PROTOCOL

In vitro culture and expansion of human limbal epithelial cells

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Limbal stem cells (LSCs) have an important role in the maintenance of the corneal surface epithelium, and autologous cultured limbal epithelial cell transplantations have contributed substantially to the treatment of the visually disabling condition known as LSC deficiency. In this protocol, we describe a method of establishing human limbal epithelial cell cultures by a feeder-free explant culture technique using a small limbal biopsy specimen and human amniotic membrane (hAM) as the culture substrate. This protocol is free of animal-derived products and involves the use of human recombinant growth factors. In addition, the recombinant cell dissociation enzyme TrypLE is used to replace trypsin and autologous serum replaces FBS. It takes ~2 weeks to establish a confluent monolayer from which ~3 × 10^6 cells can be harvested. This procedure can be adopted for both basic research purposes and clinical applications.

INTRODUCTION

The cornea is the transparent window on the ocular surface that allows light rays to pass through the anterior chamber; it contributes to over 60% of the total refractive power of an eye. The corneal surface consists of a stratified epithelial layer that is ~5–6 cells in thickness. The narrow zone of ~2-mm thickness between the cornea and the bulbar conjunctiva is known as the limbus and is widely accepted as the niche for corneal epithelial stem cells[1–3]. Limbal stem cells (LSCs) have an important role in the regular maintenance of the corneal surface epithelium. In case of any injury or during normal homeostasis, the activated stem cells from the limbus migrate centripetally to the central cornea and help in tissue regeneration. Several factors can lead to LSC deficiency (LSCD), such as chemical (alkali/acid) or thermal injury, ultraviolet and other ionizing radiation, repeated surgical interventions, extensive microbial infection and immunological disorders such as Stevens-Johnson Syndrome, and even long-term use of contact lenses in some cases. LSCD is typically characterized by the invasion of conjunctival epithelium onto the corneal surface leading to conjunctivalization, neovascularization, subepithelial scarring and symblepharon formation; this results in corneal opacity and visual impairment and varying degrees of discomfort, including redness, irritation and watering in the affected eye[4,5]. Previously, ocular surface disorders were generally managed by surgical procedures such as transplantation of human amniotic membrane (hAM)[6] or of biopsied healthy limbal tissues[7,8]. Over the past decade, transplantation of *in vitro*–cultured, autologous limbal epithelial cell sheets grown on different culture substrates (including hAM, with or without mitotically inactivated NIH 3T3 feeder cells) has gained popularity and has achieved an appreciable success rate in terms of ocular surface reconstruction and visual outcomes[9–16]. This approach also minimizes the size of donor tissue required and generates an epithelial sheet for reconstructing the diseased ocular surface, thus helping to replenish the depleted stem cell pool.

Similar to many other epithelial cell types, limbal epithelial cells also require special substrates for *in vitro* culture and expansion. Various substrates have been used for culturing limbal epithelial cells, such as fibrin gels[17], Myogel[18], plasma polymer–coated surfaces[19], biodegradable matrices made of human recombinant collagen[20], temperature-responsive culture inserts[21], mitotically inactivated feeder layers of mouse NIH 3T3 cells[9], hAM[10] and human limbal stromal fibroblasts. Limbal epithelial cells can be expanded by explant culture or by isolating them from donor tissues enzymatically and then culturing them on some of the above-mentioned substrates, either in the presence or in the absence of a mitotically inactivated NIH 3T3 feeder layer. The use of murine NIH 3T3 cells, FBS and trypsin in cultures may pose the threat of transmission of animal-derived pathogens and immunological problems during transplantation. Therefore, the use of human-derived sources such as hAM or human limbal stromal keratocytes as feeders and recombinant protein sources might overcome this issue. However, the use of synthetic and biodegradable polymers would completely avoid the issue of donor tissue–derived cryptic infections and disease transmission.

In this paper, we describe a detailed protocol for *in vitro* expansion of limbal epithelial cells (LECs) on hAM using a small limbal tissue biopsy sample (2 × 2 mm^2). This biopsy sample is either freshly harvested—autologously from the healthy eye of an individual or allogenically from a live donor—or obtained from a registered eye bank from the limbal rings of cadaveric donor corneas. The protocol is devoid of any animal-derived products; hAM is used as the culture substrate, the recombinant cell dissociation enzyme solution TrypLE replaces trypsin and culture components including human recombinant growth factors (epidermal growth factor (EGF) and insulin), and autologous serum replaces FBS. This method does not require the use of mouse NIH 3T3 fibroblast feeder cells (see **Box 1**). This protocol can therefore be adopted both for clinical applications and for basic research purposes involving the study of LSCs. It has been established on the basis of our long-term experience in LSC therapy, and has been continuously modified over the years on the basis of earlier reports from our group and from others[12–16,22–25].

BOX 1 | SALIENT FEATURES OF THIS CULTIVATION TECHNIQUE

1. It is a feeder-free explant culture system.
2. It is a xeno-free culture system that uses autologous serum, recombinant enzymes and human growth factors and is devoid of animal-derived products.
3. It is a submerged culture technique (promotes stem cell maintenance), in contrast to an airlift technique (promotes differentiation).
4. The expanded cells are transplanted at monolayer stage to allow *in vivo* stratification.
5. The use of this technique allows the reduction of total culture time from 4 to 2 weeks.

The explant culture technique used in this protocol is equally efficient in generating a limbal epithelial monolayer in comparison with suspension culture methods[26]. It does not involve any enzymatic treatment and thereby prevents any loss of residual stem cells during processing; therefore, it is a better option in the case of biopsy samples collected from a partially damaged eye in bilateral cases of LCSD. The submerged culture technique prevents early differentiation and retains more stem cells as opposed to the stratification and differentiation that is observed in extended airlift culture techniques. Human amniotic membrane is an inexpensive biological source of cell culture scaffold and is known to be hypoimmunogenic because of the incomplete expression of HLA antigens and also because it helps in reducing any immunological problems during transplantations and gets integrated into the corneal stroma over time. The expression of several growth factors and cytokines in hAM promotes epithelial growth while suppressing the initial wound-induced inflammation and corneal stromal scarring[27,28]. Furthermore, limbal epithelial sheets established on hAM can be easily handled and are amenable to suturing. Although the amniotic membrane used is from a human source, the risk of pathogen transmission can be negated after an extensive microbiological screening to confirm and certify the membrane's pathogen-free status before using it for clinical or research purposes. However, it is a thick tissue (~75–100 μm) and can therefore influence the corneal thickness as it gets incorporated into the corneal stroma after transplantation; this, in turn, can affect light refraction and the immediate visual outcome. However, our experience and reports from several groups have shown that hAM is gradually biodegraded over time (between 2 and 6 months) and does not affect long-term visual outcome.

Human amniotic membrane can be prepared from placentas (collected with the consent of the donor during cesarean deliveries to ensure minimal physical damage and sterility of the tissue) after screening for any cryptic infections such as HIV, hepatitis B, hepatitis C and syphilis. In people with LSCD, limbal tissues can be harvested for autologous applications from a healthy eye (contralateral biopsy) or from a healthy area of an affected eye (ipsilateral biopsy). The biopsy can be carried out under local or general anesthesia at the surgeon's discretion. Alternatively, limbal tissues can be collected for allogenic applications from live related donors or from donor volunteers undergoing cataract surgerys. A small limbal tissue sample of ~2 × 2 mm^2 in size and ~150–200 μm in thickness should be sufficient for explant culture covering an area of hAM of ~3 × 2.5 cm^2 in size. Limbal biopsy should be performed by a trained ophthalmic surgeon in a sterile operating environment. Cadaveric limbal tissues of donor eyes collected and preserved by a registered eye bank can also be used for this purpose. The limbal rings obtained from donor corneas (after the central cornea has been harvested for corneal transplantation) can also serve as a tissue source for limbal epithelial cells. Approvals from appropriate institutional review boards (IRBs) and ethics committees, as well as from other specific regulatory authorities, are mandatory for conducting research involving human subjects and tissues; strict compliance with the regulatory guidelines is of utmost importance. Appropriate informed consent must be obtained from volunteers before taking limbal biopsy samples. The entire protocol takes ~2 weeks to establish a uniform monolayer of limbal epithelial cells on hAM of ~3 × 2.5 cm^2 effective size.

MATERIALS

REAGENTS

- MEM Eagle's medium with alpha modification (MEM; Sigma, cat. no. M0644)
- Nutrient mixture, Ham's F-12 (F-12; Sigma, cat. no. N6760)
- DMEM powder (Sigma, cat. no. D5648)
- Sodium bicarbonate (Sigma, cat. no. S5761)
- Dulbecco's phosphate-buffered saline (PBS; Sigma, cat. no. D5652)
- L-Glutamine (Sigma, cat. no. G6392)
- HEPES (Sigma, cat. no. H4034)
- Penicillin-streptomycin (100× solution; Sigma, cat. no. P4333)
- Amphotericin B (100× solution; Sigma, cat. no. A2942)
- Human recombinant epidermal growth factor (Sigma, cat. no. E9644)
- Human recombinant insulin (Sigma, cat. no. I2643)
- Fetal bovine serum (FBS; Sigma, cat. no. I2003C)
- TrypLE (1× solution; Invitrogen, cat. no. 12604)
- Glycerol (Sigma, cat. no. G2025)
- Mycoplasma detection Kit (Roche, cat. no. 11296744001)
- Genscreen HIV-1/2 version 2 (Bio-Rad, cat. no. 72278)
- Monolisa HBs Ag ULTRA (Bio-Rad, cat. no. 72348)
- Syphilis EIA II (Bio-Rad, cat. no. 72519)
- Monolisa Anti-HCV PLUS version 2 (Bio-Rad, cat. no. 72318)
- Human amniotic membrane (cryopreserved in 50% (vol/vol) glycerol and DMEM) ▲ **CRITICAL** Institutional review board approval and informed consent from the donor are mandatory before collecting the tissue biopsy sample.
- Thioglycollate medium (powder, HiMedia, cat. no. M979)
- Nutrient agar medium (powder, HiMedia, cat. no. M001)
- Sterile Milli-Q water
- Hydrochloric acid (HCl; Fisher, cat. no. A142-212)
- Sodium hydroxide (NaOH; Fisher, cat. no. S320-1)
- Bovine serum albumin (BSA; Sigma, cat. no. A7906)
- Ringer's lactate saline solution (Claris Lifesciences)
- 1% (vol/vol) Hypochlorite solution (Loba Chemie, cat. no. 283)
- Limbal biopsy from healthy eye tissue ▲ **CRITICAL** Institutional review board approval and informed consent from the donor are mandatory before collecting the tissue biopsy sample.

EQUIPMENT

- Class II biosafety cabinet (Telstar, model: Bio II A)
- CO_2 incubator (Thermo Fisher Scientific, model: 371)
- Refrigerated centrifuge (Eppendorf, model: 5810R)
- Inverted phase-contrast microscope (CK40, with CCD camera; Olympus)
- Upright freezer (−80 °C, Forma 900 Series; Thermo Scientific, cat. no. 906)
- Hot-air oven (Binder, model: D53)

- Microplate absorbance reader (Bio-Rad, model: iMark)
- Filtering units (Steritop-GP, Millipore, cat. no. SCGPT10RE)
- Vacuum pump (Millipore, cat. no. VACPMPKIT)
- Sterile syringe filters (0.22-µm pore, Millipore, cat. no. SLGP033RS)
- VACUETTE tubes (with serum clot activator, 4 ml; Greiner Bio-One, cat. no. 454092)
- Tissue culture dishes (53 mm, TPP, cat. no. 93060)
- Sterile conical centrifuge tubes (50-ml; Tarson, cat. no. 500040)
- Microfuge tubes (Axygen, cat. no. MCT-150-C)
- Pipette aids (Gilson, cat. nos F110751 and F123602)
- Disposable plastic pipettes (Costar-Corning, cat. no. 4488)
- Disposable plastic pipette tips (Axygen, cat. nos T-1000-B-R, T-200-Y-R and T-300-R)
- Glass bottles for media and buffer storage (Schott)
- Screw-capped glass vials for cryopreservation of hAM (Schott)
- Sterile surgical blades (Bard-Parker, cat. no. 21)
- Sterile latex surgical gloves (Surgicare)
- Gas-sterilized nitrocellulose paper (Sigma, cat. no. N7892)
- Gas-sterilized polyethylene pouches for placenta collection
- Blood collection tubes for preparing serum (Greiner Bio-One, cat. no. 454092)
- Blood collection tubes for molecular diagnostics (Greiner Bio-One, cat. no. 454235)
- Water purification system (Millipore)
- Ethylene oxide gas sterilizer (Axis)
- 0.20-µm pore, DMSO-compatible sterile syringe filters (Millipore, cat. no. SLLG025SS)
- Falcon conical tubes, 15 ml and 50 ml (BD, cat. no. 352196, 352070)
- Sterile microscopic glass slides (cut to dimensions of 3 × 2.5 cm^2, Corning, cat. no. 2947-75X25)
- Glass cutter
- Syringe (20 ml)
- Sterile 24-gauge needles
- Sterile sharp- and curve-tipped forceps
- Sterile blunt-ended forceps

REAGENT SETUP

L-Glutamine (100× solution) Reconstitute one vial with 10 ml of sterile 1× PBS to prepare a 200 mM stock solution. Filter-sterilize the solution using a 0.22-µm syringe filter (low protein binding) and store at 4 °C for use within 1 week or store at −20 °C for up to 6 months.

PBS (1× solution) Dissolve the contents of a packet (for 1 liter) of PBS in 900 ml of Milli-Q water and adjust the pH to 7.2 with 1 N HCl or 1 N NaOH while stirring; adjust the final volume to 1 liter with Milli-Q water and sterilize using an autoclave. Store at 4 °C for up to 1 week.

Human recombinant EGF (2,000× stock solution; 20 µg ml^{-1}) Prepare 1× PBS solution containing 0.1% (wt/vol) BSA and filter-sterilize it using a 0.22-µm syringe filter (low protein binding). To prepare 20 µg ml^{-1} of EGF stock solution, reconstitute a vial of 200 µg of human recombinant EGF in 10 ml of sterile PBS containing 0.1% BSA. Prepare 200-µl aliquots and store at −20 °C for up to 6 months.

Human recombinant insulin (2,000× stock solution; 10 mg ml^{-1}) Prepare 1% (vol/vol) HCl solution and filter-sterilize it using a 0.22-µm syringe filter. To prepare 10 mg ml^{-1} of stock solution, reconstitute a vial of 50 mg of human recombinant insulin in 5 ml of sterile 1% HCl solution. Prepare 200-µl aliquots and store at −20 °C for up to 6 months.

Human corneal epithelial (HCE) growth medium HCE growth medium is composed of MEM/F12 (1:1) solution containing 10% (vol/vol) autologous serum, 2 mM L-Glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2.5 µg ml^{-1} amphotericin B, 10 ng ml^{-1} human recombinant EGF and 5 µg ml^{-1} human recombinant insulin. To prepare person-specific growth medium, add 5 ml of the person's serum, 0.5 ml of L-Glutamine stock solution, 0.5 ml of penicillin-streptomycin stock solution, 0.5 ml of amphotericin B stock solution, 25 µl of EGF stock solution and 25 µl of insulin stock solution and adjust the volume to 50 ml with MEM/F12 (1:1) solution; filter-sterilize the solution using a 0.22-µm syringe filter (low protein binding) and store at 4 °C for up to 1 week. Alternatively, FBS can be used in the place of autologous serum for culturing limbal epithelial cells for basic research purposes. Perform a sterility check by streaking cells onto chocolate agar and blood agar plates and by inoculating plates in thioglycollate broth before use (see **Box 2**).

Freezing solution for hAM (DMEM containing 50% glycerol) Prepare 1 liter of 1× DMEM solution, filter-sterilize it, run a sterility check and certify it (in regard to pathogen-free status) for further use. Sterilize glycerol by autoclaving. Combine equal volumes of sterile DMEM solution and glycerol in a sterile hood, mix thoroughly and prepare 30-ml aliquots in sterile glass containers for cryopreservation of the processed amniotic membranes. ▲ **CRITICAL** The hAM freezing solution should be freshly prepared and aliquotted into 30-ml sterile glass vials for immediate use.

Freezing solution for limbal epithelial cells (DMEM containing 10% DMSO and 40% FBS) To prepare 10 ml of freezing solution, combine 5 ml of sterile DMEM solution, 4 ml of FBS and 1 ml of DMSO, mix thoroughly, and filter-sterilize it using a 20-µm, DMSO-compatible syringe filter; store the solution on ice. ▲ **CRITICAL** Prepare fresh freezing solution for immediate use.

Thioglycollate broth This is an enriched, differential growth medium used primarily to differentiate aerobic, anaerobic, microaerophilic and facultative anaerobic organisms on the basis of their growth at different levels in the medium. It is a reducing medium that contains sodium thioglycollate, which reacts with molecular oxygen and keeps free oxygen levels low. This produces a range of oxygen levels in the medium that decrease with increasing distance from the surface. Anaerobic organisms grow in the lower layers and aerobic organisms grow in the top layers of the medium. To prepare 1 liter of medium, dissolve 29.7 g of powdered thioglycollate medium in ~900 ml of Milli-Q water, warm the solution to dissolve the medium completely, and

BOX 2 | STERILITY CHECK FOR GROWTH MEDIUM AND CULTURES

Mycoplasma contamination in cultured cells is detected by ELISA to check for the most common mycoplasma and/or acholeplasma species (*Mycoplasma arginini, Mycoplasma hyorhinis, Acholeplasma laidlawii, Mycoplasma orale*) that generally contaminate mammalian cell cultures. A sterility check should also be carried out to screen for both aerobic and anaerobic microorganisms that can contaminate a culture; this is done by conducting grow-out tests on freshly prepared medium or culture supernatants.

Mycoplasma detection

1. Mycoplasmas are detected in cell culture supernatants using an antibody-based ELISA kit according to the manufacturer's instructions (see REAGENTS). The antibodies used in the assay are biotin conjugated and are specific to some of the most common species of mycoplasma and/or acholeplasma. Streptavidin–alkaline phosphatase conjugate and a suitable substrate for the enzyme that can give rise to a colored product are used for signal detection.
2. The colored reaction product is quantified using a microplate (ELISA) reader. An optical density (OD) value of ≥0.2 at 405 nm absorbance is considered significant.

Aerobic and anaerobic microorganism detection

1. For the detection of aerobic and anaerobic microorganisms, inoculate a few drops of the medium or the culture supernatant into 5 ml of thioglycollate broth and then streak them onto chocolate agar and blood agar plates.
2. Incubate the cultures in a bacteriological incubator at 37 °C for ~3–4 d.
3. Observe the cultures for any growth. Approve the medium for culture use once it has been cleared by the sterility check.

adjust the pH to 7.2 with 1 N HCl or 1 N NaOH while stirring. Adjust the volume to 1 liter and sterilize by autoclaving at 15 p.s.i. at 121 °C for 15 min. The broth can be checked by a grow-out test by incubating the broth at 37 °C in a shaker incubator for 2–3 d and then certified for further use. Store the medium at room temperatures below 30 °C for up to 2 weeks. ▲ CRITICAL Do not use the medium if there are any signs of contamination and deterioration (discoloration or evaporation).

Blood agar plate This is an enriched, nonselective but differential growth medium used to identify pathogenic β-hemolytic bacterial species such as *Streptococcus* from normal nonpathogenic microflora. This medium contains 5–10% fresh sheep's blood. Blood should be collected intravenously in a sterile, heparinized bag. To prepare 1 liter of medium, dissolve 28 g of powdered nutrient agar medium in ~900 ml of Milli-Q water, warm the solution to dissolve the medium completely, and adjust the pH to 7.4 with 1 N HCl or 1 N NaOH while stirring. Adjust the volume to 1 liter and sterilize by autoclaving at 15 p.s.i. at 121 °C for 15 min. To prepare the complete medium, add 5% fresh sheep's blood when the medium is lukewarm (40–45 °C) and plate out into a 10-cm bacteriological culture plate and allow it to set inside a biosafety cabinet. Plates can be checked by grow-out test by incubating them at 37 °C for 2–3 d and then certifyied for further use. The medium is light and temperature sensitive. Store the plates in the dark at 2–8 °C for up to 2 weeks. ▲ CRITICAL Do not use the medium if there are any signs of deterioration (shrinking, cracking or discoloration), hemolysis or any contamination.

Chocolate agar plate This is a variant of the blood agar plate and is prepared similarly. It is an enriched, nonselective growth medium used for growing fastidious bacterial species. It contains 5% sheep's blood that has been lysed by heating the blood very slowly to 56 °C. The medium is light sensitive. Store the plates in the dark at 2–8 °C for up to 2 weeks. ▲ CRITICAL Do not use the medium if there are any signs of deterioration (shrinking, cracking or discoloration), hemolysis or any contamination.

PROCEDURE

Collection of limbal biopsy and blood samples ● TIMING 1 d

1| Obtain a limbal tissue biopsy specimen (2 × 2 mm^2) (**Fig. 1a**). Briefly, the procedure involves making an incision, using a no.15 blade on a Bard-Parker handle, at the conjunctiva of the eye 3 mm behind the limbus (preferably at 12 o'clock position for contralateral biopsies) and dissecting toward the limbus superficially and into the clear cornea up to 1 mm. The conjunctiva has to be excised out at the limbus just behind the pigmented line (Palisades of Vogt), preserving the limbal tissue with 1 mm of clear cornea[14].

! CAUTION For fresh tissues, biopsy should be carried out by a trained ophthalmic surgeon. For donor samples from an eye bank, preserved less than 48 h are preferred. Collect the tissues only if they have passed the routine serological testing carried out in the eye bank for any infectious conditions.

▲ CRITICAL STEP Approval from appropriate regulatory agencies and the donor's informed consent are mandatory before collecting the tissue biopsy specimen.

2| Transfer the biopsied limbal tissue to a sterile 1.5-ml microfuge tube containing 1 ml of HCE growth medium and send it to the laboratory at room temperature (22–25 °C) for immediate processing (see Step 4, **Fig. 1b**).

■ PAUSE POINT We recommend processing the limbal tissues on the same day as sample collection. However, the tissues can be stored overnight or up to 24 h at 4 °C (in case of late-hour sample collections) or at room temperature (if unavoidable during transit) without much loss of cell viability.

3| Collect ~12 ml of blood intravenously from volunteers (people with LCSD) in VACUETTE tubes containing serum clot activator (4 ml of blood in three tubes) to obtain autologous serum for culturing the limbal cells. Blood samples should be brought to the laboratory at room temperature and processed within 1–2 hours.

▲ CRITICAL STEP Alternatively, FBS can be used instead of serum for culturing limbal epithelial cells for basic research purposes. If FBS is used, blood sample collection and processing to produce autologous serum is not necessary and Steps 3–7 can be skipped.

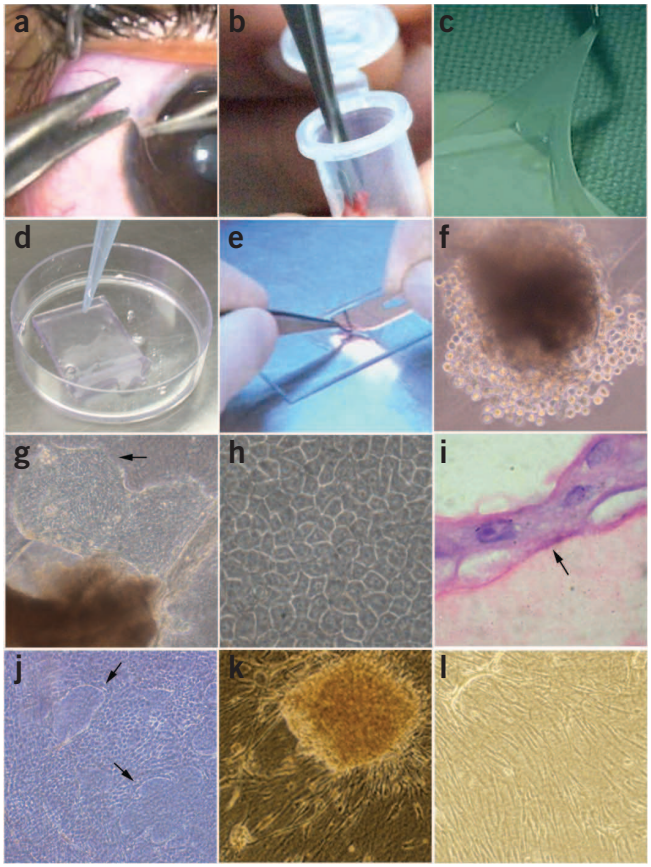

Figure 1 | Stepwise display of the procedure and culture outcomes. (**a**) Limbal biopsy (2 × 2 mm^2) from a healthy eye; (**b**) collection of tissue biopsy specimens in a sterile microcentrifuge tube containing HCE medium; (**c**) peeling of hAM from the nitrocellulose paper; (**d**) denuded hAM spread and tucked around a glass slide; (**e**) mincing of limbal tissue on a sterile glass slide; (**f**) proliferating cells migrating out from the periphery of the explant on days 2–3; (**g**) appearance of the growth zone of a limbal monolayer on hAM on days 4–5 (arrow points to an advancing growth zone); (**h**) a confluent monolayer of limbal epithelial cells on days 10–14; (**i**) hematoxylin and eosin staining of paraffinized sections of limbal epithelial cells grown on hAM (arrow points to the cell-basement membrane junction between cultured cells and hAM at the bottom); (**j**) patchy growth (arrows point to patches on hAM without cells); (**k**) explant showing only fibroblastic outgrowth; and (**l**) a failed culture with only stromal keratocytes.

! CAUTION Blood collection should be carried out by trained medical staff. Approval from appropriate regulatory agencies and the donor's informed consent are mandatory before collecting the blood samples.

4| Transport the biopsy and blood samples to the laboratory in a sterile container at room temperature for further processing. All tissue processing should be carried out in a Class II biosafety cabinet.

Preparation of autologous serum ● TIMING 2 h

5| Allow the blood sample to clot at room temperature for ~1 h.

? TROUBLESHOOTING

6| Centrifuge the tubes containing clotted blood at room temperature for 15 min at 1,250*g* for serum separation.

7| Pipette the supernatant serum into a fresh 50-ml Falcon tube and use it for preparing person-specific HCE growth medium at 10% concentration (see REAGENT SETUP); filter-sterilize the complete medium using a 0.22-μm syringe filter. About 5 ml of serum can be obtained from the 12 ml of blood sample collected. About 50 ml of complete HCE medium can be prepared using this autologous serum, which is sufficient for 2–3 weeks of maintenance of limbal cultures. Alternatively, FBS can be used at 10% (vol/vol) for preparing complete HCE medium.

? TROUBLESHOOTING

Preparation of hAM ● TIMING 1 d

8| Collect placenta from cesarean deliveries of consenting donors in a gas-sterilized pouch.

▲ CRITICAL STEP Approval from appropriate regulatory agencies and the donor's informed consent are mandatory before collecting placenta samples.

9| The volunteers donating the placental tissue must be screened for any cryptic infections such as HIV, hepatitis B, hepatitis C and syphilis with the help of commercially available kits for serological tests according to the manufacturer's instructions (see REAGENTS). However, this screening is generally completed at the maternity hospital before surgery as a part of routine presurgical testing and results can be obtained at the time of tissue collection.

▲ CRITICAL STEP If testing for cryptic infections has not been done, it is important to complete the serological testing by collecting a separate blood sample from the donors with their informed consent; use blood collection tubes suitable for molecular diagnostic purposes.

! CAUTION Only placentas from donors that have passed the viral tests should be approved for further processing. The membrane should be processed within 24 h of tissue collection.

10| Store the placenta in a sterile container at 4 °C until ready for further processing.

11| Place the placenta in a sterile pan and wash with Ringer's lactate saline solution containing 2× antibiotics (penicillin, streptomycin, amphotericin B); aspirate out the wash fluid into a liquid waste container with one-third of its volume filled with 1% (vol/vol) hypochlorite solution.

12| Repeat Step 11 until the wash solution is clear and all the residual blood is washed off completely.

13| After the initial prewash, transfer the placenta to another sterile pan aseptically and carry out further processing within a clean and sterile Class II biosafety cabinet.

14| Peel the amniotic membrane using blunt forceps to separate the amnion and chorion.

15| Spread the amniotic membrane with the epithelial side down and clean the basal side with a sterile swab to remove the mucus layer. Keep the membrane wet by intermittent addition of sterile Ringer's lactate saline solution containing antibiotics.

16| Once a clean transparent area of ~2 inches × 2 inches (5 × 5 cm^2) has been processed, attach a piece of gas-sterilized nitrocellulose paper (5 × 5 cm^2 size) to the basal sticky side of the membrane.

▲ CRITICAL STEP Stick the amniotic membrane to the nitrocellulose paper perfectly without trapping any air bubbles or leaving any gaps in between. The presence of air bubbles between the membrane and the paper will interfere with the tight adherence of hAM to the charged surface of nitrocellulose paper.

17| Cut the amniotic membrane around the nitrocellulose paper and turn it over, leaving the epithelial side facing upward.

18| Cut the nitrocellulose paper with the attached amniotic membrane into two smaller pieces of 5 × 2.5 cm^2 in size.

19| Roll the cut-out pieces of nitrocellulose paper attached to amniotic membrane with the epithelial side facing inward; insert the rolls into sterile glass vials containing ~30 ml of hAM freezing solution, or enough to cover the entire membrane roll.

20| Repeat Steps 16–19 until the entire membrane is processed. About 40–50 vials of hAM of 5 × 2.5 cm^2 size can be prepared with one donor placenta if the tissue remains intact (i.e., without any internal tears during processing).

21| Assign the vials a single lot number and check the lot for sterility using a sample vial (see **Box 2**); document the results.

22| Label the vials with details indicating donor ID, date of preparation and sterility status; store them at −80 °C.
▲ **CRITICAL STEP** Ensure complete sterility and handle the amniotic membrane with the utmost care. Use only sterile equipment throughout the procedure.
■ **PAUSE POINT** The hAM kept in freezing solution can be stored at −80 °C for up to 6 months.
? TROUBLESHOOTING

De-epithelialization of hAM ● TIMING 1 h

23| Thaw the processed and cryopreserved hAM (5 × 2.5 cm^2) slowly at room temperature and use it immediately after thawing. Avoid repeated freezing and thawing.

24| Place a small sterile glass slide (3 × 2.5 cm^2 size, cut from a regular glass slide of 7.5 × 2.5 cm^2 with a glass cutter and sterilized by autoclaving) at the center of a 60-mm^2 culture dish.

25| With the help of sharp- and curved-tip forceps, peel the hAM from the underlying nitrocellulose paper (**Fig. 1c**).

26| Gently transfer the amniotic membrane onto the surface of the glass slide with the epithelial side facing upward.
▲ **CRITICAL STEP** Handle the membrane with care; slide it gently without creating any tears in the membrane. The excess storage medium sticking to the membrane will enable it to slide smoothly onto the glass slide.
? TROUBLESHOOTING

27| Spread the hAM onto the glass slide smoothly, without creating any folds.

28| Add 1 ml of TrypLE onto the surface of the membrane and incubate it for 30 min at 37 °C.

29| Add ~2–3 ml of sterile 1× PBS solution to the membrane and flush out the loosened epithelium by gently triturating it with a blue tip attached to a 1-ml pipette aid.

30| Aspirate out the cell debris and wash the membrane thoroughly with PBS (1×).

31| Repeat Steps 29–30 twice to peel off the entire epithelial layer and to wash cells off the membrane completely. Aspirate out the saline solution completely.

32| Observe the membrane under a phase-contrast microscope to ensure complete de-epithelialization.

33| Ensure that the de-epithelialized hAM is spread onto the glass slide smoothly (without any folds), as the membrane tends to float and move during the wash steps (**Fig. 1d**).
? TROUBLESHOOTING

34| Lift the glass slide with blunt forceps and tuck the excess membrane (~1 cm) lengthwise on either side under the glass slide using another set of curved-tip forceps in order to secure the membrane to the slide when it is placed in culture medium. The effective culture area of the hAM on the slide will therefore be ~3 × 2.5 cm^2.

35| Transfer the glass slide with the denuded and tucked-in membrane to a new dish before using it as a substrate for culturing the limbal explants.
▲ **CRITICAL STEP** The glass slide with the membrane will be slippery to handle. Care must be taken in this step.

Explant culture of limbal tissues on de-epithelialized hAM ● TIMING 1 h

36| Rinse the limbal tissue from Step 2 with 1× PBS containing 2× antibiotics.

37| Repeat Step 36 once.

38| Place the limbal tissue in a sterile dish with a few drops of HCE medium to keep it wet while processing.

39| Chop the tissue into finer pieces of ~0.4–0.5 mm (~20 pieces, according to the convenience of handling) using a sterile no. 21 surgical blade (**Fig. 1e**).

40| Pick the tissue bits with a 24-gauge sterile needle and explant them onto the denuded hAM surface (from Step 35).

41| Distribute the explants evenly with enough spacing (~5 mm apart) to cover the entire membrane surface.

42| After explantation, cover the dishes and incubate them in a humidified CO_2 incubator maintained at 37 °C with a 5% CO_2 supply for 20–30 min. This incubation promotes adhesion of explants onto the membrane surface.

43| After 30 min of incubation, add a few drops of HCE medium onto the explants and add several drops around the slide to create a moist chamber; place the dishes back in the CO_2 incubator for ~8–10 h or overnight to allow good adherence of the tissue explants to the membrane.

44| After 8–10 h of incubation, flood each of the culture dishes with 4 ml of HCE medium containing 10% autologous serum and culture them at 37 °C in a CO_2 incubator for ~2 weeks until a complete epithelial sheet is generated. Alternatively, HCE medium with 10% FBS can be used.
▲ **CRITICAL STEP** Do not allow the explant tissue to dry while processing, as this will substantially affect the viability and quality of the culture. To avoid any dislodging, add medium to the dish without disturbing the explants.
? TROUBLESHOOTING

Maintenance of limbal epithelial cell cultures ● TIMING 2 weeks

45| Change the medium in the culture dishes every alternate day by replacing 2 ml of spent HCE medium with 2 ml of fresh HCE medium.

46| Monitor the growth of cells under an inverted phase-contrast microscope (see **Box 3** and **Fig. 1f,g**).

47| Repeat Steps 45–46 for approximately 2 weeks (as indicated in Step 45, change HCE medium once every 2 d).
? TROUBLESHOOTING

48| A uniform monolayer of limbal epithelium on hAM will be established by the end of 2 weeks (**Fig. 1h**).
? TROUBLESHOOTING

49| Wash the hAM with the limbal epithelial monolayer using 2 ml of 1× PBS solution.

50| Aspirate out the wash solution and repeat Step 49 once, and then aspirate the wash solution again. The limbal epithelial cells are now ready either for clinical or basic research applications. Approximately 3×10^6 cells can be obtained from a confluent culture covering an effective culture area of ~3×2.5 cm^2 of hAM. These cells can be directly fixed along with the amniotic membrane, paraffin embedded, and sectioned and processed for immunohistochemistry, or they can be trypsinized and used for fluorescence-activated cell sorting characterization or for cryopreservation.

BOX 3 | MONITORING OF GROWTH

After an average of ~1–3 d in culture, clusters of rounded cells migrate out of the explants and can be seen as clusters at the edge of the explant (**Fig. 1f**). The migrating cells attach and spread on the membrane and form a monolayer with a growing edge that can be clearly seen by 4–5 d (**Fig. 1g**). The growth zones of the adjacent explants gradually merge to form a uniform and confluent monolayer (with a typical cobblestone or honeycomb pattern) covering the entire membrane surface within a period of ~10–14 d of culture (**Fig. 1h**).

● TIMING

Steps 1–4, Collection of limbal biopsy and blood samples: 1 d
Steps 5–7, Preparation of autologous serum: 2 h
Steps 8–22, Preparation of hAM: 1 d
Steps 23–35, De-epithelialization of hAM: 1 h
Steps 36–44, Explant culture of limbal tissues on de-epithelialized hAM: 1 h
Steps 45–50, Maintenance of limbal epithelial cell cultures: 2 weeks

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Absence of blood clotting	Blood samples collected in heparin-coated tubes	Collect blood in tubes containing serum clot activator
7	Red coloration of the serum	Lysed RBCs	Properly collect and store blood samples to avoid lysis of RBCs
	Not enough serum	Low total volume of blood sample (<10 ml)	Collect a larger volume of blood sample. Alternatively, a certified lot of FBS can be used instead of serum
22	Contamination of hAM vials	hAM not handled under sterile conditions	Discard the entire lot of processed hAM
26, 33	Tearing of hAM	Slow disintegration due to long-term storage	Use a new vial, preferably from a different lot
44	Detachment of explants	Improper attachment of explants and/or drying of explants during processing	Mince the limbal tissue with a few drops of HCE medium to prevent drying of the tissue
47	Insufficient and/or patchy epithelial growth (**Fig. 1j**)	Improper attachment of explants and uneven explantation on hAM	Mince the limbal tissue with a few drops of HCE medium to prevent drying of the tissue and explant evenly spaced tissue on hAM to cover the entire surface area
48	Fibroblastic growth (of stromal keratocytes) is predominant (**Fig. 1k,l**)	No residual stem cells in the explanted tissue	Repeat the procedure with another tissue sample (from live or cadaveric sources)

ANTICIPATED RESULTS

By adopting this protocol, a uniform sheet of limbal epithelial monolayer (**Fig. 1h**) (~3 × 2.5 cm^2) can be established on hAM *in vitro* from a smaller limbal biopsy specimen (~2 × 2 mm^2). The entire culture protocol is totally xeno free and takes ~2 weeks. Hematoxylin and eosin staining of paraffinized sections of limbal epithelial cells grown on hAM shows the presence of a 1- to 2-cell-thick epithelial layer above the hAM layer (**Fig. 1i**). Detachment of explants, uneven placement of explants on hAM and drying of the tissue while processing may result in insufficient or patchy growth (**Fig. 1j**). A complete absence of any residual stem cells in the explant may result in cultures comprising only the fibroblastic stromal keratocytes (**Fig. 1k,l**).

Limbal epithelial cells cultured following the above-described protocol have been characterized by histology, immunohistochemistry, electron microscopy and flow cytometry to assess the morphological features of cultured cells and the expression of several cell-type–specific markers. The epithelial cells of the monolayer established good cell-cell contact by forming tight junctions (**Fig. 2a**), gap junctions and desmosomes and were well adhered to the hAM at the basal side. They expressed the corneal epithelial marker cytokeratin CK12 (**Fig. 2b**); basal cells stained positive for stem cell markers p75 (S.G., unpublished data) and p63 (the 4A4 antibody is specific to pan-p63 and marks the transiently amplifying cells and putative stem cells) (**Fig. 2c**); patches of cells expressing the stem cell marker ABCG2 were also detected in whole-mount preparation (**Fig. 2d**). In addition, the expanded cells also expressed other corneal epithelial markers such as Pax6, K3, K19 and p75 and the proliferating cells incorporated the BrdU (5-bromo-2-deoxyuridine) label (A.F. and S.G., unpublished data). Pulse-chase time-course experiments with BrdU labeling showed that ~11% of expanded cells retained the label at the end of 2 weeks and ~3% at the end of 3 weeks in culture (A.F., unpublished data). Fluorescence-activated cell sorting profiling of expanded cells showed that ~2% of the total cell population was ABCG2 positive and ~1% was integrin-β1

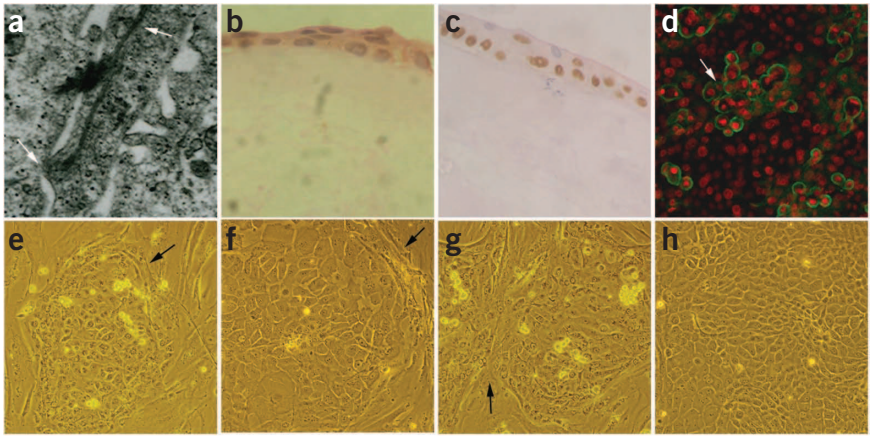

Figure 2 | Characterization of *in vitro*–expanded limbal epithelial cells on hAM. (**a**) Electron microscopic image of cell-cell junctions between two neighboring cells (arrows point to tight junctions between two cells); (**b**) immunohistochemistry on paraffinized sections of human limbal epithelial cells grown on hAM for the expression of corneal epithelial marker CK12; (**c**) immunohistochemistry on epithelial stem cell marker p63; (**d**) immunocytochemistry on whole-mount preparations for the expression of the stem cell marker ABCG2 stained in green and counterstained with propidium iodide, which marks cell nuclei in red (arrow points to a cluster of ABCG2-positive cells); (**e**) arrows pointing to a clone of human limbal cells primarily expanded on hAM and subcultured on inactivated human limbal stromal cells; (**f**) growth zone of a clone; (**g**) merging of two clones; and (**h**) a completely established monolayer.

positive, which represents the stem cell pool. However, we found that ABCG2-positive cells belonged to a subset of aldehyde dehydrogenase (ALDH)-dim cells, but not ALDH-bright cells (S.T., unpublished data). This observation is in agreement with a recent report on LSCs[29]. As reported previously, hAM-expanded cells can be trypsinized and further expanded on mitotically inactivated human limbal stromal fibroblast feeders to form distinct cell clones resembling holoclones (**Fig. 2e**), meroclones and paraclones[30]. We observed a cloning efficiency of ~3% in hAM-expanded cells; the clones merged to give rise to a uniform monolayer (**Fig. 2f–h**) (I.M., S.S. and S.G., unpublished data). A recent report from Pellegrini's group shows that cultures with more than 3% p63 bright cells (expressed by the holoclones) allow corneal regeneration in 80% of patients as opposed to 11% in case of cultures with <3% p63 bright cells[31]. Therefore, it is recommended that expanded cells need to be validated using several of the approaches discussed above before considering them for clinical applications; this would help in assessing potential clinical outcomes objectively.

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