S., M.T. and A.J.Q supervised the project and provided critical advice; K.S. provided reagents (LN511E8); and R.H. and A.J.Q wrote the manuscript.

Competing financial interests

The authors declare that they have no competing financial interests.