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ISOLATION OF CORNEAL AND LIMBAL CELLS FROM HUMAN DONOR CORNEA

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Abstract

Objective: Isolation of corneal cells while preserving their specific phenotypes is crucial for regenerative medicine applications. Here, we aimed to evaluate corneal cell isolation techniques from human corneal tissue.

Methods: We isolated corneal cells and limbal stem cells from human donor corneas obtained from the eye bank by employing both enzymatic and explant cell culture approaches and monitored the morphological characteristics of the isolated cells.

Results: Explant culture demonstrated suitability for the isolation of corneal endothelial and epithelial cells, while both explant and enzymatic culture processes yielded successful results for limbal and corneal stromal tissues. The optimal conditions for enzymatic culture were determined to be 2 mg/mL enzyme solution for 30 minutes at 37 °C. On the other hand, the feasibility of explant cultures was increased by re-transferring and culturing tissue specimens to isolate an enlarged quantity of cells in an extended culture periods. However, this approach resulted in morphological changes in the isolated cells. The earliest passage at which isolated primary cells (keratocyte and limbal stem cells) could be immortalized was Passage 1, enabling subculturing and cell stock creation. On the other hand, isolated primary limbal stem cells demonstrated the capacity to differentiate into corneal epithelial and keratocyte cells.

Conclusion: This study provides a comprehensive and detailed presentation that will serve as an informative resource in the literature for corneal cell culture, corneal tissue engineering, and drug delivery studies.

Keywords: *Cornea, primary human corneal cells, limbus, limbal stem cells, differentiation, immortalization.*

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Introduction

The cornea, situated in the outermost region of the eye, is a transparent and avascular tissue crucial for optimal vision, facilitating the transmission of incoming light to the lens.[1] Comprising five layers—epithelium, Bowman's membrane (BM), stroma, Descemet's membrane (DM), and endothelium—in a hierarchical arrangement from outermost to innermost, it embodies specialized cells with distinct functions within three of these layers: the epithelium, stroma, and endothelium. Conversely, BM and DM are collagen-rich membranes devoid of cells, primarily serving to delineate layers and offer sites for cellular adhesion.[2]

The epithelium layer, the outermost of the three cellular layers within the cornea, comprises corneal epithelial cells, forming approximately 50µm thickness composed of 5-7 layers of cells.[3] Serving as a selective passage for water and other substances from tear to the corneal stroma, this layer directly interfaces with the external environment[3]. Corneal epithelial cells are interconnected via desmosomes and junctions, with intercellular spaces measuring 10-20 nm, thereby providing a diffusion barrier and shielding against external factors.[4] Regeneration within the corneal epithelium layer involves differentiation of limbal epithelial stem cells residing in the limbus epithelial layer into transit amplifying cells, subsequently developing into corneal epithelial cells.[5] Extensive literature demonstrates utilization of enzymatic and explant culture methods for isolating human corneal epithelial cells. Enzymatic culture involves trypsin[6,7] or dispase[8,9] separation of cells, followed by culture, passaging, and amplification. Conversely, the explant culture method entails placing corneal epithelial tissue strips, segregated from other corneal tissues, into culture flasks, and observing cell migration from these tissue strips.[10,11]

The stroma, constituting the thickest layer of the cornea, is comprised of collagen fibrils and 70% water.[12] These collagen fibrils, highly organized, endow the stroma with distinctive biomechanical and optical characteristics. Collagen type I (COL-I) predominates, followed by collagen type IV, forming precisely ordered fibrils, spatially arranged by glycosaminoglycans (GAGs).[13,14] Keratocyte cells (KCs) within the stroma synthesize collagens, GAGs, and other extracellular matrix (ECM) components, characterized by a dendritic morphology and interconnected network.[15] Derived from mesenchymal stem cells (MSCs) in the limbal stroma, KCs are quiescent and lack active cell cycles under healthy physiological conditions.[16] Upon injury or pathology KCs become activated, transitioning into stromal fibroblasts and subsequently myofibroblasts to repair damaged areas. This reparative process often leads to scar formation as myofibroblasts produce ECM molecules to fill the injured site, resulting in stromal opacity.[17] Various studies in the literature detail the isolation of KCs from human stromal tissue utilizing both explant and enzymatic methods. Enzymatic isolation typically employs collagenase type I due to its specificity for COL-I, the predominant ECM protein in the stroma.[18,19] Conversely, explant culture techniques involve direct culture of stromal tissue devoid of other corneal components, facilitating cell migration from tissue fragments.[20,21] Comparative analysis of KC-specific markers of cells isolated via explant and enzymatic methods from either central or peripheral stromal regions revealed no significant differences.[22]

The innermost layer of the cornea is known as the endothelial layer, comprising a monolayer of polygonal cells crucial for maintaining corneal transparency through fluid pumping and active ion coupling.[23] Unlike other corneal layers, the endothelium lacks regenerative capacity, leading to an annual cell loss rate of 0.3-0.6%.[24] Due to their limited proliferation potential, isolation and passage of corneal endothelial cells pose significant challenges, resulting in scarce literature on the subject. Enzymatic isolation methods typically involve the use of collagenase to separate endothelial cells, primarily targeting collagen type IV within DM.[25,26] Alternatively, trypsin-based approaches for endothelial cell detachment from DM are employed, albeit with associated cell damage.[23,27] While explant culture techniques involve culturing endothelial layers with DM, achieving cell proliferation is hindered by the endothelial cells' low proliferation capacity.[28,29] The substrate upon which corneal endothelial cells grow significantly influences their viability. Coating the growth surface with ECM molecules such as collagen, laminin, and fibronectin enhances endothelial cell viability and proliferation.[23,30,31]

The limbus, situated between the sclera and cornea, serves as a crucial reservoir of stem cells for corneal regeneration.[32] Within limbus, both limbal epithelial stem cells (LESCs) in the limbal epithelium and MSCs in the limbal stroma are found.[32,33] Disruption of the highly ordered collagen fibril organization occurring at the limbus contributes to opacity.[4] Studies have demonstrated that MSCs isolated from the limbus possess the capacity for adipogenesis, chondrogenesis, and osteogenesis, highlighting their potential role in corneal regeneration.[34]

Since the cornea, and particularly the limbus, are tissues of very small dimensions, dissection and cell isolation are challenging and require specialized expertise. The isolation of these cells holds significant importance for studies in regenerative medicine, tissue engineering, wound healing, and drug development. Therefore, the isolation and immortalization of cells that preserve their phenotype and genotype, as well as the differentiation potential for stem cells, are of great significance. Although various studies in this field exist in the literature, there is no study that clearly compares the advantages and disadvantages of techniques side by side. In this study, as a first major step, corneal and limbal cells were isolated using both explant and enzymatic techniques, and the advantages and disadvantages of these techniques were assessed for each cell type. Additionally, this study, for the first time, demonstrates the effect of the passage used for immortalization on proliferation of corneal keratocytes and the relationship between tissue shrinkage during explant culture and proliferation capacity. In this study, human donor corneas sourced from eye bank underwent dissection, and the corneal layers were separated for cell isolation, employing both enzymatic and explant culture techniques. The isolated cells were subsequently subcultured, and their morphologies were assessed. Notably, no previous literature has reported a comprehensive investigation isolating cells from all corneal layers simultaneously from human donor corneas. In addition, the feasibility of continuous and high-volume cell isolation was evaluated through the re-transfer of tissues that provided cell outgrowth. As a second major step, immortalization of primary corneal keratocytes and limbal stem cells and most importantly, testing the differentiation capacity of limbal stem cells into corneal cells were conducted. Therefore, this study presents a comprehensive and comparative analysis of corneal cell isolation techniques from human corneal tissue, filling a significant gap in the existing literature.

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Methods

Dissection and Cell Culture of Corneal Layers

10 human donor corneas were obtained from Haydarpasa Numune Training and Research Hospital (under Ethical Approval 771/11/2019#20 and SUNUM-2019-82) (Supplementary Information Table S1). The tissues were washed several times with 50 µg/mL Gentamicin (Pan Biotech, Aidenbach, Germany) containing phosphate buffer saline (PBS) and then rinsed with sterile PBS. The dissection tools, plates, and pipette tips used were sterilized, and throughout the procedure, they were frequently washed with 70% ethanol and rinsed with sterile PBS. The scleral tissue was dissected using a sterile scalpel to completely remove it, leaving only the limbus and corneal tissues. The dissection process was performed under a stereo microscope (Leica M165 C, Leica Microsystems, Wetzlar, Germany) while keeping the tissues inside a sterile petri dish. Following the dissection, culture procedures were immediately initiated to minimize cell loss.

Corneal Epithelial Cells

The corneal epithelial layer was grasped with forceps and lifted from the surface. Explant tissue pieces for culture were placed in a 6-well plate where the cell-containing surface facing upwards. They were then kept in a cell culture incubator for 15 minutes to allow tissue strips to adhere to the surface of the culture plate. At the end of the incubation, growth medium was gently added to prevent the tissue pieces from detaching from the surface. During the culture process, three different growth media were tested, namely serum free media that is Keratocyte Serum-Free Media (KSFM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.005 µg/mL epithelial growth factor (EGF) (Gibco), 5 µg/mL bovine pituitary extract (BPE) (Gibco) and 1% Penicilin-Streptomycin Solution (Pan Biotech), complete media that is Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) (Pan Biotech) supplemented with 10% fetal bovine serum (FBS) (Pan Biotech) and 1% Penicilin-Streptomycin Solution (Pan Biotech), and complete media with EGF that is DMEM-F12 (Pan Biotech) supplemented with 10% FBS (Pan Biotech), 1% Penicilin-Streptomycin Solution (Pan Biotech), and 10 ng/mL EGF (Peptrotech, Cranbury, NJ, USA). Samples isolated via explant culture and cultured with different growth media were monitored via Primovert Cell Culture Light Microscope (Carl Zeiss AG, Oberkochen, Germany).

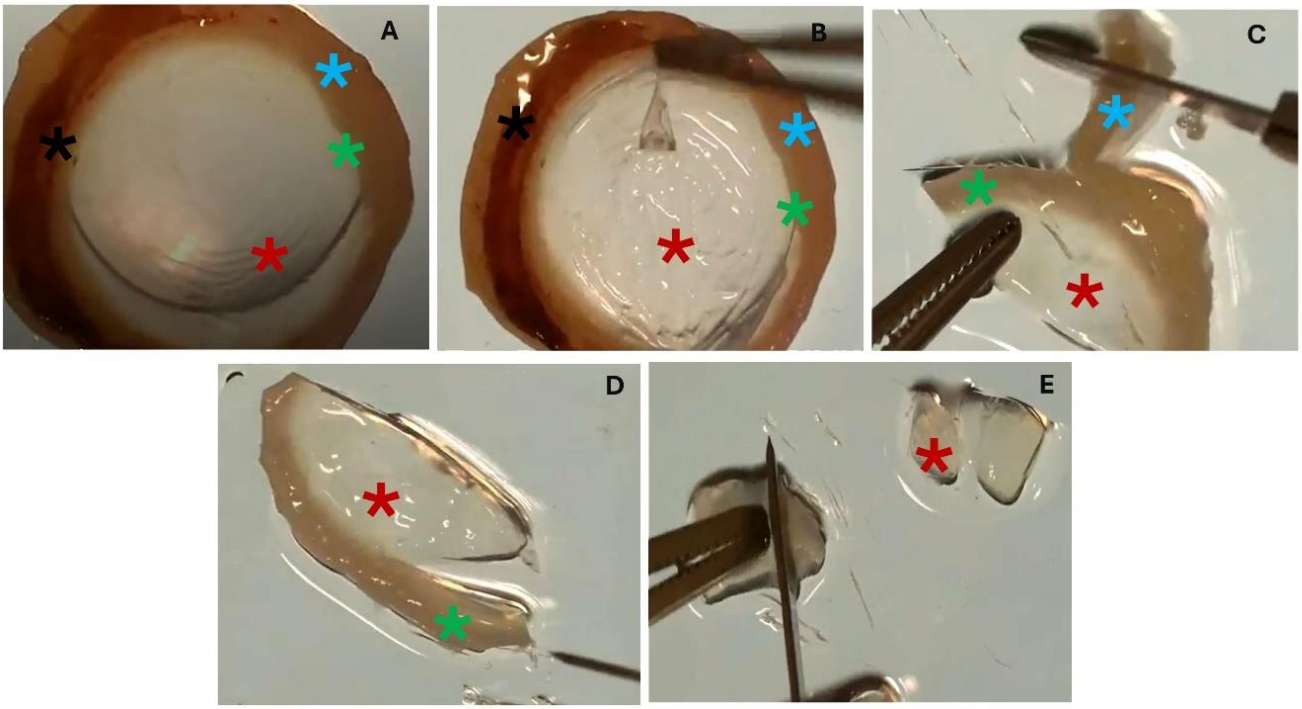


Figure 1. Dissection of corneal and limbal tissues for cell culture processes. (A) The inner face of the cornea. (B) Collection of corneal epithelial layer. (C) elimination of the scleral tissue from limbus and cornea. (D) Corneal and limbal tissue. (E) Separated corneal and limbal tissue and cutting of corneal stroma into small pieces (*: trabecular meshwork, *: sclera, *: limbus, *: cornea).

Corneal Endothelial Cells

The corneal endothelial layer was grasped with forceps and pulled off to collect from the tissue. In the classic explant culture approach, samples were placed in a 6-well plate coated with FNC Coating Mix (Athena Enzyme Systems, Baltimore, MD, USA) and kept in a cell culture incubator for 15 minutes. The addition of growth media was done carefully to avoid dislodging the tissue strips from the surface. The growth medium was Opti-MEM supplemented with 5% FBS (Pan Biotech), 1% Penicillin-Streptomycin Solution (Pan Biotech), 2.5 mM L-Glutamine (Pan Biotech), 50 µg/ml Bovine Pituitary Extract (Gibco), 0.08% chondroitin sulfate (Carbosynth, Compton, Berkshire, UK), 200 µg/ml CaCl2 (Sigma Aldrich, St. Louis, Missouri, ABD), and 1.3 mM L-ascorbic acid (Sigma Aldrich). In the alternative explant culture approach, tissue strips were placed in a 6-well plate and Opti-MEM (Gibco) supplemented with 8% FBS (Pan Biotech) was added. The plate was then incubated overnight at 37°C under 5% CO2. After incubation, the samples were washed with Hank's Balanced Salt Solution (HBSS) (Pan Biotech) and incubated in 0.02% Ethylenediaminetetraacetic Acid (EDTA) (Sigma Aldrich) solution for 1 hour. After centrifugation of tissue strips at 300×g for 5 minutes, the pellet was resuspended in growth media for seeding.

In the enzymatic culture procedure, the collected endothelial tissue strips were placed in a 15 mL falcon tube and incubated at 37°C with 0.25% Trypsin / 0.02% EDTA Solution (Pan Biotech) for 5 minutes under shaking. Mechanical agitation

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was then quickly performed by pipetting for a few seconds and trypsin enzyme was inactivated with FBS. After centrifugation at 300×g for 5 minutes, the pellet was resuspended in growth medium and seeded into a 6-well plate coated with FNC Coating Mix. Medium changes were performed every other day to observe cell emergence from tissue pieces. Opti-MEM (Gibco) supplemented with 5% FBS (Pan Biotech), 1% Penicillin-Streptomycin Solution (Pan Biotech), 2.5 mM L-Glutamine (Pan Biotech), 50 µg/ml Bovine Pituitary Extract (Gibco), 0.08% chondroitin sulfate (Carbosynth), 200 µg/ml CaCl2 (Sigma Aldrich), and 1.3 mM L-ascorbic acid (Sigma Aldrich) was used as growth medium.

Corneal Stromal Keratocyte Cells

The donor cornea tissue was dissected to remove the corneal epithelium, corneal endothelium, and limbus completely. The remaining corneal stromal tissue was cut into small pieces using a scalpel. For explant culture, samples were placed in a 25 cm^2 flask and incubated at 37°C in a cell culture incubator for 15 minutes to allow the tissue pieces to adhere to the surface of the flask. After the incubation period, growth media was added gently by avoiding detaching of tissue pieces. For enzymatic isolation, the obtained tissue pieces were placed in a 15 mL falcon tube and incubated at 37°C with a solution of Collagenase I (Merck, Darmstadt, Germany) prepared in growth media, under gentle shaking. Three different protocols were applied for enzymatic isolation, which were incubation for 1 hour in 4 mg/mL enzyme concentration, 1 hour in 2mg/mL enzyme concentration, and 30 minutes in 2 mg/mL enzyme concentration. After incubation in the enzyme solution, centrifugation at 300×g for 5 minutes was performed, and the pellet was resuspended in growth media and seeded into 25 cm2 flasks. In both enzymatic and explant culture processes, the growth media was DMEM-F12 (Gibco) supplemented with 5% FBS and 1% Penicillin-Streptomycin Solution (Pan Biotech), and culture period was maintained with medium changes every 2 days and cells obtained at passage 0 were harvested by trypsinization for subculturing.

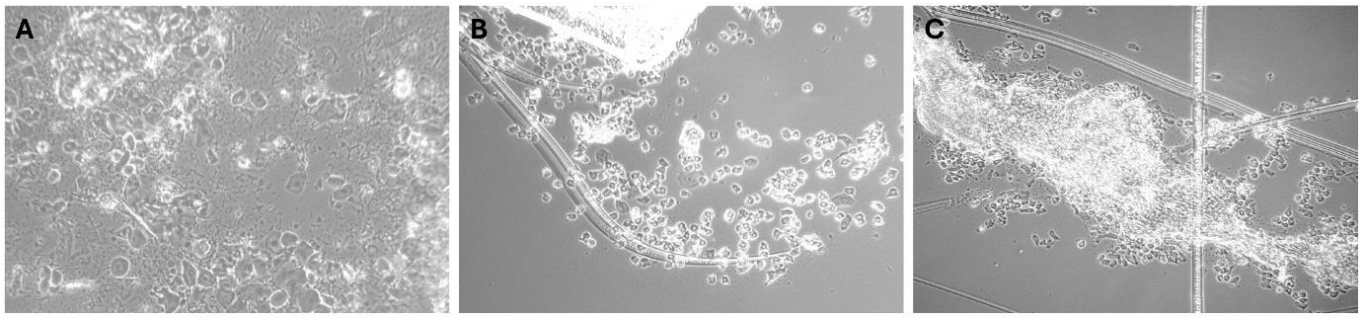


Figure 2. Corneal epithelial cells isolated by explant culture by using different culture medias; (A) serum free media, (B) complete media, and (C) complete media with EGF (10X Magnification).

Limbal Epithelial Stem Cells

Limbal epithelial cell isolation was performed using two techniques. In the first technique, the collected limbal epithelial layer with BM was incubated with 1.2 U/mL Dispase II (Sigma Aldrich) in KSFM (Gibco) for 30 minutes under shaking. The cells were then centrifuged, and KSFM (Gibco) supplemented with 0.005 µg/mL EGF (Gibco) and 5 µg/mL BPE (Gibco) was added to the pellet and cells were seeded into cell culture plate.

In the second technique, the limbal tissue separated from the corneal tissues was cut into small pieces and placed onto cell culture plates with the limbal epithelial layer facing upwards. A drop of FBS was added to the tissue pieces, which were then incubated overnight at 37°C and 5% CO2. The culture was continued by adding growth medium, which consisted of DMEM-F12 (Pan Biotech) supplemented with 5 ng/ml EGF, 5 mg/ml-5 mg/ml-5 ng/ml insulin-transferrin-sodium selenite (ITS) (Pan Biotech), 0.5 mg/mL hydrocortisone (Sigma Aldrich), 0.5% Dimetilsülfoksit (DMSO) (Sigma Aldrich), 1% Penicillin-Streptomycin Solution (Pan Biotech), and 5% FBS (Pan Biotech).

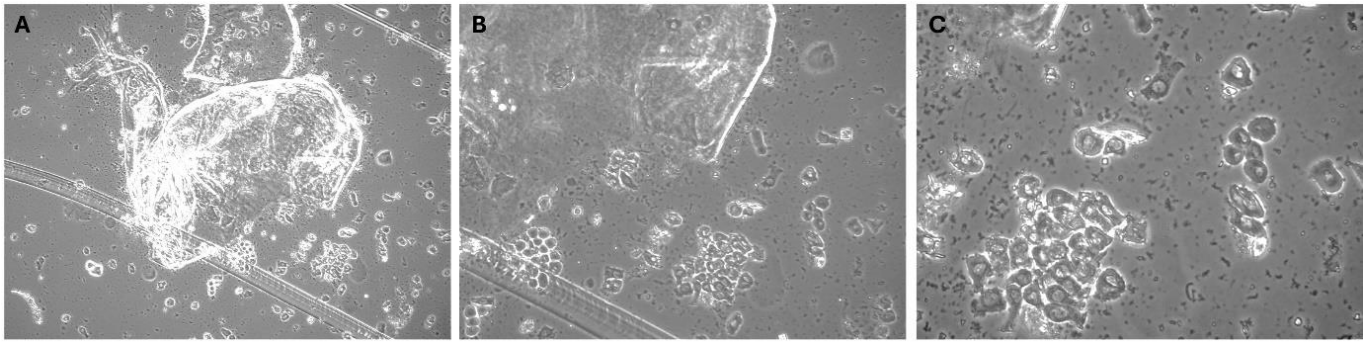


Figure 3. Corneal endothelial cells isolated by explant culture after 15 days culture period. (A) 10X magnification, (B) 20X magnification, (C) 40X magnification.

Limbal Stromal Stem Cells

For the isolation of limbal stromal cells, both explant and enzymatic culture techniques were applied. In explant culture, limbal stromal tissue, separated from other tissues, was dissected into small pieces and placed onto culture plates. After incubating for 15 minutes in a cell culture incubator to allow tissue adhesion to the surface, the culture process was initiated by adding growth medium consisting of DMEM-F12 (Pan Biotech) supplemented with 5% FBS (Pan Biotech) and 1% Penicillin-Streptomycin Solution (Pan Biotech).

In enzymatic culture, the dissected limbal tissues were incubated in a solution of Collagenase I (Merck) enzyme

prepared in growth medium. Three different procedures were applied: incubation for 1 hour in 4 mg/mL Collagenase I solution, 1 hour in 2 mg/mL Collagenase I solution, and 30 minutes in 2 mg/mL Collagenase I solution. Following the enzymatic process, the cells were centrifuged, growth medium was added onto pellet, and cells were seeded on culture plate.

Immortalization of Cells

Viable cells in passage 0 and passage 1 were immortalized with SV40, when the culture reached approximately 80% confluency. Briefly, transfection with Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and pSG5 Large T-SV40gp6 (Addgene, Watertown, MA, USA) was conducted using the protocol outlined below. After collecting the medium, the culture plate was washed with sterile 1X DPBS, and Opti-MEM medium was added onto the cells. The mixture of 125 µL of Opti-MEM (Gibco) and 5µL of Lipofectamine 3000 Reagent was prepared into an Eppendorf tube, and diluted lipofectamine solution was vortexed for 2-3 seconds. In another Eppendorf tube, diluted DNA solution containing 5 µg SV40 plasmid, 10 µL P3000 Reagent, and 250 µL Opti-MEM was prepared. The diluted DNA solution was added to the diluted lipofectamine solutions (125 µL each) and incubated at room temperature for 15 minutes. Then, it was gently added onto the cells in the culture plate and incubated for 2-4 days. The cells that were successfully immortalized continued to live, while the cells that failed to immortalize died and were eliminated through media changes.

Continuous Cell Acquisition through Tissue Transfer

After observing cell emergence from the cultured tissue pieces and proliferation of the emerged cells covering the surface of the culture plate, the tissue was gently grasped from the surface using forceps and washed with PBS. Subsequently, it was placed onto a new culture plate. The tissue was incubated in a cell culture incubator for 15 minutes to ensure adhesion to the surface. Then, growth medium was added to continue the culture process. As long as cell emergence continued, the transfer of tissue and cell culture procedures were continued.

Differentiation of Limbal Cells to Corneal Cells

After cell isolation by incubating with 2 mg/mL Collagenase I enzyme solution for 30 minutes, limbal cells were subcultured until passage 2. The growth medium used was DMEM-F12 (Pan Biotech) supplemented with 5% FBS (Pan Biotech) and 1% Penicillin-Streptomycin Solution (Pan Biotech). At passage 3, cell culture with different media to trigger differentiation was initiated. Three different media were evaluated; complete media was DMEM-F12 supplemented with 5% FBS and 1% Penicillin-Streptomycin Solution, serum free epithelial media was KSFM supplemented with 0.005 µg/mL EGF, 5 µg/mL BPE, and 1% Penicillin-Streptomycin Solution, and serum free keratocyte media was KSFM supplemented with 0.005 µg/mL EGF, 5 µg/mL BPE, 500 ng/mL hydrocortisone, 0.005 mg/mL insulin, and 1% Penicillin-Streptomycin Solution. The cells at passage 4 were characterized using immunocytochemistry (ICC) to evaluate their differentiation capacities. For the ICC procedure, cells fixed with 4% paraformaldehyde were permeabilized and blocked by incubating them in a solution containing 0.1% Triton X-100 and 3% bovine serum albumin (BSA) in PBS for 35 minutes. Primary antibodies diluted in 3% BSA in PBS were applied and incubated at room temperature for 1 hour. Similarly, secondary antibodies diluted in 3% BSA in PBS were applied and incubated at room temperature in the dark for 1 hour. The primary antibodies used in the study were Anti-Cytokeratin 3 (Abcam, Cambridge, UK, Ab68260, 1/50) (CK-3), Zonula occludens-1 Polyclonal antibody (Thermo Fisher Scientific, 18-7430, 1/250) (ZO-1), Anti-Collagen I (Abcam, Ab90395, 1/2000) (COL-I), Anti-Keratocan (Abcam, Ab230175, 1/200) (KERA), and Anti-Ki67 (Abcam, Ab15580, 1/100) (Ki67). The secondary antibodies used were Goat Anti-Mouse IgG H&L (Abcam, Ab175473, 1/500) and Goat Anti-Rabbit IgG H&L (Abcam, Ab150077, 1/500). Additionally, Cytopainter Phalloidin iFlour 647 (Abcam, Ab176759, 1/1000) was used for actin filament staining. After completion of the staining process, stained cell samples were mounted using Mounting Medium with DAPI (Sigma) on glass slides. Samples were then sealed with nail polish to prevent drying and observed under a confocal microscope (Carl Zeiss, Oberkochen, Germany, LSM710).

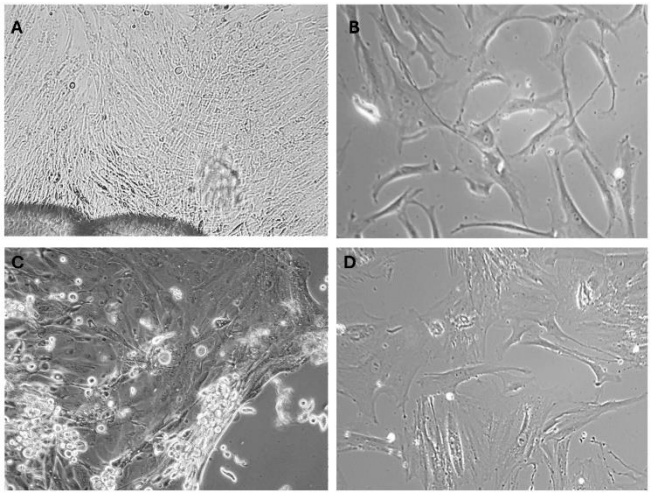


Figure 4. Morphological properties of isolated primary and immortalized corneal KCs. Morphology of KCs at passage 0 obtained from explant culture (A), isolated using 2 mg/mL collagenase for 1 hour (B), and isolated using 2 mg/mL collagenase for 30 minutes (C), after 1 week of culture. KCs isolated using 2 mg/mL collagenase for 30 minutes after subculture in passage 1 (D) following 2 weeks of culture (10X Magnification).

Results

Dissection and Cell Culture of Corneal and Limbal Layers

The tissue obtained from the eye bank comprises sclera, limbus, and corneal layers. Firstly, the scleral tissue was excised and removed. Subsequently, the corneal epithelial and endothelial layers were held with forceps and peeled off the surface. The remaining corneal stroma and limbal tissues were also dissected (Figure 1).

The dissection procedure described above was performed on 10 different donor corneas. The donor corneas were obtained from male and female donors aged between 55 and 74 years, with endothelial cell counts ranging from 1000 to 4635 cells per mm² (Table S1).

Corneal Epithelium

Explant culture was applied to the collected epithelial tissue strips using three different culture media. Based on the 15-day culture results (Figure 2), it can be stated that while the cell morphology derived from the tissue strips remains unchanged depending on the culture medium, but the cell density varies. The highest cell output was observed in the

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serum and EGF-supplemented medium, whereas the lowest cell output was observed in the serum-free medium.
Epithelial cell isolation was performed on one cornea by using both serum-free media and complete media with EGF and on five corneas by using complete media. Nonetheless, cells subcultured to passage 1 were unable to maintain viability. Therefore, immortalized cell line couldn't be obtained.

Corneal Endothelium
Three different techniques were applied for cell isolation from corneal endothelial tissue strips: explant culture, mechanical isolation, and enzymatic culture. The data obtained after a 15-day culture process indicate that the isolation of corneal endothelial cells was not successful with mechanical and enzymatic isolation techniques. According to the results (Figure 3), it can be stated that in explant culture application, endothelial cells were observed to emerge from tissue strips adhering to the surface to the culture plate, and these cells exhibited a hexagonal cell morphology similar to that in the natural tissue.
Even though, endothelial explant culture protocol was applied to eight donor corneas, none of the cells subcultured to passage 1 maintained viability and therefore, immortalized cell line could be obtained.

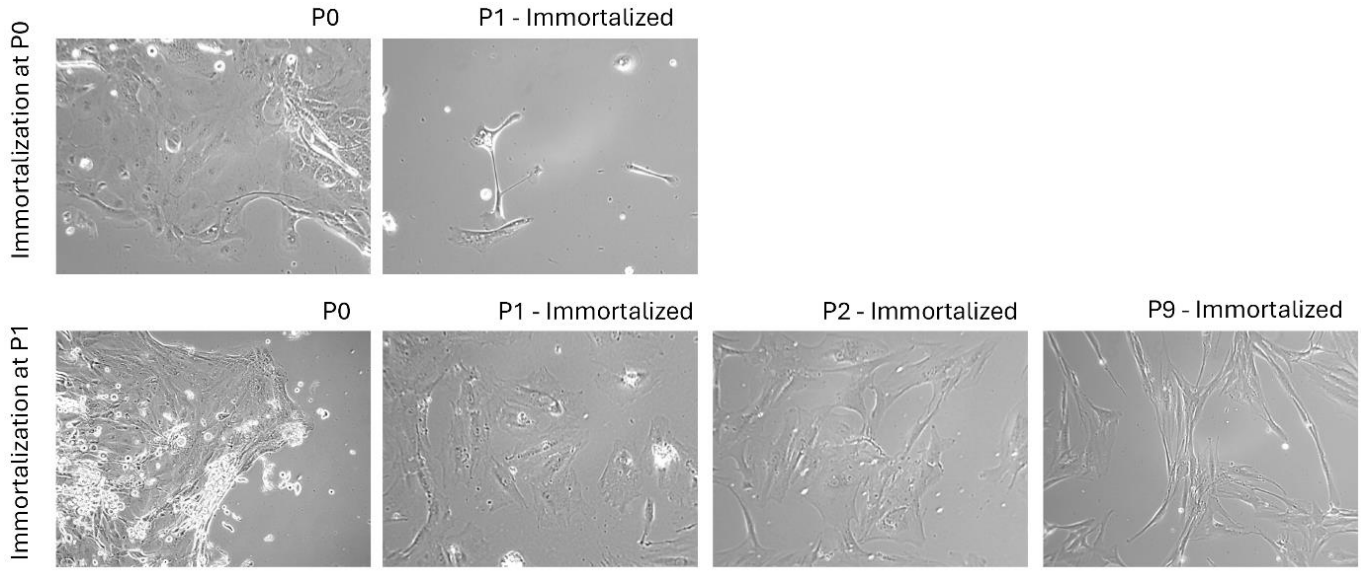


Figure 5. Morphologies of corneal stromal cells immortalized at passage 0 and 1 (P: passage) (10X Magnification).

Corneal Stroma
For the isolation of corneal stromal KCs, explant and enzymatic culture procedures were employed. Intensive cell output was obtained in the corneal stromal tissue with the explant culture process (Figure 4A). The explant culture process was applied to three donor corneas, but cell outgrowth was observed from only one cornea.
In the enzymatic culture process, cell isolation was not achieved with 1-hour isolation at 4mg/mL enzyme solution. Following this unsuccessful trail on one cornea, the enzyme concentration was reduced to 2 mg/mL (Figure 4B), a low cell density was obtained. However, the density of the isolated cells was low. Therefore, the isolation time was reduced to 30 minutes and high-density cell isolation was achieved (Figure 4C). Thereon, five donor corneas were used to obtain keratocytes with only one cornea resulting in positive outcome. The primary keratocyte cells isolated in 2 mg/mL enzyme solution in 30 minutes maintained their viability during subculture (Figure 4D). Therefore, it can be concluded that while explant culture remains advantageous, isolation of KCs with a 30-minute reaction time using a 2 mg/mL Collagenase I enzyme solution is also successful.
Corneal stromal cells isolated by incubation in a 2 mg/mL enzyme solution for 30 minutes were subjected to SV40 immortalization process at passage 0 and passage 1 (Figure 5). Cells that were highly confluent at passage 0 could not maintain viability after the immortalization process. When the immortalization process was applied at passage 1, a high amount of stress vesicles was accumulated in the immortalized cells. However, the amount of stress vesicles decreased by subculturing. Cells immortalized in passage 1 were subcultured up to passage 17, with a decrease in proliferation capacity was observed in higher passages. Based on these results, it can be stated that immortalizing primary corneal stromal cells after at least one subculturing provides successful outcomes.

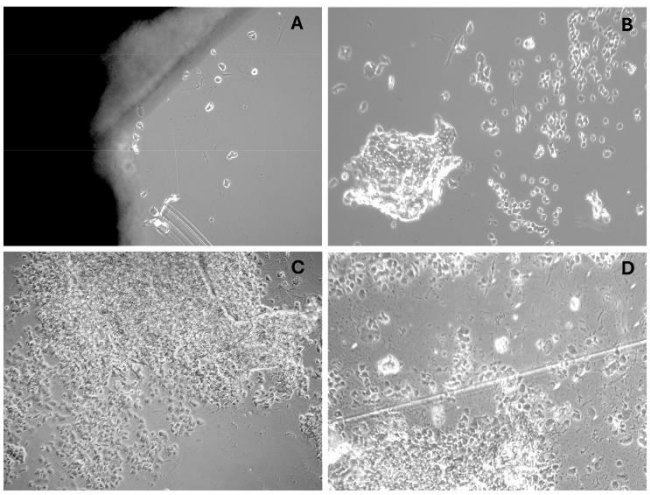


Figure 6. Morphologies of corneal stromal cells immortalized at passage 0 and 1 (P: passage) (10X Magnification).

Limbus
Limbal tissue is the source to isolate both limbal epithelial stem cells in the limbal epithelial layer and MSCs in the limbal stromal layer.

Limbal epithelial stem cells
Two different techniques were applied for the isolation of limbal epithelial cells. In the first technique, tissue pieces

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were incubated overnight with FBS droplets before adding the culture medium to initiate the explant culture process. After one week of culture, cell emergence from the tissues was observed (Figure 6A), and an increase in cell density was noted after 15 days of culture (Figure 6B). In the second technique, enzymatic culture was employed to isolate cells, where cell clusters were observed after one week of culture (Figure 6C), and cells proliferated throughout the 15-day culture period (Figure 6D). It was observed that cells isolated by both techniques exhibited epithelial cell morphology. These findings suggest that both explant and enzymatic culture techniques are viable for isolating limbal epithelial cells.

Limbal stromal stem cells

Explant and enzymatic culture procedures were employed for the isolation of limbal stromal stem cells. Tissue outgrowth from limbal tissue was observed after a culture period of 15 days (Figure 7A). In the enzymatic culture technique, isolation procedures were conducted using 4 mg/mL Collagenase I enzyme solution for 1 hour (Figure 7B), 2 mg/mL Collagenase I enzyme solution for 1 hour (Figure 7B), and for 30 minutes (Figure 7C). Cellular growth was observed in all three enzymatic isolation procedures.

Explant culture was applied to four corneas, and cell outgrowth was observed in one of them. Enzymatic isolation with 1-hour incubation using 4 mg/mL and 2 mg/mL enzyme solutions was performed on one cornea each, resulting in the acquisition of cell populations. The isolation process using 2 mg/mL enzyme solution for 30 minutes was applied to three corneas, yielding successful cell isolation in all cases, followed by subculturing. Cells immortalized at passage 1 maintained their proliferative capacity up to passage 14 (Figure 7E-F).

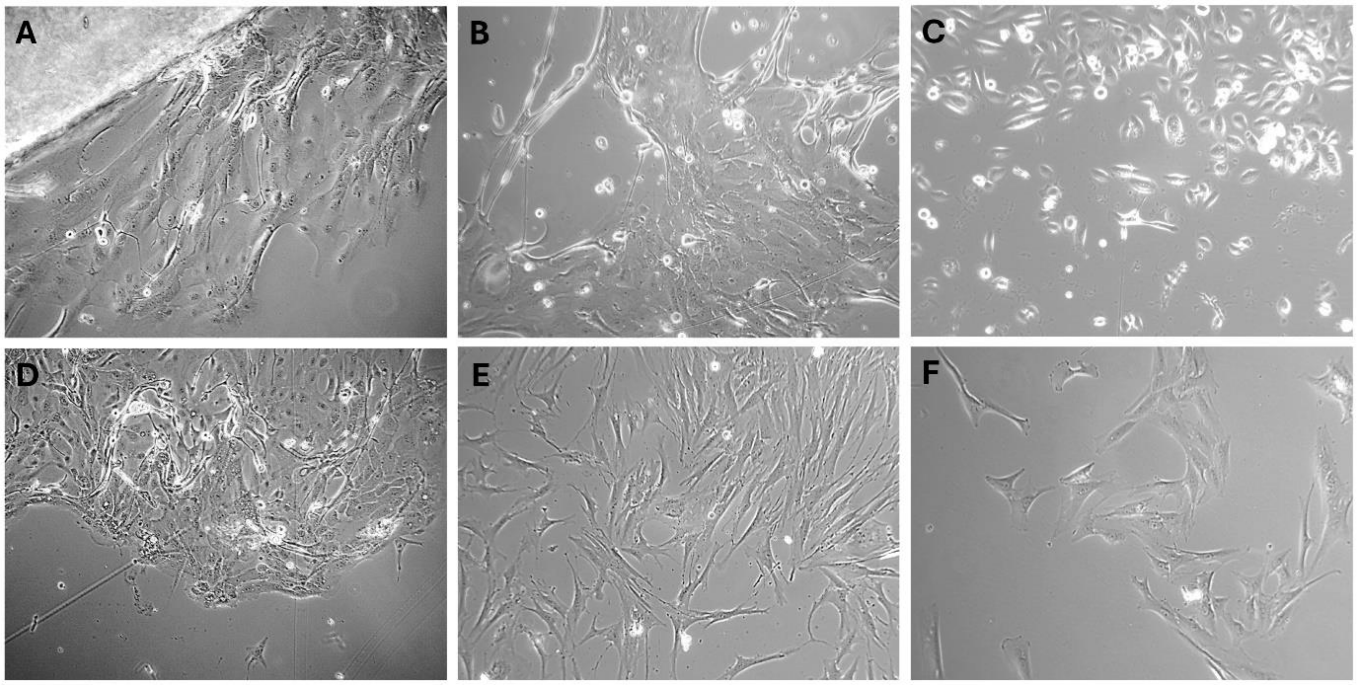


Figure 7. Corneal stromal stem cells isolated by explant culture (A) and enzymatic culture by applying 4mg/mL enzyme solution for 1 h (B), 2 mg/mL enzyme solution for 1h (C), 30 min (D) and immortalized cell at passage 2 (E) and passage 14 (F) after 15 days culture period (10X Magnification).

Continuous Cell Acquisition through Tissue Transfer

After observing cell outgrowth from the limbal tissue pieces used in the explant culture and subsequent proliferation of the emerged cells, tissue pieces were removed from the surface and transferred to a new culture plate to observe cell outgrowth once again. Based on the results, it can be stated that the transferred tissue piece undergoes shrinkage over the course of the process (Figure 8). Additionally, while the cells emerging from the tissue pieces were initially dense, their proliferation capacity decreased during transfers, and cellular changes due to stress were observed.

Differentiation of Limbal Cells to Corneal Cells

Limbal cells isolated using enzymatic culture method were cultured up to passage 2, and then culture media (complete, serum free epithelial, and serum free keratocyte medias) was changed at passage 3 to assess their differentiation potential. The cells maintained and subcultured in these growth medias were characterized using the ICC technique at passage 4. While the cells cultured in serum free keratocyte medium could not maintain viability, those in complete and serum free epithelial medias were viable, as evidenced by the ICC results (Figure 9).

The obtained results indicate that the corneal epithelial cell markers CK-3 and ZO-1 are expressed in both cell groups, but higher CK-3 expression is observed in the serum free epithelial media group. While the corneal keratocyte markers COL-I and KERA are expressed in both cell groups, COL-I expression is higher in the complete media group. Additionally, the expression of Ki67, a proliferation marker, is higher in the complete media group, and a homogeneous dendritic cell population is present.

Discussion

The cornea, a transparent and avascular tissue, provides two-thirds of the total refractive power of the eye.[2] The cornea has five main layers, which are epithelial, endothelial, and stromal KCs, with specific characteristics and functions.[2] The dissection and isolation of this small tissue are challenging that require expertise. However, the isolation of these cells is essential in various research fields, such as the treatment of corneal diseases, drug formulation, and tissue engineering. In this context, the isolation of corneal cells from donor corneas that are unsuitable for transplantation is of great importance. This study aimed to

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provide a detailed explanation of the dissection, isolation, subculture, and immortalization processes of corneal and limbal cells, using 10 human donor corneas obtained from an eye bank that were unsuitable for transplantation.

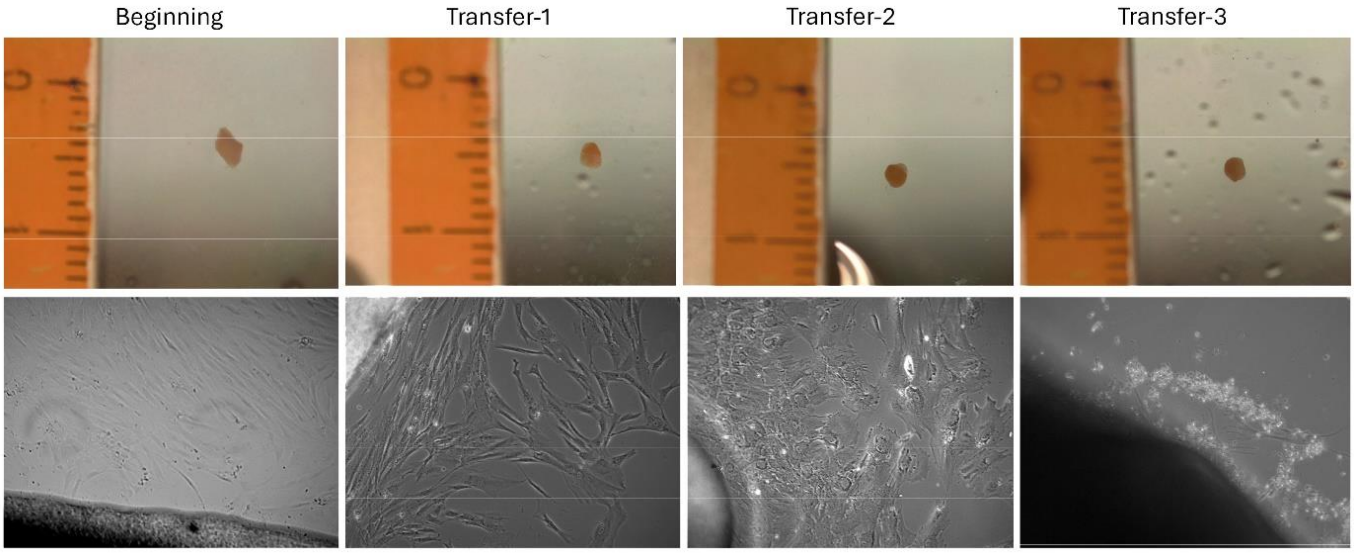


Figure 8. Transferred limbal tissue on cell culture. The tissue size decreases, and the morphology of the obtained cells exhibits changes induced by stress (10X Magnification).

The isolation of primary corneal epithelial cells through explant culture resulted in cell outgrowths in all three culture medias. The cells exhibit a rounded morphology, which is consistent with corneal epithelial cell morphology (Figure 2).[35] Additionally, in the serum-free medium, there was a lower cell outgrowth compared to the complete media (Figure 2A and 2B). When comparing complete medias among themselves, it was observed that the sample cultured in the complete media with EGF exhibited a higher cell outgrowth (Figure 2B and 2C). These findings indicate that serum and growth factor support in primary corneal epithelial cell culture enhance cell outgrowth from tissue strips. Factors such as donor age, smoking, and diabetes can affect the health of corneal cells and, consequently, their isolation.[36] The donor's health may lead to corneal opacification, which could prevent the viability of the cells isolated from these tissues during the culture process. In this study, opacification was observed in three corneas obtained from the eye bank, and corneal epithelial cells isolated from these tissues could not be subcultured (Table S1, Corneas #5, #6, and #8).

Another critical parameter is the endothelial cell count and viability, which determines the suitability of a donor cornea for transplantation, as it reflects the quality of vision after transplantation.[37] Since the corneal tissues used in this study were unsuitable for transplantation, the failure of the isolated cells to proliferate during the subculture process is an expected outcome. Accordingly, while endothelial cell outgrowths were observed from tissue strips in the explant culture (Figure 3), the results of enzymatic and mechanical isolation were unsuccessful. Corneal endothelial cells constitute a specialized cell population with a low proliferative capacity in the body. These cells are predetermined in a specific number during the embryonic period and do not proliferate throughout life or have very limited proliferative capacity.[38] This also restricts cell proliferation in primary cultures. Due to the low proliferation capacity, ECM support is applied in corneal endothelial cell culture, where proteins such as collagen, fibronectin, and laminin are preferred.[39] In the conducted study, the culture plate surface was coated with FNC Coating Mix as ECM support, which contains fibronectin, collagen type I, and albumin proteins.[40] It was observed that explant culture was more successful in the isolation of human corneal endothelial cells compared to other isolation techniques. According to the literature, the two main parameters affecting corneal endothelial cell isolation are donor age and the isolation technique. The study by Choi et al. demonstrated that the isolation success rate for donors aged 31-60 is 38.8%.[29] The donor corneas used in this study fall within this age range, directly influencing the outcome. Another study showed that among 13 explant culture isolation studies 4 were successful, while among 24 studies of enzymatic isolation using Trypsin-EDTA, only 2 reported success.[29] This result is attributed to the fact that enzymatic and mechanical isolation methods offer rapid isolation but can cause damage to the cells. On the other hand, although the isolation process takes longer in explant culture, the cells within their natural ECM structure allows for better preservation of phenotype and viability.[41]

Explant and enzymatic isolation techniques can successfully applied for corneal keratocyte isolation.[42] The results indicate that both explant (Figure 4A) and enzymatic (Figure 4B, 4C, and 4D) cultures yield successful outcomes in corneal KC isolation in this study as well. However, prolonged isolation times or high enzyme concentrations in explant culture adversely affect cell isolation. Increased enzyme concentration and prolonged isolation incubation time can lead to a reduction in cell viability and proliferation.[43] Therefore, it is necessary to determine the optimal conditions for isolation. This study demonstrates that, for the isolation of corneal keratocyte cells, incubation in a 2 mg/mL collagenase solution for 30 minutes represents the optimal isolation condition.

According to the information provided, primary cells have limited replicative capacity and undergo aging over time.[44] To address this issue, immortalization processes are applied to achieve long-term culture. The process of immortalization can be achieved by affecting the maintenance of telomeric length and cell cycle mechanisms.[45] In addition to immortalizing cells, the most commonly used technique, SV40, enhances proliferation capacity and cell viability while preserving primary cell characteristics. This technique ensures the continuity of the cell cycle by inhibiting p53 and retinoblastoma (Rb) proteins.[46] There are limited options available on the market for immortalized corneal cells. Options include cells immortalized with the HPV16 E6/E7 gene[47] and primary cells.[48] In line with this knowledge, the isolation of corneal KCs was conducted in this study, followed by their immortalization (Figure 5). However,

when the immortalized cells at passage 0 were subcultured, cell viability and proliferation did not continue. This was attributed to the high sensitivity of the cells at passage 0 to the stress induced by the immortalization process, leading to cell death.[49] Nevertheless, when the same process was applied to cells at passage 1, successful subculture was observed. Throughout subculture, it is noted that immortalized cells contained stress vesicles, which can be attributed to the stress imposed by the immortalization process on the cells.

In the limbus region, known as the palisades of Vogt, situated between the corneal and scleral tissues, LESCs are found, and these cells play a role in the regeneration of the corneal epithelial layer.[50] In this study, both explant (Figure 6A and 6B) and enzymatic (Figure 6C and 6D) culture techniques were employed for LESC isolation. The results indicate that cells isolated using both techniques exhibit a rounded morphology similar to limbal epithelial cells. However, upon subculture, the cells lost their viability, despite reports in the literature of successful isolations using these techniques.[51,52] It is known that the endothelial cell count per mm² of the corneas used in these procedures was zero, and visible opacification was observed in the corneas (Table S1, Corneas #7-#8). Based on this result, it can be suggested that the number of endothelial cells in the donor corneal tissue may serve as a potential indicator for the success of cell isolation. According to the literature, the endothelial cell count, which is a parameter for corneal transplantation, provides information about the overall health of the cornea.[37]

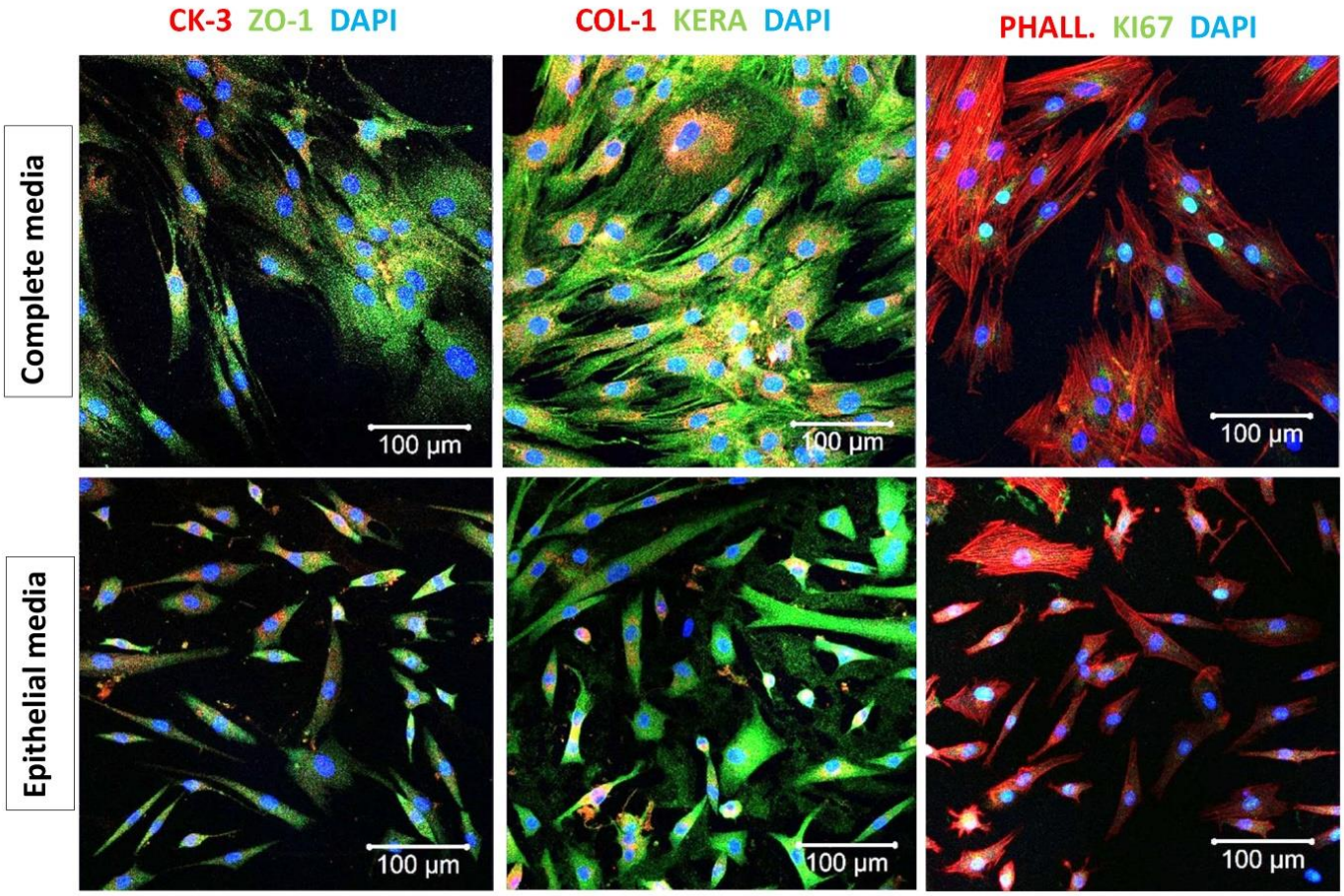


Figure 9. ICC results of limbal cells cultured on complete and serum free epithelial medias.

MSCs present in the limbal stroma contribute to corneal and limbus regeneration in a fibroblast-like morphology.[50] In this study, intense cell outgrowth was observed in explant culture (Figure 7A), with cells exhibiting a fibroblast-like morphology consistent with MSCs. However, this procedure was applied to four corneal tissues, with successful isolation achieved in only one. As previously explained, a low endothelial cell count provides information about the overall health of the cornea and directly affects the success of cell isolation.[37] In light of this information, it can be suggested that the reason for the unsuccessful isolations is the low endothelial cell count (1000-1300 cell/mm2) in two of the corneas (Table S1, Corneas #1 and #4). The other two corneas (Table S1, Cornea #5 and #6) had a higher endothelial cell count of 2700 cell/mm2. However, it's also known that advanced donor age reduces the likelihood of successful cell isolation from tissue. As such, cells were isolated from only 38.8% of donor tissues aged between 31 and 60 years.[29] In this study, the donor corneas #5 and #6 were from donors over the age of 70, which may explain the unsuccessful outcome. Similarly, cell isolation was achieved through enzymatic culture; however, with a high enzyme concentration, there was a high density of dead cells, and morphological changes occurred over prolonged culture periods (Figure 7B and 7C). Cells obtained through a 30-minute isolation process in a 2 mg/ml enzyme solution exhibited both high cell density and a cell population consistent with MSC morphology (Figure 7D). The literature shows that successful limbal cell isolation has been achieved with longer isolation times and higher concentrations of enzyme solutions.[43] However, it is known that during the enzymatic isolation process, the damage incurred by primary cells can lead to the loss of phenotype preservation in later passages.[53] Based on these findings, it can be concluded that the optimal isolation condition for corneal and limbal stromal cells via enzymatic culture is 30 minutes in a 2 mg/ml enzyme solution.

The conducted explant culture studies indicate that after cell outgrowth from the tissue piece, these emerged cells attach to the

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surface of the culture plate and proliferate along the surface. Based on this information, limbal tissue transfer was performed to evaluate the reusability of the tissue with cell outgrowth, and the morphology of the obtained cells was assessed after each transfer (Figure 8). The results obtained show that the tissue undergoing cell outgrowth exhibits shrinkage throughout the transfer processes. Additionally, while the cells before transfer showed a fibroblast-like morphology, they changed to a morphology containing dendritic structures with a high degree of stress vesicles after the first transfer. By the third transfer, cell outgrowth had almost ceased. This study, conducted for the first time, demonstrated that while it is possible to achieve additional cell outgrowth by transferring donor tissue that had previously yielded cells to a new surface, the isolated cells undergo morphological changes over time. Therefore, the use of cells isolated through tissue transfer in tissue engineering, therapeutic applications, and drug development studies during in vitro stages may not be suitable.

As it is known, the limbus serves as a source of stem cells for the cornea. Limbal stem cells play a key role in corneal regeneration. Therefore, the isolation of limbal stem cells from donor tissue is critical for research areas such as developing treatments for corneal diseases, regenerative medicine, and tissue engineering.[54] In Turkey, the company LimbuStem, led by Dr. Özlem Bulut Selver, conducts clinical treatments for corneal and limbal diseases through limbal stem cell transplantation.[55,56] Considering this information, the potential of isolated limbal stem cells to differentiate into corneal cells was evaluated. For this purpose, cultured limbal stem cells were cultured and subcultured in three different culture media and characterized by applying ICC technique (Figure 9). The expression of CK-3, a marker for corneal epithelial cells, was highest in cells cultured in serum-free epithelial medium. This could be attributed to the presence of EGF in serum-free epithelial medium, which supports the transformation into epithelial cells.[57] The expression of COL-I and KERA, markers for corneal KCs, was higher in cells grown in complete medium. Additionally, cells cultured in complete medium exhibited a higher expression of the proliferation marker Ki67 and displayed a dendritic cell morphology. This result may be due to the large amount of growth factors provided to the medium by FBS. It has been reported that limbal stem cells differentiated in medium supplemented with 10% FBS show positive expression of corneal keratocyte markers.[58] In this study, differentiation was achieved with 5% FBS supplementation. Therefore, it can be concluded that FBS supplementation as low as 5% is sufficient for the differentiation of limbal MSCs into corneal keratocytes.

Limitations

This study was conducted using ten human donor corneas that were not eligible for transplantation, and the findings should be interpreted within this limitation.

Conclusion

The isolation of cells from human corneal and limbal tissues is a challenging process that requires expertise. Although various applications and studies exist in the literature, there is no comprehensive source that thoroughly explains the isolation of all corneal and limbal cell types in detail. In this study, the isolation of all cell types from human donor corneal and limbal tissues is performed, and the outcomes encountered during these procedures are explained in detail, along with their underlying causes. It has been demonstrated in this study that the most critical factor in endothelial and epithelial cell isolation is the donor endothelial cell count. Additionally, for the successful isolation of limbal and corneal stromal cells, the optimal method is a 30-minute enzymatic digestion with a 2 mg/mL enzyme solution. The potential of limbal stem cells to differentiate into corneal cells has been proven, and this differentiation can be achieved through the supplementation of the culture medium. With the presented data, this source provides a detailed and comprehensive guide on the isolation and culture of corneal and limbal cells.

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Conflict of Interest

The authors declare no conflict of interest.

Compliance of Ethical Statement

This research was carried out under Ethical Approval 771/11/2019#20 and SUNUM-2019-82.

Author Contributions

S.C.: Study idea/Hypothesis; S.C., S.B.S.: Design; S.B.S.: Data Collection, Analysis, Literature review, Writing; E.T.V.: Tissue Collection; S.C.: Critical review.

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