

Isolation and ex vivo Expansion of Human Limbal Epithelial Progenitor Cells

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[Abstract] Limbal stem cell transplantation has been used successfully to treat patients with limbal stem cell deficiency all over the world. However, long term clinical results often proved less satisfactory due to the low quality of the graft or inadequate properties of transplanted cells. To enhance the *ex vivo* expansion of human limbal epithelial stem or progenitor cells (LEPC) by preserving stem cell phenotype and to improve subsequent transplantation efficiency, cell-matrix interactions *ex vivo* should mimic the condition *in vivo*. The laminin isoforms preferentially expressed in the limbal niche can be used as a culture matrix for epithelial tissue engineering. We recently published the expansion of LEPC on various laminin isoforms and observed that laminin alpha 5-derived matrices support the efficient expansion of LEPC compared to tissue culture plates and other laminin isoforms by preserving stem/progenitor cell phenotype. Here, we describe an optimized protocol for the isolation of LEPC from cadaveric corneal limbal tissue by collagenase digestion and efficient expansion of LEPC using recombinant human laminin-511 E8 fragment (LN-511E8) as culture substrate.

Key words: Limbal epithelial progenitor cells, Laminin, Limbal stem cells, Cornea, Isolation, Expansion

[Background] Limbal epithelial stem/progenitor cells (LEPC), responsible for the continuous renewal of the corneal epithelium, reside in a highly specialized and complex environment. It comprises specific extracellular matrix components and supporting limbal niche cells at the corneo-scleral limbus (Schermer *et al.*, 1986; Cotsarelis *et al.*, 1989; Ordonez *et al.*, 2012; Mei *et al.*, 2012). Damage or injury to this stem/progenitor cell reservoir can lead to corneal neovascularization, chronic inflammation, and stromal scarring associated with corneal opacity and loss of vision (Kenyon and Tseng, 1989; Sangwan and Tseng, 2001; Dua *et al.*, 2010). Cultured limbal epithelial transplantation (CLET) has been applied as a therapeutic approach in various clinical centers and the overall success rates of autologous CLET for unilateral cases with a follow-up period of at least 24 months were reported to amount to 72-76% (Rama *et al.*, 2010; Tsai *et al.*, 2010; Utheim *et al.*, 2013; Holland *et al.*, 2015). In the case of bilateral, 34 months of follow-up showed a 70% success rate after allogeneic central penetrating limbo-keratoplasty in conjunction with conjunctivoplasty, mitomycin C, and amniotic membrane transplantation (Eberwein *et al.*, 2012). However, long-term corneal regeneration often proved less satisfactory. This might be caused by the low quality of the graft or inadequate properties of transplanted progenitor cells (Daya *et al.*, 2005; Rama *et al.*, 2010; Pellegrini *et al.*, 2014; Nakamura *et al.*, 2016). These limitations underscore the need for developing novel standardized LEPC culture techniques that ensure the

preservation of the stem/progenitor cell phenotype and function during cultivation and after transplantation.

Laminins (LNs) are the best-characterized extracellular matrix constituents present in basement membranes (BM) of adult stem cell niches, including the limbal stem cell niche, where they influence cell behaviors, such as cell adhesion, differentiation, and phenotype stability. Various reports have shown heterogeneity of LN isoform expression in BMs of ocular surface epithelia. Especially LN chains $\alpha 5$, $\beta 1$, $\beta 2$, and $\gamma 1$ are preferentially localized to the BM of the limbal epithelium compared to that of the corneal epithelium (Ljubimov *et al.*, 1995; Schlötzer-Schrehardt *et al.*, 2007; Poliseti *et al.*, 2017) (Figure 1). We have reported earlier that LN alpha 5-containing isoforms as well as the bioactive fragment of human LN-511 (LN-511 E8) support efficient *ex vivo* expansion of LEPC (Poliseti *et al.*, 2017).

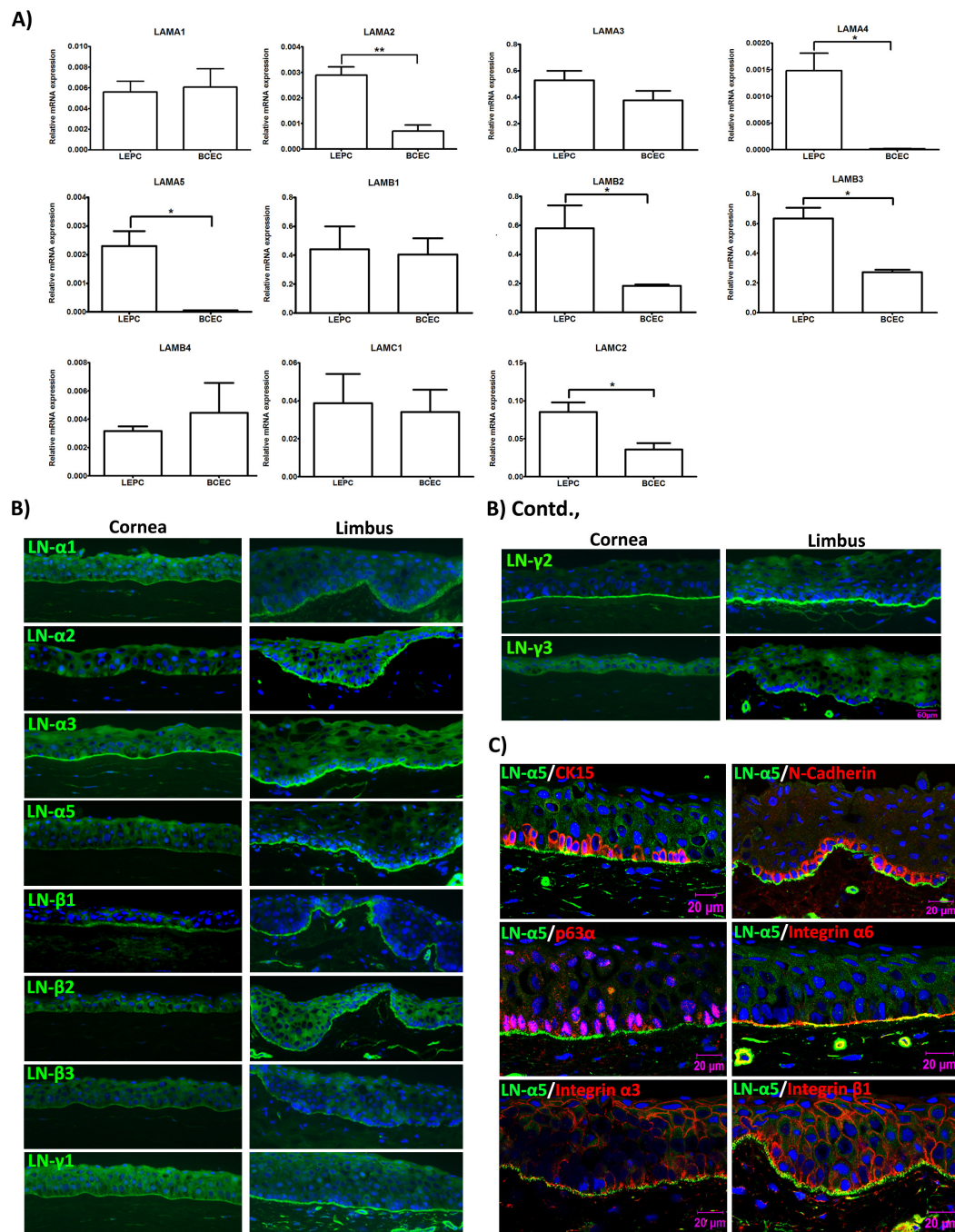


Figure 1. Expression of laminin chains in the limbal stem cell niche *in situ*. A. Quantitative real-time polymerase chain reaction (qRT-PCR) primer assays showing higher expression levels of laminin $\alpha 2$ (LAMA2), $\alpha 4$ (LAMA4), $\alpha 5$ (LAMA5), $\beta 2$ (LAMB2), $\beta 3$ (LAMB3), and $\gamma 2$ (LAMC2) in microdissected limbal epithelial stem/progenitor cell (LEPC) clusters compared with basal corneal epithelial cell (BCEC) populations; laminin $\alpha 1$ (LAMA1), $\alpha 3$ (LAMA3), $\beta 1$ (LAMB1), $\beta 4$ (LAMB4), $\gamma 1$ (LAMC1) showed no differential expression patterns. B. Immunofluorescence analyses of corneoscleral tissue sections showing differential staining patterns of laminin $\alpha 2$, $\alpha 5$, $\beta 2$, $\beta 3$, $\gamma 2$, and $\gamma 3$, but similar staining patterns of laminin $\alpha 1$, $\alpha 3$, $\beta 1$, and $\gamma 1$ in the basement membranes of corneal and limbal epithelia; laminin $\alpha 4$ was largely negative in epithelial basement membranes. C.

Immunofluorescence double labeling of laminin (LN) $\alpha 5$ (green) and cytokeratin (CK)15, N-Cadherin, p63 α , integrin $\alpha 6$, integrin $\alpha 3$, and integrin $\beta 1$ (red); nuclear counterstaining with DAPI (blue); scale bar = 20 μ m. Reprinted from Polisetti *et al.*, 2017, licensed under a CC BY 4.0.

Here, we present an optimized protocol for isolation and expansion of LEPC. The isolation protocol is similar to the protocol provided by Chen *et al.* (2011) with few modifications, but we expanded LEPC on LN-511E8-coated plates to improve proliferation and preservation of a stem/progenitor cell phenotype (Polisetti *et al.*, 2017).

Materials and Reagents

1. Micropipette tips (0.5-20 μ l, 100-200 μ l, 1,000 μ l) (Greiner Bio-One)
2. 60 mm cell culture dish (Corning, Falcon®, catalog number: 353004)
3. 100 mm cell culture dish (Corning, Falcon®, catalog number: 353003)
4. Syringe filter 0.2 μ m (VWR, catalog number: 28145-501)
5. Disposable Scalpel blades No. 10 (pfm Medical ag, Feather®, catalog number: 201000010)
6. Serological pipettes (5 ml, 10 ml) (Corning, Stripette™)
7. 15 ml conical tubes (Greiner Bio-One, catalog number:188271)
8. T75 flasks (Corning, catalog number: CLS430641)
9. Reversible cell strainers (Stem Cell Technologies, catalog number:27215)
10. Cell filter 20 μ m (Sysmex Partec GmbH, Görlitz, Germany)
11. Collagenase A (Sigma-Aldrich, Roche Diagnostics, catalog number: 10103578001)
12. Dulbecco's Phosphate buffered saline (DPBS) (Pan Biotech, catalog number: P04-361000)
13. 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Gibco®, catalog number: 25200056)
14. 0.05% Trypsin-EDTA (Thermo Fisher Scientific, Gibco®, catalog number: 25300054)
15. Dulbecco's Modified Eagle Medium (DMEM) high glucose (Thermo Fisher Scientific, Gibco™, catalog number: 11960044)
16. Fetal Bovine Serum (FBS) (Thermo Scientific, Gibco™, catalog number: 10082147)
17. Penicillin-Streptomycin/Amphotericin B Mixture (Pan Biotech, catalog number: P06-07300)
18. Keratinocyte serum-free medium (KSFM) (Life Technologies, catalog number: 10724-011)
19. Keratinocyte supplements, contains bovine pituitary extract, human recombinant EGF (Life Technologies, catalog number: 37000-015)
20. iMatrix 511(Nippi, catalog number: 892012)
21. 70% Ethanol
22. Cytokeratin (CK) 15 (Invitrogen, catalog number: MA5-11344)
23. p63 α (Abcam, catalog number: ab32353)
24. CCAAT/enhancer-binding protein delta (Abcam, catalog number: ab139730)
25. CK19 (Abcam, catalog number: ab52625)
26. CK3 (Invitrogen, catalog number: MA1-5763)

27. CK12 (Abcam, catalog number: ab185627)
28. Connexin-43 (Abcam, catalog number: ab235282)
29. Collagenase solution (see Recipes)
30. KSFM complete medium (see Recipes)
31. Laminin coated plates (see Recipes)

Equipment

1. Pipette aid
2. Micropipette (P20, P200, P1000)
3. Microdissection Scissors
4. Forceps
5. Scissors
6. Hemocytometer
7. Biosafety cabinet
8. CO₂ incubator
9. Centrifuge
10. Phase contrast inverted microscope with a camera (Objectives 4x, 10x, 20x)
11. Freezer -20 °C
12. Refrigerator 2-8 °C

Software

1. CapturePro 2.10.0.1 (JENOPTIC Optical systems GmbH)

Procedure

A. Dissection of limbus

1. Organ-cultured corneoscleral tissue with appropriate research consent and ethics approval may be provided by a cornea bank if the corneas are not suitable for transplantation due to low corneal endothelial cell density ($< 2,200$ cells/mm²), or the presence of neurological disorders or malignancies in the donor. Donor cornea remnants after corneal endothelial transplant preparation are also a valuable source if appropriate research consent and approval have been obtained.
2. Place the corneoscleral tissue in a 60 mm culture dish and wash with DPBS twice. Cut the tissue into 12 three-clock hour sectors using a scalpel blade and forceps.
3. Make incisions at 1 mm before and beyond the anatomical limbus to get limbal segments (see Video 1).



Video 1. Limbal Epithelial Progenitor Cell Isolation

B. Isolation of limbal epithelial progenitor cells

1. Place the limbal segments into a 60 mm dish containing 4 ml of collagenase A (2 mg/ml) and cut the limbal segments into smaller pieces with the scalpel blade. Incubate at 37 °C with 5% CO₂ overnight to digest the stromal collagen (get limbal epithelial cell clusters).
2. After overnight incubation, triturate several times with 1 ml pipette and observe for the presence of cell clusters and single cells in the microscope (Figure 2). The cell clusters are supposed to consist of limbal epithelial progenitor cells, stromal, and melanocyte niche cells.

Note: In case of incomplete digestion of limbal segments after overnight incubation and trituration, re-incubate for 2 more hours in the same solution at 37 °C with 5% CO₂ for complete digestion. On the other hand, over digestion of tissue might adversely affect cell viability and the quality of cells.

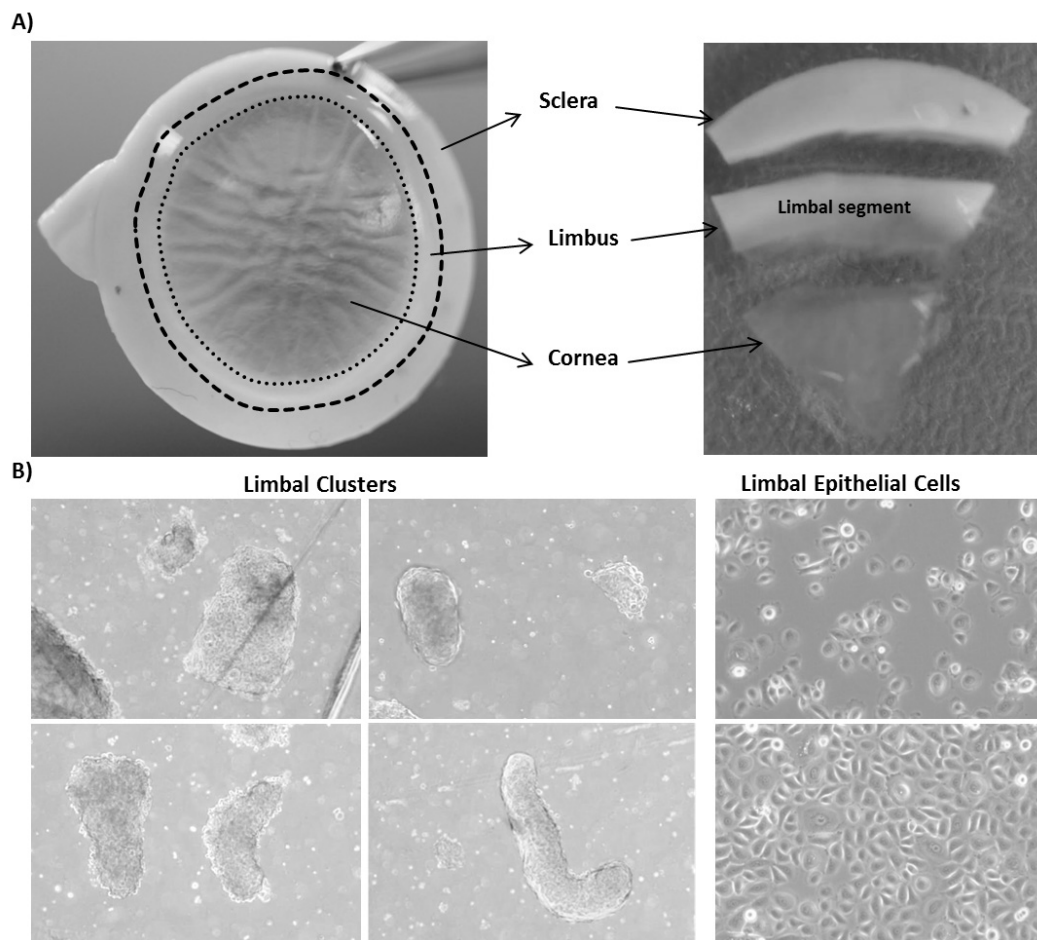


Figure 2. Isolation of limbal epithelial progenitor cells. A. The corneal scleral rim (left) was cut into sectors and each sector was trimmed off 1mm before and after the limbal region (right). B. Different sizes of limbal clusters and single cells (left) formed after overnight incubation of limbal segments in collagenase solution (x40 magnification). Limbal cluster-derived limbal epithelial cells cultured in KSFM media at 50% confluence (right, top) and 100% confluence (right, bottom) (x40 magnification).

3. Separate cell clusters from single cells by using reversible cell strainers with a pore size of 37 μm . The single cells pass through the cell strainer and the clusters are retained. Wash the strainers with PBS twice to remove single cells. Reverse the strainer and place it on a 15 ml Falcon tube or on a 60 mm dish. Add 0.25% trypsin-EDTA (5 ml) to flush clusters into petri dish or tube and incubate at 37 $^{\circ}\text{C}$ for 10-15 min to dissociate the clusters into single cells.
Note: In place of reversible cell strainers, 20 μm cell filters can be used. In the video, we used 20 μm filters.
4. After incubation, triturate the cell suspension with a 1ml pipette. Inhibit trypsin digestion using 5ml of DMEM containing 10% FBS. Transfer the cell suspension into a 15 ml Falcon tube and centrifuge at 200 x g for 5 min.

5. After centrifugation, resuspend the cell pellet in 1 ml of KSFM complete media for feeder-free cultures. These cells can also be used for clonal assays on feeder layers.

C. Expansion of limbal epithelial progenitor cells

1. Seed the limbal cluster-derived single cells (1×10^3 cells/cm²) onto LN511E8-coated plates (flasks or plates depending on cell number obtained. Preparation of laminin-coated plates described in Recipe 3.

Note: Do not allow the laminin-coated flasks to dry.

2. Cultivate for 7 to 10 days at 37 °C with 5% CO₂ in KSFM complete medium to expand limbal epithelial cells. Change media every 2 days.

Note: If any stromal fibroblast contamination occurs after 7 days in culture, remove the stromal fibroblasts, which tend to adhere less firmly to the LN matrix, by using 0.05% trypsin-EDTA. Remove the media and wash it with DPBS. Incubate with 4 ml of 0.05% trypsin-EDTA for 2 min. Wash the flasks with DMEM containing 10% FBS followed by one wash with KSFM. Add 12 ml of KSFM complete medium and continue with culture

3. To verify the expanded population for the presence of stem/progenitor cell population, various stem/progenitors markers (Cytokeratin (CK) 15, p63 α , CCAAT/enhancer-binding protein delta, CK19) and differentiated markers (CK3, CK12, Connexin-43) can be used by means of Reverse Transcription Polymerase chain reaction and by immunocytochemistry.

D. Sub-cultivation of limbal epithelial progenitor cells

1. At 70 to 80% confluency, remove the media and wash once with DPBS.
2. Add 4 ml of trypsin-EDTA (0.05%) and incubate at 37 °C with 5% CO₂ for 5 min.
3. After incubation, add 4 ml of DMEM containing 10% FBS to inhibit trypsin action and mix well.
4. Transfer the cell suspension into 15 ml tube and centrifuge at 200 x g for 5 min. Dissolve the cell pellet in KSFM complete medium and use cells for the application of choice or for subculturing.

Note: Over confluence (more than 80%) and prolonged trypsin digestion (more than 5 min) adversely affect cell viability and the quality of cells during sub-culturing. Always passage cells at 70 to 80% confluence. Avoid prolonged incubations in trypsin.

Data analysis

The conditions provided in this protocol have been optimized to isolate and expand LEPCs. A detailed analysis of the isolation and expansion of the limbal epithelial progenitor cells can be found at Poliseti *et al.*, 2017.

Recipes

1. Collagenase solution

Reagent	Collagenase A
Components and Preparation	5 mg Collagenase A 220 ml of DMEM High Glucose 25 ml of fetal calf serum 5 ml of Penicillin-Streptomycin/Amphotericin B Mix Mix well by inverting
Method of Sterilization	Sterile Filter with 0.2 µm filter
Notes	Prepare 10 ml aliquots
Storage	-20 °C

2. KSFM Media

Medium	Keratinocyte Serum-Free Medium (KSFM) complete
Components and Preparation	500 ml of KSFM Basal Medium 25 mg of BPE (add entire amount) 2.5 µg of EGF (add entire amount) 5 ml of Penicillin-Streptomycin/Amphotericin B Mix Mix well by inverting
Method of Sterilization	None
Notes	Prepare aliquots if needed Protect from light
Storage	1 month at 2-8 °C

3. Laminin coated plates

- Dilute 75 µl of iMatrix-511 in 8 ml of 1x DPBS
- Coat the T75 flask with 8 ml of dilute iMatrix-511 (0.5 µg/cm²) solution for 1 h at 37 °C, 5% CO₂ in the incubator, or at 4 °C overnight
- After incubation, remove the iMatrix-511 solution and add 10 ml of KSFM complete medium

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Competing interests

The authors declare that they have no competing interests.

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