

DEVELOPMENT OF CORNEAL TISSUE SUBSTITUTES

by

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ABSTRACT

DEVELOPMENT OF CORNEAL TISSUE SUBSTITUTES

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Keywords: Cornea, Corneal Tissue Engineering, Isolation and Culture of Corneal Cells

The cornea, a transparent, avascular, dome-shaped tissue at the eye's outermost layer, is vital for vision. Corneal transplantation, or keratoplasty, is the primary treatment for corneal diseases. However, the success of this method is significantly influenced by factors such as donor cornea health, donor age, and the risk of tissue rejection. Therefore, as presented in this thesis, the research in corneal tissue engineering is of utmost importance, offering potential solutions to these challenges.

Within the scope of this thesis, corneal and limbal cells were isolated from human donor corneas using various methods. Characterization processes were conducted on the isolated primary cells to assess their suitability for corneal tissue engineering. Additionally, the impact of immortalization on primary corneal cells was evaluated, determining that immortalized passage 12 corneal cells are most suitable for corneal tissue engineering. Furthermore, the characterization of corneal cell lines confirmed their suitability for corneal tissue engineering studies.

Corneal epithelial cells were cultured on transparent PGS membranes to mimic corneal layers and performed thorough characterization procedures. The obtained data strongly indicate the feasibility of mimicking epithelial and endothelial layers with PGS membrane and corneal cells, offering hope for future advancements in corneal tissue engineering.

The thickest layer of the cornea, the stromal layer, was successfully mimicked using cross-linked RADA hydrogels containing PGS nanoparticles. The results from the 3D hydrogel culture of corneal keratocyte cells demonstrated the promise of the tissue scaffold created using this method, paving the way for potential future applications in corneal tissue engineering.



ÖZET

KORNEA DOKU İKAMELERİNİN GELİŞTİRİLMESİ

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Tez Danışmanı: Dr. Sibel Çetinel

Anahtar Kelimeler: Kornea, Kornea Doku Mühendisliği, Korneal Hücrelerin İzolasyonu ve Kültürü

Kornea gözün en dış kısmında bulunan transparan, damarsız, saat camı şeklinde bir dokudur. Işık korneadan geçerek lense iletildiği için kornea, gözün görme kapasitesinde yüksek öneme sahiptir. Korneal hastalıkların tedavisi için keratoplasti denilen kornea nakli en çok tercih edilen yöntemdir. Ancak kornea naklinde donör kornea sağlığı, donör yaşı, doku reddi riski gibi parametreler etkilidir. Bu sebeple kornea doku mühendisliği çalışmaları yüksek önem taşımaktadır.

Bu tez çalışması kapsamında insan donör korneasından korneal ve limbal hücreler çeşitli yöntemlerle izole edilmiştir. İzole edilen primer hücreler üzerinde karakterizasyon işlemleri yürütülerek kornea doku mühendisliğine uygunlukları belirlenmiştir. Ayrıca immortalizasyon sürecinin primer korneal hücreler üzerindeki etkisi değerlendirilerek immortalize pasaj 12 korneal hücrelerin kornea doku mühendisliğinde kullanımının en

uygun olduđu belirlenmiřtir. Buna ek olarak, h cre hattı korneal h crelerin karakterizasyonları yapılarak kornea doku m hendisliđi  alıřmalarında kullanıma uygun oldukları belirlenmiřtir.

Korneal tabakaların taklit edilmesi amacıyla, transparan PGS membran  zerinde korneal epitel h creleri k lt r edilerek karakterizasyon iřlemleri y r t lm řtir. Elde edilen veriler, PGS membran ve korneal h crelerle epitel tabakasının taklit edilmesinin m mk n olduđu g stermektedir. Korneanın en kalın tabakası olan stroma tabakası  apraz bađlanmış RADA ve PGS nanopartik l hidrojel kullanılmıřtır. 3 boyutlu hidrojelde y r t len korneal keratocyte h cre k lt r  sonu ları g stermektedir ki  apraz bađlanmış RADA-PGS nanopartik l hidrojel ve korneal keratocyte h creleri birlikte kullanılarak oluřturulan doku iskelesi korneal stromanın taklit edilmesi i in uygundur.



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“Hayatımın en büyük şansı olan kıymetli annem ve babama...”

“To my dear mother and father, who are the greatest blessings of my life...”

TABLE OF CONTENTS

1. Introduction	1
1.1. General Biological Properties of the Cornea	1
1.1.1. Corneal Epithelium	2
1.1.2. Bowman's Layer	4
1.1.3. Stroma	4
1.1.4. Descemet's Membrane	8
1.1.5. Endothelium	9
1.2. Cornea Tissue Engineering	10
1.2.1. Main Parameters for CTE	11
1.2.2. Previous Research on CTE Applications	13
2. Materials and Methods	23
2.1. General Cell Culture Techniques	23
2.1.1. Throwing and Seeding of Corneal Cells	23
2.1.2. Subculturing and Freezing of Corneal Cells	23
2.1.3. SV40 Immortalization	24
2.2. General Cell Characterization Techniques	25
2.2.1. Immunocytochemistry (ICC)	25
2.2.2. Gene Expression	28
2.2.3. Trans Epithelial Electrical Resistance (TEER) Measurement	31
2.2.4. Collagen Staining	32
2.2.5. Biocompatibility Assays	32
2.3. Dissection of Human Donor Cornea	33
2.4. Isolation of Primary Corneal and Limbal Cells	33
2.4.1. Corneal Epithelial Cells	34
2.4.2. Corneal Keratocyte Cells	35
2.4.3. Corneal Endothelial Cells	36
2.4.4. Limbal Cells	37
2.5. Creation of Cell Stocks and Characterization of Corneal Cell Lines	40

2.5.1. hCEC	40
2.5.2. hKC	40
2.5.3. hCEnC	41
2.6. Co-culture of Corneal Cells	41
2.6.1. Selection of Optimum Culture Media for Co-culture	41
2.6.2. Determination of the Effect of Co-culture on Corneal Cells	42
2.7. Cornea Tissue Engineering Studies	44
2.7.1. Corneal Epithelium Tissue Engineering	44
2.7.2. Corneal Stroma	45
3. Results and Discussions	45
3.1. Dissection of Human Donor Cornea	45
3.2. Isolation of Primary Corneal and Limbal Cells	50
3.2.1. Corneal Epithelial Cells	50
3.2.2. Corneal Keratocyte	52
3.2.3. Corneal Endothelial Cells	65
3.2.4. Limbal Cells	67
3.3. Creation of the Cell Stocks and Characterization of Cell Line Corneal Cells	78
3.3.1. hCEC	78
3.3.2. hKC	90
3.3.3. hCEnC	93
3.4. Co-culture of Corneal Cells	95
3.4.1. Selection of Optimum Cell Culture Media for Co-culture of Corneal Cells	96
3.4.2. Determination of the Effect of Co-culture Process on Corneal Cells	97
3.5. Tissue Engineering	103
3.5.1. Corneal Epithelium Tissue Engineering	103
3.5.2. Corneal Keratocyte Tissue Engineering	107
4. Conclusion	109
5. References	111

LIST OF FIGURES

Figure 1. The main anatomy of the cornea and five distinct corneal layers, which are epithelium, Bowman's membrane, stroma, Descemet's membrane, endothelium ^[4]	2
Figure 2. Corneal epithelium and limbus. Limbal stem cells differentiate into corneal epithelial cells by asymmetric division (TAC: transient amplifying cells, PMC: post-mitotic cells, TDC: terminally differentiated cells) (Reproduced with permission from Tseng et al. ^[7] . © Elsevier).....	3
Figure 3. Collagen fibril organization of the corneal stroma. (A) highly ordered collagen fibril organization on the stroma layer, (B) Collagen fibril structure, (C) TEM image of healthy human corneal stroma, and (D) keratoconus human corneal stroma. (K: keratocyte, PG: proteoglycan, CF: collagen fibril) (Reproduced with permission from Cheng et al. ^[14] © Royal Society, Massoudi et al. ^[13] © Springer) ^[13-15]	5
Figure 4. General properties of corneal keratocytes. All corneal cells contain specific AQPs to provide water and pressure balance (A). There are many pressure effects on the cornea, which are balanced (B). In the scar situation on the corneal stroma, KCs differentiate into myofibroblast and activated fibroblast cell types (C). Limbus contains stem cells, one of which, CSSC, is the source for KC (D) (Reproduced with permission from Ahearne et al. ^[23] © Mary Ann Liebert, Inc., Hertsenberget al. ^[22] © Elsevier, Mohan et al. ^[21] © Elsevier) ^[19, 21-23]	8
Figure 5. Fundamental parameters to be considered in CTE studies.....	11
Figure 6. The results of <i>in vivo</i> studies conducted with LiQD artificial cornea in rabbits and mini-pigs ^[84]	22
Figure 7. The immortalization process was conducted using Lipofectamine 3000 and the SV40T plasmid ^[85]	25
Figure 8. Dissection processes of corneal and limbal tissues. (A) Anterior surface of the cornea, (B) Posterior surface of the cornea, (C) Posterior surface of the cornea before collecting the trabecular meshwork, (D) Posterior surface of the cornea after collecting the trabecular meshwork, (E) Collection of corneal epithelial and endothelial layers, (F) Removal of scleral tissue, (G) Limbal and corneal stroma tissues, (H) Separation and cutting of limbal and corneal stroma into small pieces (C: cornea, L: limbus, S: sclera, TM: trabecular meshwork).	49
Figure 9. Morphological results of primary hCECs cultured for 15 days in three different media using the explant culture technique (10X magnification).	51
Figure 10. KC cells isolated from Cornea 1 using the explant culture technique. Passage 0 cells at 12 days (A), 18 days (B), 26 days (C), and 32 days (D) after culture initiation, and passage 1 cells after 1 week (E) and 2 weeks (F) of culture.	53

Figure 11. The primary KCs obtained from different donor corneas and their cell morphologies. Morphologies of passage 0 (A) and passage 1 (B) KCs obtained with 1-hour isolation in a 2 mg/mL Collagenase I solution after 15 days of culture.	
Morphologies of passage 0 KCs obtained from Cornea 3 (C), Cornea 6 (D), Cornea 9 (E), and Cornea 10 (F) with 30 minutes of isolation in a 2 mg/mL Collagenase I solution after 15 days of culture (10X Magnification).	55
Figure 12. Immortalization and subculturing of primary KCs at different passages (10X Magnification).	57
Figure 13. The ICC results of primary passage 2 and immortalized passages 9, 12, and 15 KCs with main KC markers (P: passage).	59
Figure 14. Gene expression results for main corneal keratocyte markers and myofibroblast marker of primary and immortalized KCs (n=2) (Relative Quantification, Mean $\pm$ SEM).	61
Figure 15. The expression results of the stem cell marker VIM and the MSC marker CD90 in primary and immortalized KCs (n=2) (Relative Quantification, Mean $\pm$ SEM).	62
Figure 16. Morphologies of CEnCs isolated using the explant culture technique. (A) CEnCs isolated from Cornea #1, (B) Cornea #2, (C) Cornea #4, (D) Cornea #5, (E) Cornea #6, (F) Cornea #7, (G) Cornea #8, and (H) Cornea #10 after 2 weeks of culture (10X magnification).	66
Figure 17. Morphological appearances of LESC cells isolated from Cornea 7 via enzymatic culture: (A) at passage 0 after 2 weeks of culture, (B) at passage 0 one week after immortalization, and (C) at passage 1 after one week of culture with immortalized cells (10X magnification).	68
Figure 18. Morphologies of LESC cells isolated from Cornea 8 via explant culture: (A) after 1 week of culture at passage 0, (B) after 2 weeks of culture at passage 0, and (C) after 1 week of culture at passage 1 (10X magnification).	69
Figure 19. CSSC isolation via explant culture. Morphologies of CSSCs at passage 0 after 15 days of culture: (A) Cornea-1, (B) Cornea-4, (C) Cornea-5, (D) Cornea-6. Morphologies of CSSCs at passage 1: (E) Cornea-5 and (F) Cornea-6 (10X magnification).	70
Figure 20. Morphologies of CSSCs isolated from Cornea-2 (A), Cornea-7 (B), Cornea-3 (C), Cornea-9 (D), and Cornea-10 (E) after 15 days of enzymatic culture (10X Magnification).	71
Figure 21. Morphologies of Cornea-10 CSSCs at primary passage 2 (A) and immortalized passages 2 (B), 7 (C), 9 (D), 12 (E), and 14 (F) (10X Magnification).	72
Figure 22. ICC results of immortalized KCs in passages 9 and 12.	73
Figure 23. Gene expression results of primary passage 4, immortalized passages 9 and 12 CSSCs (n=1) (Relative Quantification, Mean $\pm$ SEM).	76
Figure 24. ICC results of CSSCs cultured in serum supplemented and epithelial serum free medias.	77
Figure 25. The effect of the tissue transfers on CSSC morphology (10X Magnification).	78

Figure 26. Summary of the study: Investigations were conducted to characterize the fundamental properties of human corneal epithelial cells (hCECs) cultured under control and ALI conditions in both serum-free and complete media.	79
Figure 27. Evaluation of CK expression during the 2 and 4-week culture periods. (A) Z-stack images obtained from ICC assay (Red: CK3, x and y axis: 400 μ m). (B) CK3, (C) CK12, (D) CK14 gene expression profiles (n=2) (Relative Quantification, Mean $\pm$ SEM).	81
Figure 28. Stratification capacities of hCECs cultured under control and ALI conditions in two different media after 4 and 2 weeks of culture. (A) Z-stack images obtained from ICC application of DAPI-stained samples (Blue: nucleus). (B) Thickness data obtained from measurements of z-stack images of cell groups (n=6).	83
Figure 29. Ki67 proliferation marker results for hCECs cultured under ALI and control conditions in serum-free and complete media for 2 and 4 weeks: (A) ICC z-stack images (green: Ki67) and (B) gene expression results (n=2) (Relative Quantification, Mean $\pm$ SEM).	84
Figure 30. Tight junction integrity profiles of hCECs cultured under control and ALI conditions in serum-free and complete media for 2 and 4 weeks of culture: (A) ICC images (blue: nucleus, green: ZO-1), (B) ZO-1 gene expression profile (n=2) (Relative Quantification, Mean $\pm$ SEM), (C) TEER measurement results (n=3).	87
Figure 31. The BM production capacity of hCECs was assessed using collagen-stained samples. Photographs of collagen-stained samples (A) and microscope images (10X Magnification) (B) are shown. Gene expression results for COL4A2 (C) are presented (n=2) (Relative Quantification, Mean $\pm$ SEM).	88
Figure 32. ICC results of hKCs at passage 2 using main KC and myofibroblast markers.	91
Figure 33. Gene expression results of hKC at passage 2 using main corneal and limbal marker genes (n=2) (Relative Quantification, Mean $\pm$ SEM).	93
Figure 34. ICC results of hCECs at different passages using main endothelial markers, Na-K ATPase and ZO1, proliferation marker Ki67, and actin filament stain phalloidin.	94
Figure 35. The culture set-up of the study. Single and co-culture were applied to determine the effect of co-culture on corneal cells.	96
Figure 36. The graph illustrates the viability percentages of corneal cells cultured in different media. The dark gray bars indicate the control group, consisting of cells cultured singly, while the light gray bars represent cells co-cultured. The media formulations tested include: M1: Keratinocyte Serum-Free Medium (KSFM), M2: Endothelial-SFM (Endo-SFM), M3: Prigrow Medium, M4: M1-M2-M3 at a 1:1:1 ratio, M5: M1-M2 at a 1:1 ratio, M6: M2-M3 at a 1:1 ratio, M7: M1-M2 at a 1:1 ratio (n=6).	96
Figure 37. The results of ICC staining using corneal epithelial-specific markers CK3 and ZO1 for hCECs are presented. hCECs were cultured under the following conditions after a two-week culture period: single-culture in M1 and M2 media and co-culture in M2 medium.	98
Figure 38. TEER measurements were conducted for HCECs cultured for two weeks under single-culture conditions in M1 and M2 media and under co-culture conditions in M2 medium (n=3).	99

Figure 39. The results of ICC performed using corneal endothelial-specific markers Na-K ATPase and ZO1 for hCEnCs are presented. The hCEnCs were cultured under both single- and co-culture conditions in M2 media after a two-week culture period.	100
Figure 40. TEER measurements were conducted for hCEnCs cultured for two weeks under both single-culture and co-culture conditions in M2 media (n=3).	101
Figure 41. The results of ICC performed using corneal keratocyte-specific markers COL I and KERA for hCKCs are presented. The hCKCs were cultured under single-culture conditions in M3 and M2 media, as well as under co-culture conditions in M2 medium, after a two-week culture period.	102
Figure 42. Transparency properties of PGS membranes. (A) UV-Vis spectroscopy results of PGS membranes polymerized for 50 minutes, (B) for 75 minutes, and (C) for 105 minutes, along with photographs taken after 48 hours of crosslinking.	104
Figure 43. Viability percentage values of hCECs cultured on 50, 75, and 105 min polymerized PGS membranes (n=4).	105
Figure 44. ICC results of hCECs after 15 days cultured on PGS membranes pre-polymerized at different periods.	106
Figure 45. Viability percentages of hKCs after 15 days culture period on RADA peptide hydrogel, crosslinked and uncrosslinked RADA – PGS-NP hydrogels (n=2).	108
Figure 46. ICC results of 2D cultured hKCs as control group and 3D cultured hKCs on hydrogel. (A) 3D distribution of cells, (B) COL I staining, (C) KERA staining.	109

LIST OF TABLES

Table 1. The antibodies used in the study and their fundamental characteristics.	26
Table 2. Main informations about reaction cycles of RT-PCR.	29
Table 3. Primers were used in the study.....	30
Table 4. Growth medias used in the culture studies of primary corneal epithelial cells.	34
Table 5. The details of the culture medias used in the co-culture study.	42
Table 6. The main information of human donor corneas, which are donor age and gender, endothelial cell count and tissue pictures.....	45
Table 7. Gene expression results of primary and immortalized hKCs by using main limbal cell markers (n=2) (Relative Quantification, Mean $\pm$ SEM).	63
Table 8. The gene expression profiles of primary and immortalized KCs for CEC and CEnC specific genes (n=2) (Relative Quantification, Mean $\pm$ SEM).....	64
Table 9. The summary of the ALI culture and media effect on the hCECs.	89

LIST OF ABBREVIATIONS

ACS	Adipose derived mesenchymal stem cell
ALDH	Aldehyde dehydrogenase
ALI	Air liquid interphase
AQP	Aquaporin
ASD	Anterior segment dysgenesis
BM	Basement membrane
BL	Bowman's layer
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
CEC	Corneal epithelial cell
CEnC	Corneal endothelial cell
CK	Cytokeratin
CSSC	Corneal stromal stem cell
CTE	Cornea tissue engineering
CLP	Collagen like peptide
CLP-PEG-MPC	Collagen like peptide -PEG- 2methacryloyloxyethyl phosphorylcholine
DL	Descemet's membrane
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EDC/NHS	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide / N-Hydroxysuccinimide
EGF	Epithelial growth factor

ESC – Embryotic stem cell
FBS – Fetal bovine serum
FECD – Fuch’s endothelial corneal dystrophy
GELMA – Methacrylated gelatin
HBSS – Hank’s balanced salt solution
hCEC – Human corneal epithelial cell
HEMA – 2-hydroxyethyl methacrylate
hKC – Human keratocyte cell
hCEnC – Human corneal endothelial cell
hTERT – Human telomerase reverse transcriptase
ICC – Immunocytochemistry
KC – Keratocyte
KSFM – Keratocyte serum free media
LESC – Limbal epithelial stem cell
LSCD – Limbal stem cell deficient
MSC – Mesenchymal stem cell
OD – Optical density
PBS – Phosphate buffer saline
PCL – Poly ethylene glycol
PCR – Polymerase chain reaction
PECL – Poly(ϵ -caprolactone)-poly(ethylene glycol)
PEG – Poly(ethylene glycol)
PEGDA – Diacrylated PEG
PGS – Poly(glycerol sebacate)
PGS-NP – PGS nanoparticle
PVA – Polyvinyl alcohol
RT-PCR – Real time PCR
TEER – Trans epithelial electrical resistance
TAC – Transit amplifying cell
ZO – Zonula occludens

α SMA – Alpha smooth muscle actin



1. Introduction

The cornea is a transparent tissue located at the outermost part of the eye, playing a crucial role in clear vision. Numerous tissue engineering studies have been conducted in this field. Below are detailed explanations of the fundamental properties of the cornea, the critical parameters for corneal tissue engineering, and literature reviews on these topics.

1.1. General Biological Properties of the Cornea

The cornea is the transparent tissue located on the outermost part of the eye that allows light to be transmitted to the inner layers of the eye (**Figure 1**). Along with the lens, it ensures that clear images are conveyed to the retina. The transparency of the corneal tissue is maintained by the high organization of collagen fibers within its structure and the absence of blood vessels. Because of the lack of blood vessels, nutrients, and oxygen are delivered to the cornea via diffusion through the tear fluid. The cornea has a unique shape similar to that of a glass watch, which helps to focus light precisely onto the retina ^[1]. Changes in the shape of the cornea, such as in astigmatism and keratoconus, lead to blurred vision. In a healthy human eye, the corneal thickness is approximately 540 μ m. Conditions such as keratoconus, Fuchs' endothelial dystrophy, Down syndrome, eye injuries, and diabetes can alter corneal thickness and result in visual impairment ^[1-2].

The cornea consists of five layers, from outermost to innermost: epithelium, Bowman's layer, stroma, Descemet's layer, and endothelium (**Figure 1**). The epithelium, stroma, and endothelium layers contain cells, whereas Bowman's and Descemet's layers do not contain cells and provide support and adhesion ^[3]. Each corneal layer has specific material compositions and functions, detailed below.

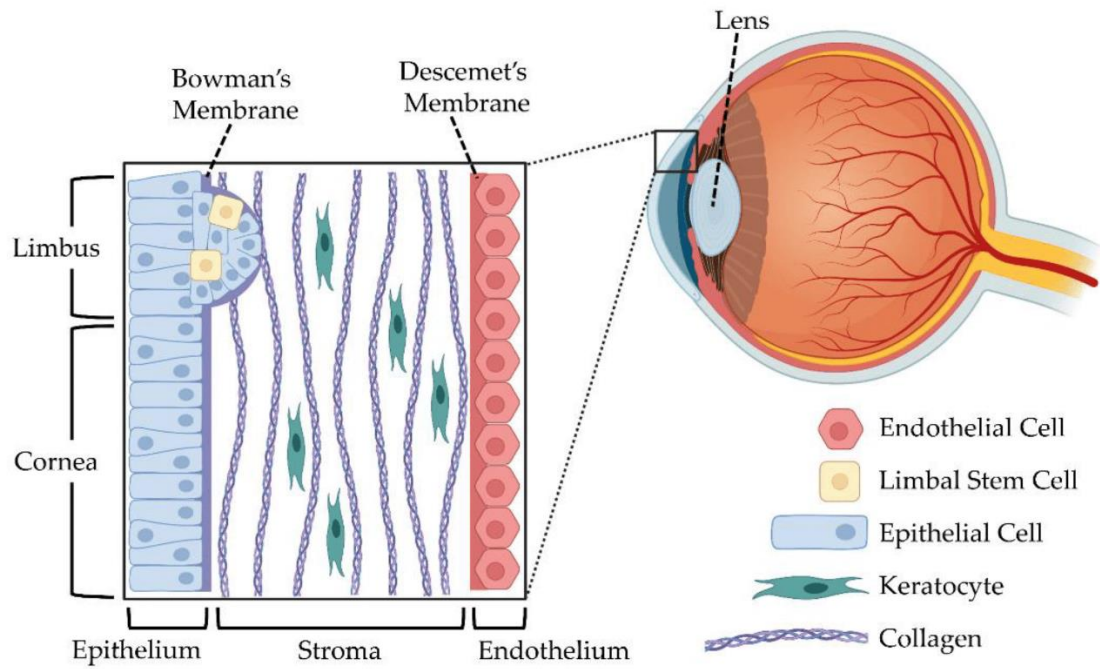


Figure 1. The main anatomy of the cornea and five distinct corneal layers, which are epithelium, Bowman's membrane, stroma, Descemet's membrane, endothelium (Reproduced from Jameson, J.F. et al. with permission from © MDPI) ^[4].

1.1.1. Corneal Epithelium

The outermost layer of the cornea, the epithelium, is 50µm thick and composed of 5-7 layers of corneal epithelial cells. The basal cells, located at the bottom layer, continuously regenerate, ensuring the renewal of the epithelium. The cells move upward through the stratification of the constantly dividing basal cells. The middle layer contains polygonal cells that retain tear fluid to prevent dryness, nourish the cornea, and protect against infections. The uppermost layer comprises flattened cells that help maintain corneal transparency. These cells are arranged in a regular pattern and form tight junctions, minimizing intercellular spaces and ensuring transparency ^[5]. Corneal epithelium regeneration is facilitated by stem cells located in the limbal epithelium, known as limbal epithelial stem cells (LESCs) ^[6]. LESCs maintain the stem cell population in the limbus through asymmetric division and provide a cell source for the corneal epithelium ^[6]. As LESCs differentiate, they transform into transit amplifying cells (TACs), which migrate

from the Palisades of Vogt, a curved region, towards the cornea, completing their differentiation into corneal epithelial cells (CECs) ^[6-7] (**Figure 2**).

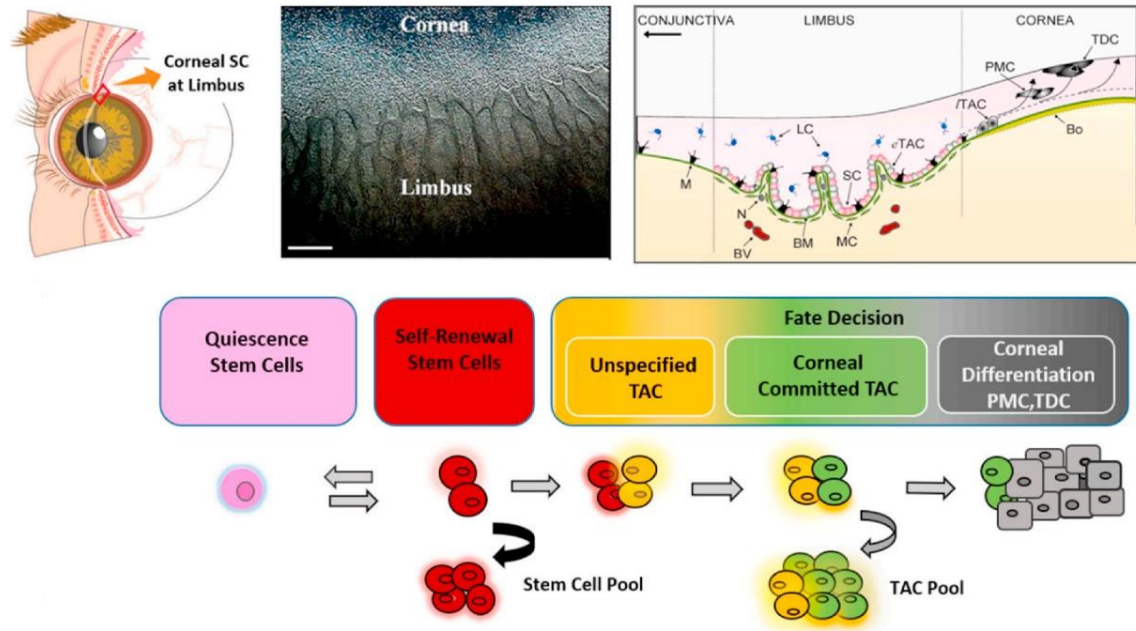


Figure 2. Corneal epithelium and limbus. Limbal stem cells differentiate into corneal epithelial cells by asymmetric division (TAC: transient amplifying cells, PMC: post-mitotic cells, TDC: terminally differentiated cells) (Reproduced from Tseng et al. ^[7] with permission from © Elsevier).

One of the primary characteristics of corneal epithelial cells is their specificity in cytokeratin (CK) expression. CKs are keratin-containing intermediate filaments found in epithelial cells, which play roles in mechanical strength, cell shape, and organelle transport within the cell. The CK types produced vary depending on the epithelial cell type and function. LSCs express high levels of CK-5 and -14, while the expression of CK-3 and -12 increases as they differentiate into CECs ^[8]. Another essential feature of corneal epithelial cells is minimizing gaps between cells through junctions. Intercellular junctions formed by structures such as claudins, occludin, and zonula occludens (ZO) regulate the passage of water and macromolecules from tears to the inner layers of the eye ^[9]. The corneal epithelium, with its high junction-containing structure, differs from other corneal layers and the limbal epithelium. For example, CECs express connexin-43 gap junction protein, while limbal cells do not ^[8].

CECs are located on the basement membrane (BM), which controls the passage of materials from the epithelium to the stroma and provides a surface for epithelial cell adhesion. The BM comprises laminins, collagens, heparan sulfate proteoglycans, and nidogens. BM laminins such as laminin-111, -332, and -511 provide attachments for CECs. In the BM, collagen types IV, VII, XVII, and XVIII facilitate CEC adhesion and proliferation. Hemi-desmosomes formed between the BM and CECs are created by collagen type XVII in the BM and integrin $\alpha 6\beta 4$ in CECs ^[10].

1.1.2. Bowman's Layer

Bowman's layer (BL) is located just beneath the BM, forming a regular connection between the epithelium and the stroma. This layer, which is 8-12 μm thick, comprises randomly oriented collagen fibrils. BL consists of collagen type I, III, V, and XII. Additionally, proteoglycans such as heparan sulfate and perlecan found in BL are positioned between collagen fibers, increasing their water-retaining capacity ^[11]. With its semi-permeable nature, BL regulates the passage of water and macromolecules into the stroma.

1.1.3. Stroma

The stroma, which constitutes approximately 90% of the cornea, contains extracellular matrix (ECM) molecules, keratocyte cells (KCs), and a high-water content. ECM molecules include proteoglycans, collagens, and glycoproteins. Within the stroma, ECM molecules are organized in a highly structured manner, minimizing light scattering and enhancing mechanical resistance against trauma. Proteoglycans contribute to the water-retaining capacity of the highly hydrated stromal layer, maintaining hydration. Glycoproteins present in the stromal ECM facilitate the interaction between KCs and the ECM ^[12].

The highly organized distribution of collagen in the stroma plays a significant role in maintaining clear vision by minimizing light scattering as it passes through the cornea. The primary types of collagens found in the stroma are type I and type V, with type I being the most abundant. In the corneal stroma, there are fibril-forming collagens (type I, III, and V), fibril-associated collagens (type XII and XIV), and network-forming collagens (type IV, VI, and VIII) [12-13]. The fundamental characteristic of the stroma, composed of small-diameter collagen fibrils, is stabilized by other collagens, proteoglycans, and glycoproteins, resulting in a highly ordered structure [12, 14]. This organized architecture of stromal collagens is disrupted in the presence of corneal diseases (**Figure 3**) [15].

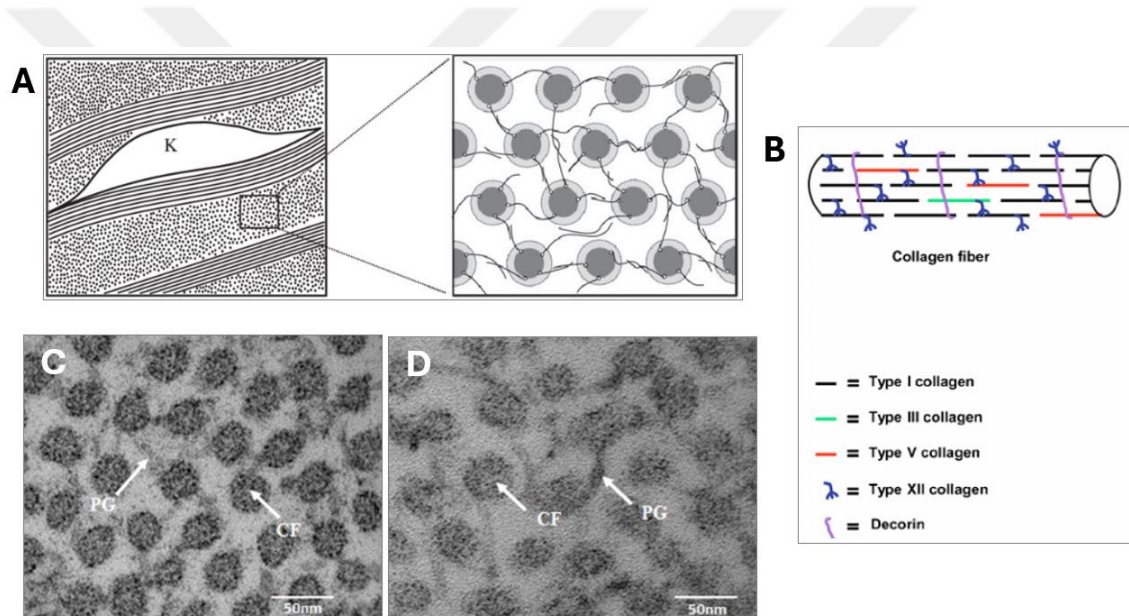


Figure 3. Collagen fibril organization of the corneal stroma. (A) highly ordered collagen fibril organization on the stroma layer, (B) Collagen fibril structure, (C) TEM image of healthy human corneal stroma, and (D) keratoconus human corneal stroma. (K: keratocyte, PG: proteoglycan, CF: collagen fibril) (Reproduced from Cheng et al. with permission from © Royal Society, Massoudi et al. with permission form © Springer, Alkanaa et al. with permission from © Nature) [13-15].

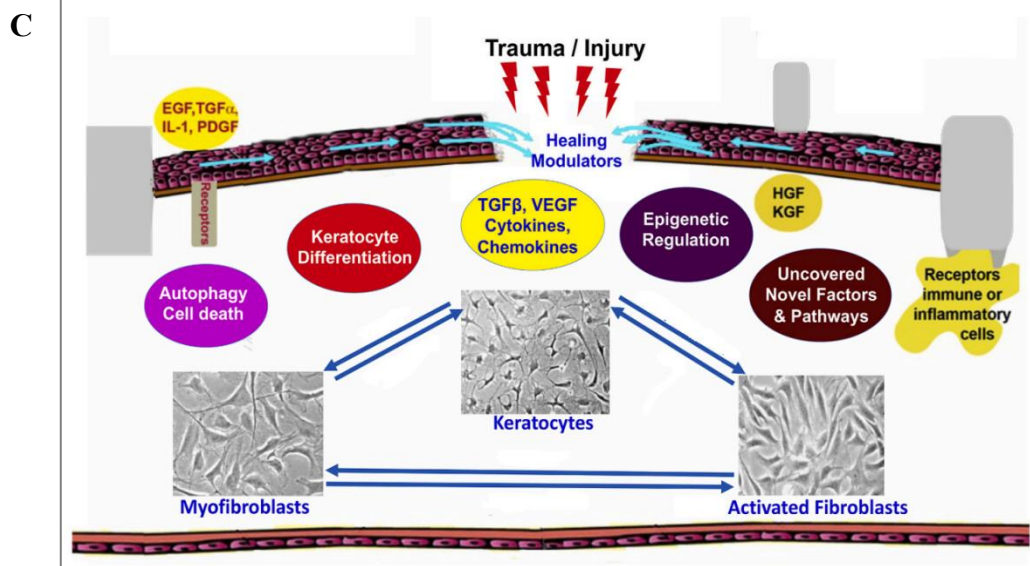
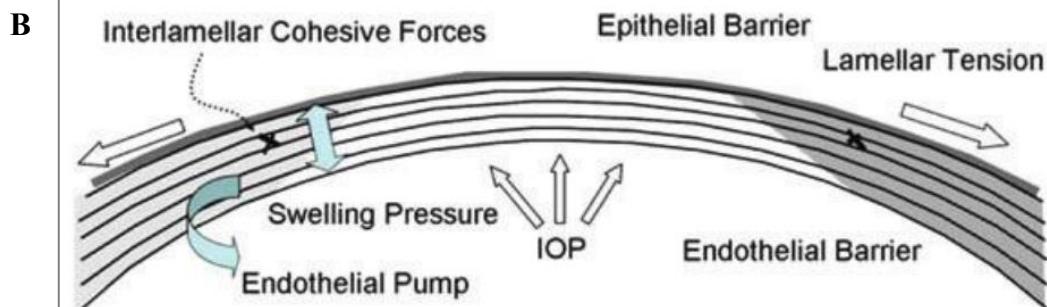
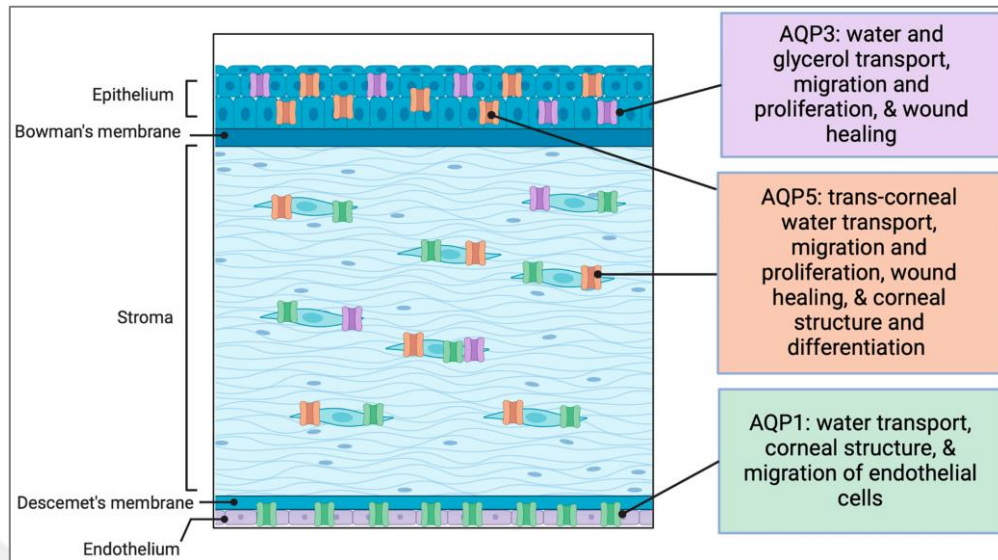
Proteoglycans are another ECM molecule involved in forming highly organized collagen fibrils. Corneal stroma proteoglycans, such as decorin, biglycan, lumican, keratocan, and fibromodulin, perform functions such as fibril formation, stroma hydration, providing

attachment areas for growth factors, and supporting cell growth. Keratocan, the most abundant proteoglycan in the corneal stroma, regulates the spacing between collagen fibrils ^[12].

The corneal stroma contains keratocytes (KCs) that are mitotically inactive and exhibit a dendritic morphology ^[16]. The maintenance of the corneal stroma is facilitated by the production of extracellular matrix (ECM) molecules by KCs. These dendritic KCs interact with ECM molecules through numerous lamellipodia, a cellular structure that helps minimize light scattering ^[17]. The most highly expressed ECM molecules by KCs are collagen type I and keratocan. Additionally, they express the crystallin protein aldehyde dehydrogenase (ALDH) ^[17-18]. ALDH is an enzyme that oxidizes aromatic and aliphatic aldehydes and plays a role in the defense mechanism against ultraviolet light and reactive oxygen species and reducing light scattering ^[17]. Water balance on the cornea is provided by water channels called aquaporins (AQPs), in which all corneal cells contain specific AQPs (**Figure 4-A**). The maintenance of hydration in the highly water-containing corneal stroma is of critical importance, and AQP plays a vital role in this process ^[19]. By regulating AQP function, intraocular pressure balance is maintained, preventing the formation of edema (**Figure 4-B**) ^[20].

KCs constitute approximately 3% of the stromal layer and are in a quiescent state. When injury occurs in the stroma, these cells transition from their quiescent state to an active state and surround the scar area. Activated KCs differentiate into myofibroblast cells. During this differentiation, the cells lose their dendritic morphology, acquire a fibroblast-like morphology, and exhibit muscle-like features such as forming actin-myosin bundles. Additionally, myofibroblast cells express high levels of alpha smooth muscle actin (α -SMA), while the expression of KC-specific proteins decreases. The ECM molecules produced by myofibroblast cells in the scar area disrupt the highly organized ECM structure of the corneal stroma, leading to a reduction in visual clarity (**Figure 4-C**) ^[21].

The Palisades of Vogt in the limbus serve as a source of stem cells for the cornea. The limbal stroma in this region contains mesenchymal stem cells known as corneal stromal stem cells (CSSCs) (**Figure 4-D**). These stem cells provide a cellular source and regenerative capacity for the corneal stroma, as they can fully differentiate into keratocytes. Like other mesenchymal stem cells, CSSCs are characterized by the expression of CD90⁺, CD105⁺, and CD75⁺ markers ^[22].



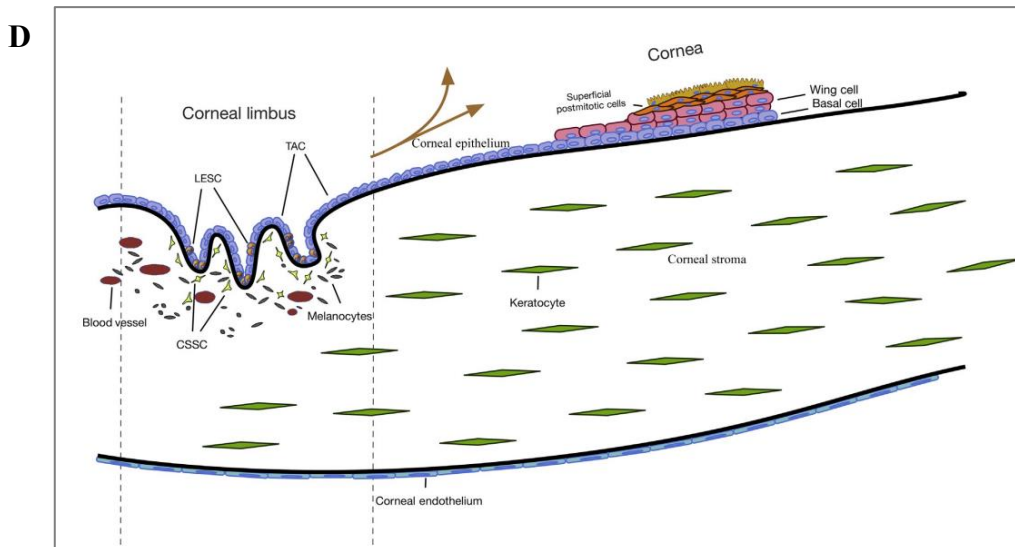


Figure 4. General properties of corneal keratocytes. All corneal cells contain specific AQPs to provide water and pressure balance (A). There are many pressure effects on the cornea, which are balanced (B). In the scar situation on the corneal stroma, KCs differentiate into myofibroblast and activated fibroblast cell types (C). Limbus contains stem cells, one of which, CSSC, is the source for KC (D) (Reproduced from Ahearne et al. with permission from ©Mary Ann Liebert, Inc., Hertsenberg et al. with permission from ©Elsevier, Mohan et al. with permission from ©Elsevier, Melnyk et al. with permission from © MDPI) [19, 21-23].

1.1.4. Descemet's Membrane

Descemet's layer (DL) is a membrane composed of collagens produced by endothelial cells, connecting the stroma and endothelium layers. The DL is approximately 3 μm thick at birth and has a banded structure. With aging, the DL becomes thicker, but there are no bands in this added thickness. In a 70-year-old individual, the DL consists of 3 μm banded and 10 μm unbanded layers. The DL contains collagen types IV and VIII, fibronectin, laminins, proteoglycans, and nidogens. Collagen type VIII is the predominant collagen type in the DL, providing elasticity. Collagen type IV enhances the mechanical strength of the DL. Proteoglycans stabilize the collagen fibers, while glycoproteins play a role in cell-matrix interactions [24].

1.1.5. Endothelium

The endothelium layer is the innermost part of the cornea, consisting of a monolayer of corneal endothelial cells (CEnCs). A human cornea has approximately 6000 CEnCs per mm² in the first month after birth. The number of CEnCs decreases with age at about 0.6% per year. This decrease occurs because corneal CEnCs are arrested in the G1 phase of the cell cycle and are thus mitotically inactive. The loss of CEnCs is compensated by the remaining cells expanding cytoplasmically to cover the area of the dead cells. Corneal failure can develop when the total number of CEnCs in the human cornea falls below 1000 [25].

CEnCs, which form a monolayer on Descemet's membrane, are connected by tight junctions [25]. Junction proteins, claudin, and ZO, are involved in the formation of these leaky intercellular tight junctions [25-26]. The corneal endothelium maintains corneal transparency by pumping water and ions from the cornea toward the inside of the eye. The efficiency of this function is enhanced by the tight junction structures between CEnCs. Additionally, these tight junctions prevent excessive fluid passage into the cornea, thereby balancing the pressure. However, conditions such as aging, injury, infections, and diabetes can disrupt the tight junction structures between CEnCs [27].

CEnCs highly express Na-K ATPase. This active transport mechanism pumps Na⁺ ions out of the cells and K⁺ ions into the cells, creating an electrochemical gradient. This electrochemical gradient generates osmotic pressure, facilitating the movement of water from the cornea into the interior of the eye. Through this mechanism, the water balance in the cornea is maintained, and intraocular pressure is regulated [25].

To maintain corneal function and structure, CEnCs express two main types of collagens, which are collagen type IV and type VIII [28]. The primary type of collagen produced by CEnCs is collagen type IV, which provides an adhesion surface for CEnCs and forms a mechanical connection between the endothelium and stroma. Mutations in collagen type IV can lead to corneal opacity and diseases such as anterior segment dysgenesis (ASD). Collagen type VIII, one of the fundamental collagens of DM, facilitates cell adhesion and

connections with other collagens. Mutations in this protein can lead to diseases such as Fuchs' endothelial corneal dystrophy (FECD) ^[13].

1.2. Cornea Tissue Engineering

Tissue engineering integrates biology, engineering, and materials science to repair or replace defective or damaged tissues and organs. Cornea tissue engineering (CTE) is a branch of tissue engineering with objectives such as reducing the need for corneal transplantation, preventing vision loss, and advancing the treatment of corneal diseases. Keratoplasty, also known as corneal tissue transplantation, is the most common treatment method used in the management of irreparable corneal diseases ^[29]. In this procedure, a healthy cornea from a donor is transplanted into the recipient's cornea. Several parameters are considered in the selection of the donor cornea for transplantation. Donor corneas preferably come from individuals under the age of 75 ^[30]. Corneas from donors with infectious or systemic diseases are deemed unsuitable for transplantation. Additionally, corneas with high intraocular pressure or eye injuries are not suitable for transplantation. Apart from these factors, the suitability of a donor cornea for transplantation is determined by the endothelial cell count. An adult donor human cornea should have approximately 2000-2200 cells/mm². During the keratoplasty procedure, approximately 10% of the cells are lost because of the mechanical force applied ^[31]. Additionally, up to 70% cell loss can occur within five years after keratoplasty ^[32].

Although keratoplasty is an effective treatment to prevent vision loss, it presents several significant disadvantages post-operation. The most critical disadvantage is immune rejection, which can lead to damage to the transplanted cornea and even loss of the eye ^[33]. Additionally, in some cases, the quality of vision may not fully return to its original state after keratoplasty, which can be because of the quality of the donor cornea, complications during surgery, or other health issues present in the patient or donor ^[34]. Since the cornea is an exposed tissue, there is a high risk of infection following keratoplasty ^[35]. Finding a suitable donor may require a long waiting period, which can reduce the success of postoperative outcomes ^[36]. These considerations highlight the

significant importance of CTE, a continually advancing field with promising future developments.

Several parameters need to be considered in CTE studies. These parameters are detailed below.

1.2.1. Main Parameters for CTE

Several fundamental parameters must be considered in cornea tissue engineering studies, detailed below (**Figure 5**).

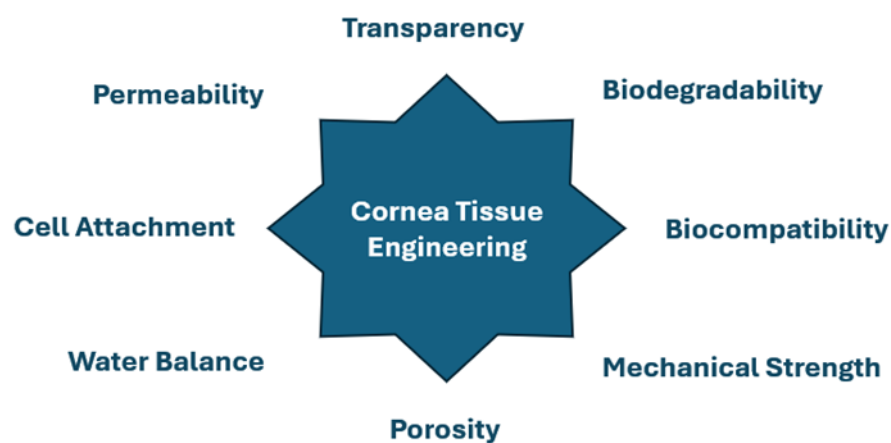


Figure 5. Fundamental parameters to be considered in CTE studies.

Biodegradability: Biodegradable scaffolds provide a structure that can form the corneal framework and generate new tissue. Over time, the body degrades these tissue scaffolds while the newly formed corneal tissue takes its place. The degradation rate of the scaffold must be proportional to the rate of new tissue formation. Rapid scaffold degradation can reduce the mechanical strength between the natural tissue and the scaffold, potentially damaging the surrounding healthy tissue. Additionally, the by-products formed during degradation must be non-toxic ^[37].

Biocompatibility: Biocompatible tissue scaffolds are structures that are compatible with body tissues and do not cause any toxic reactions. Biocompatible tissue scaffolds do not induce immune reactions, and do not cause immunological rejection ^[38]. *In vitro* cytotoxicity tests and *in vivo* animal assays can be conducted to assess the biocompatibility of tissue-engineered scaffolds.

Mechanical Strength: Tissue-engineered corneas should resist factors such as intraocular pressure, eyelid movement, tear film motion, and eye movement pressure. Their mechanical properties should be similar to natural corneal tissue and compatible with surrounding tissues. The Young's modulus measures the cornea's deformation under pressure, with the Young's modulus of a human cornea being approximately 100 kPa - 57 MPa. The cornea has a flexible structure, and the tensile strength of a human cornea is approximately 3 - 6 MPa ^[39]. The key factors influencing the cornea's mechanical strength include collagen content, corneal topography, thickness, corneal cells, and water content. The watch glass-like shape and high-water content of the cornea increase the mechanical resistance and evenly distribute intraocular pressure ^[40].

Cell Attachment: In tissue-engineered artificial corneal tissue, corneal cells must adhere, proliferate, and express their key markers. Similar to the natural cornea, different layers should contain different types of cells, and the tissue scaffold should allow the passage of nutrients, water, and biomaterials (such as growth factors, exosomes, etc.) while preventing the migration of different cell groups.

Permeability: The cornea consists of 70-80% water. When the water content is too low in a tissue-engineered scaffold, it loses its flexibility and may fracture. On the other hand, if the water content is too high, it can impair vision clarity, similar to when edema occurs in natural tissue ^[41]. Additionally, the cornea is permeable to molecules such as glucose and drugs. For example, the human cornea exhibits higher glucose permeability compared to porcine and bovine corneas, with a diffusion coefficient of $3 \pm 0.2 \times 10^{-6} \text{ cm}^2/\text{s}$ ^[42].

Transparency: The cornea is a transparent tissue, and its transparency is directly associated with the collagen fibers in the stroma. The corneal stroma comprises highly organized collagen type I fibers ^[43]. With this high level of organization in a 3D structure, light entering the cornea is transmitted through the lens with minimal scattering. Additionally, water balance and transparency in the cornea are maintained through the function of Na/K ATPase ^[41].

Porosity: Porosity is a desired characteristic in tissue engineering because it allows for attachment, motility, and cell proliferation features. The cornea is a multi-layered tissue, and nutrient and signal transduction between layers are essential for the integrity of the structure. In cornea tissue engineering, it is crucial for different cells to remain in specific layers, so pore size is also essential, as these pores need to allow for cell infiltration ^[41].

1.2.2. Previous Research on CTE Applications

Many studies have been conducted on CTE. These studies include the individual mimicry of corneal layers, the combined mimicry of epithelium and stroma layers, and whole-thickness CTE studies. Detailed information regarding these studies is provided below.

CTE Studies for Corneal Epithelium Layer

In corneal epithelium tissue engineering studies, the corneal epithelium, which is the outermost layer of the cornea and is exposed to the external environment via tears, is often targeted. Amniotic membrane is frequently utilized in CTE studies for corneal epithelium. Amniotic membranes not only exhibit biocompatibility but also demonstrate anti-inflammatory and antimicrobial properties ^[44]. *In vivo* studies utilizing amniotic membrane in CTE have been conducted in pig, goat, rat, and rabbit models. It has been observed that transplantation of tissue-engineered scaffolds mimicking human embryonic

stem cells and amniotic membrane into rabbits resulted in the acquisition of epithelial and limbal phenotypes after eight weeks [45].

Natural polymers such as gelatin, collagen, silk fibroin, and alginate are commonly preferred in corneal epithelium tissue engineering studies because of their low immunogenicity, non-cytotoxicity, and advanced biocompatibility properties [46]. A study using methacrylated gelatin - methacrylated hyaluronic acid double network hydrogel and rabbit corneal epithelial cells in corneal epithelium tissue engineering demonstrated improved mechanical properties (41 kPa compressive strength) and optical properties (>85% transparency) in the resulting structure. High viability and increased expression of CK-3 and -12 were observed in *in vitro* studies [47]. In a study by Liu et al. (2014), a freeze-dried collagen film with a micro-rough surface scaffold and human corneal epithelial cells showed increased cell proliferation over five culture periods and had a tensile strength of 9.91 MPa, Young's modulus of 18.9 MPa, and an elongation at break of 53.6%. In a related study by the same group in 2019, following keratoplasty in rabbits, re-epithelialization was observed 12 days post-surgery, with regained corneal transparency, and no neovascularization, tissue rejection, or keratoconus were observed [48]. In an *in vitro* study conducted by Hong et al. (2018), a tissue scaffold obtained by embedding decellularized human corneal stroma collagen gel supported cell proliferation and the expression of corneal epithelial-specific markers in human corneal epithelial cells. *In vivo* studies using a limbal epithelial stem cell-deficient mouse model, the scaffold demonstrated approximately 0.56 N suture retention strength and resistance to enzymatic degradation [49].

Synthetic polymers with high mechanical strength properties are used in corneal epithelium tissue-engineered scaffolds. Therefore, the combination of synthetic polymers with high mechanical strength and the biocompatibility of natural polymers is observed in the literature. In a study by Xiaomin Sun et al., a tissue scaffold was obtained by combining collagen and polycaprolactone (PCL). It exhibits similarities with native cornea with approximately 80% water absorption, approximately 1.1 MPa tensile strength, and 60% transparency. *In vitro* studies conducted with human corneal epithelial cells have shown cell attachment and proliferation [50]. In another study, a transparent and flexible scaffold was obtained by modifying the silk fibroin membrane with polyethylene glycol (PEG). *In vitro* studies conducted with LSCs have demonstrated corneal epithelial differentiation of cells. *In vivo* studies conducted with limbal stem cell-deficient

(LSCD) rabbit models indicate that LESC/PEG-modified silk fibroin membrane prevents blood vessel formation in the cornea and rescues corneal epithelial defects ^[51].

CTE Studies for Corneal Stroma Layer

The stroma is the thickest layer of the cornea and possesses both mechanical strength and transparency. In the literature, methacrylated gelatin (GELMA) hydrogels are frequently used in corneal stroma tissue engineering studies. KCs loaded into 3D printed GELMA hydrogels have demonstrated mechanical strength (20 kPa) close to that of native cornea (27 kPa). Additionally, KCs within these tissue scaffolds, which possess 80% transparency, have been shown to express KC-specific genes such as collagen type I, collagen type V, and decorin. GELMA exhibits biocompatibility, transparency, and mechanical durability, and *in vivo* studies have shown that it promotes healing processes within 8 weeks of being transplanted into the rabbit stroma ^[52]. To enhance mechanical properties and achieve cell distribution similar to natural tissue, a silk nanofibril-GELMA-alginate scaffold has been designed, providing suitable transparency (85%), elastic modulus (36.2 kPa), and tensile strength (3.8 MPa). *In vitro* studies with corneal stromal fibroblast cells have shown that the silk nanofibril-GELMA scaffold increases cell viability twofold compared to scaffolds made solely of silk nanofibrils ^[53].

Synthetic polymers can be designed to have mechanical properties similar to natural tissues and are often used in conjunction with natural polymers in tissue engineering. Accordingly, various studies in the literature on corneal stroma tissue engineering exist. For instance, in GELMA hydrogel, poly (ϵ -caprolactone)-poly (ethylene glycol) (PECL) has been used to mimic the collagen fibrils found in natural corneal tissue. The resulting tissue scaffold demonstrated cell proliferation and stromal ECM production, while also achieving suitable properties for CTE, including light transmittance (74%), tensile strength (3.47 MPa), compressive modulus (121.3 kPa), and swelling ratio (13.9) ^[54].

In another study, stromal ECM was combined with PCL and electrospun in different orientations. *In vitro* studies showed that KC migration was induced in radially and perpendicularly aligned ECM-PCL fibers ^[55]. The combination of PCL with lyophilized

silk fibroin in electrospun scaffolds was explored in various ratios to achieve optimal mechanical properties, wettability, and KC viability. In aligned 50:50 PCL:silk fibroin combinations, Young's modulus of 2.62 MPa, an elongation percentage of 14.92%, a tensile strength of 4.96 MPa, and approximately 70% transparency were achieved. *In vitro* studies with human KCs on this scaffold showed nearly 100% cell viability after a 72-hour culture period ^[56]. A scaffold obtained by 3D printed polyvinyl alcohol (PVA) and chitosan demonstrated water absorbance of 361%, optical transmittance of 88%, tensile strength of 0.72 MPa, and elongation percentage of 220%. *In vitro* studies with mesenchymal stem cells (MSCs) showed nearly 100% cell viability after a 7-day culture period ^[57].

CTE Studies for Corneal Endothelial Layer

The corneal endothelium, the innermost layer of the cornea, plays a crucial role in maintaining corneal hydration and pressure balance and directly interfaces with the aqueous humor. Both natural and synthetic polymers are utilized in corneal endothelium tissue engineering studies.

The use of decellularized tissues in corneal endothelial tissue engineering has shown successful cell culture outcomes. *In vitro* studies have demonstrated that induced pluripotent stem cell (iPSC)-derived corneal endothelial cells cultured on decellularized DM obtained from porcine corneas exhibit high proliferation rates. The decellularized porcine DM has been found to have transparency similar to that of natural human corneas (90%) ^[58]. *In vivo* studies using crosslinked amniotic membrane in cats and monkeys have achieved normal histological structure, 90% transparency, and a tensile strength of 24.5 MPa ^[59].

Gelatin is frequently used in tissue engineering with its biocompatibility and biodegradation properties. To support the proliferation of CEnCs, a gelatin scaffold modified with heparin has been shown to sustain fibroblast growth factor release for approximately 20 days while promoting cell proliferation. *In vivo* studies indicate that a

scaffold transplanted into rabbit eyes forms connections with the corneal stroma and maintains transparency without inflammation for up to three weeks [60]. Gelatin-based scaffolds reinforced through methacrylation enhance mechanical strength and support *in vitro* CEnC proliferation, increasing the expression of CEnC-specific proteins such as ZO-1 and Na-K ATPase [61]. A tissue scaffold created with electrospun GELMA fibers and a 2-hydroxyethyl methacrylate (HEMA) matrix exhibits 70% transparency, 518 MPa tensile strength, and a Young's modulus of 7 kPa. This scaffold shows no cytotoxic effects in CEnC cultures and is biocompatible with tear film proteins (human serum albumin, human immunoglobulins, lysozyme) [62]. Another natural polymer frequently used in corneal endothelial tissue engineering is silk fibroin. However, silk fibroin membranes are highly elastic, making transplantation challenging. *In vitro* studies of a silk fibroin scaffold combined with glycerin have demonstrated that glycerin-silk fibroin scaffolds support CEnC adhesion and proliferation while providing adequate transparency [63].

Synthetic polymers are preferred in corneal endothelial tissue engineering to provide suitable mechanical and surface properties. In studies utilizing poly-ε-lysine hydrogel for CEnC culture, appropriate transparency (99%), Young's modulus (0.11 MPa), and tensile strength (0.04 MPa) were achieved, but with low cell viability. To enhance cell viability, hydrogel modification with RGD and DGEA has been shown to increase cell proliferation [64]. In this context, combining natural and synthetic polymers offers significant advantages for corneal endothelial tissue engineering. PCL, PCL/collagen, PCL/gelatin, and PCL/chitosan scaffolds produced via electrospinning have been used *in vitro* studies with CEnCs. Results indicate that PCL/collagen and PCL/gelatin scaffolds offer significantly higher cell viability compared to PCL scaffolds alone (97% and 98%, respectively) [65]. Furthermore, another study demonstrates that a membrane scaffold prepared by combining the natural polymer chitosan and the synthetic polymer PCL provides higher CEnC proliferation and ECM production compared to scaffolds containing only chitosan [66].

CTE Studies for Corneal Epithelium – Stroma Layers

There is an interaction between HCECs and KCs in natural tissue, which has been confirmed in co-culture studies. In the study by Gossellin et al., collagen type IV loaded onto silk film with surface patterns, and HCECs were cultured on top, while CSSCs were cultured underneath using a transwell co-culture system. *In vitro* assays have shown that in co-culture, CSSCs transform into KCs, and over a 6-week co-culture period, there was increased expression of keratocyte and epithelial-specific genes ^[67]. Some studies in literature have aimed to mimic both the epithelium and stroma layers together. These studies primarily aim to create an epithelium layer on top of a layer with similar thickness and physical properties to the stroma. In the study by Lace et al., a porous poly- ϵ -lysine scaffold was created, in which corneal fibroblast cells proliferated, and a monolayer of CEC cells was obtained on top ^[68]. In the study by Wu et al., *in vitro* studies were conducted with HCECs and KCs on an aligned PVA/collagen scaffold produced by electrospinning. These studies demonstrated that HCEC and KC attachment and proliferation were facilitated on this scaffold. Furthermore, the obtained aligned scaffold enabled ordered KC growth. With a tensile strength of 3.581 MPa and approximately 53% transmittance, it was concluded that the use of this tissue scaffold in corneal tissue engineering is suitable ^[69].

In the literature, *in vivo* studies have been conducted in corneal epithelial-stroma tissue fabricated with both natural and synthetic polymers. In the study by He et al., a corneal tissue scaffold was obtained by blending GELMA and diacrylated PEG (PEGDA) using 3D bioprinting. *In vitro* studies with L929 mouse fibroblast cells on the obtained tissue scaffold showed cell adhesion and proliferation. Additionally, a tensile strength of 82.2 kPa, tensile modulus of 77.2 kPa, and 96-99% transparency was achieved. Following the printing of a bi-layer dome-shaped scaffold with rabbit CECs and rabbit adipose-derived mesenchymal stem cells (ACS) laden orthogonally aligned fibrous stroma, *in vivo* rabbit keratoplasty demonstrated re-epithelization and stromal regeneration ^[70]. In the study by Simpson et al., focusing on the risk of rejection and inflammation, a collagen-like peptide (CLP)-PEG hybrid scaffold was created using an inflammation suppressor, 2-methacryloyloxyethyl phosphorylcholine (MPC). The CLP-PEG-MPC scaffold, providing 80% transparency and Young's modulus of 0.044 MPa, was non-toxic and supported cell proliferation *in vitro* studies with HCECs. *In vivo* studies with alkali burn mini pigs showed that the tissue scaffold reduced neovascularization, haze, and corneal

swelling, while also promoting fast recovery of corneal sensation and nerve regeneration [71].

In corneal epithelium-stroma tissue engineering studies, decellularized tissues are also utilized. In the study by Wang et al., using decellularized porcine cornea, a transparent, injectable, and biocompatible hydrogel was obtained to target the regeneration of focal corneal defects. *In vitro* studies with human corneal fibroblast cells on the tissue scaffold, which provided 75-90% transparency and a storage modulus of 357 Pa, showed cell proliferation and ECM synthesis. *In vivo* studies conducted in a rabbit model demonstrated restoration of the stroma and epithelium layers 12 weeks after surgery [72]. In a fibrin-agarose gel, primary human KCs proliferated for 28 days, and the tissue scaffold maintained approximately 90% transparency over the same period [73]. After transplantation into New Zealand rabbits, a nanostructured fibrin-agarose scaffold containing human allogenic KCs and CECs preserved its transparency and provided proper structural integrity with the whole cornea [74]. In another study, a hydrazone-crosslinked hyaluronic acid containing dopamine moiety tissue scaffold exhibited suitable mechanical properties for CTE, with a storage modulus of 483.7 Pa, a loss modulus of 11 Pa, and 98% transparency. Human ASCs encapsulated within this tissue scaffold and modified with collagen and laminin on the surface were then seeded with LESC on the upper surface. The tissue adhesive function provided by dopamine moieties in the scaffold enabled sutureless implantation capacity, demonstrated in an *ex vivo* porcine organ model, thereby obtaining an artificial cornea housing two types of regenerative corneal stem cells [75].

CTE Studies for Whole Thickness Cornea

After corneal transplantation surgery, known as keratoplasty, various studies in the literature focus on artificial whole-thickness cornea tissue engineering to address potential postoperative issues. Hydrogels created using collagen, a natural polymer, are commonly preferred in this field. In the study by Liu et al., a hydrogel tissue scaffold made by crosslinking recombinant human collagen type I and type III was evaluated as a

whole-thickness artificial cornea. In this study, recombinant human collagens were crosslinked using different ratios of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-Hydroxysuccinimide (EDC/NHS) (0.3-1.0). The crosslinked collagen hydrogel samples achieved 80-92% white light transmission, whereas this value is around 87% in healthy human corneas. Tensile strength and elastic modulus measurements showed that collagen III hydrogel has higher mechanical strength than collagen type I. Collagen type I forms large-diameter fibrils, while collagen type III forms small-diameter fibrils, resulting in tightly packed hydrogel scaffold with high mechanical strength. Additionally, the *in vitro* biodegradation of collagen type III hydrogels is much slower compared to collagen type I hydrogels. *In vitro* experiments with HCECs showed that hydrogel scaffolds did not exhibit toxic effects. After transplantation into Yucatan mini pigs, the collagen type I hydrogel tissue scaffolds maintained optic clarity after 12 months, and regeneration occurred in all corneal layers ^[76]. Another study by the same group indicates that phase 1 clinical trials were conducted with crosslinked recombinant human collagen type III. Observations conducted over 12 months on 10 patients with corneal diseases from different age groups show that the implant supports epithelial and nerve regeneration, provides vision clarity, and reduces revascularization. However, thinning and fibrosis formation in the implant were observed in some patients ^[77].

In many tissue engineering studies, combining natural and synthetic polymers is preferred. Rafat et al. aimed to obtain an artificial whole-thickness cornea by combining the bioactive properties of natural polymers with the physical properties of synthetic polymers. The PEG-stabilized carbodiimide crosslinked collagen-chitosan hydrogel obtained exhibited 220 kPa ultimate stress, 45% ultimate elongation, and 1508 kPa elastic modulus. Additionally, glucose diffusion of $2.8 \times 10^{-6} \text{ cm}^2/\text{s}$, albumin diffusion of $1.67 \times 10^{-7} \text{ cm}^2/\text{s}$, and 90% water content were observed in this scaffold, compared to $2.6 \times 10^{-6} \text{ cm}^2/\text{s}$, $1 \times 10^{-7} \text{ cm}^2/\text{s}$, and 78%, respectively, in healthy human corneal tissue. *In vitro* tests with HCECs showed no toxic effects of the scaffold, and biodegradation and immunocompatibility properties were similar to those of natural corneal tissue observed through rat subcutaneous implantation. *In vivo* studies by implanting the scaffold into Yucatan mini pigs showed integration with host tissue and regeneration across all corneal layers ^[78]. This study demonstrates the advantage of natural and synthetic polymers in whole-thickness cornea tissue engineering. Considering the literature studies conducted in this direction, it is observed that various studies are carried out in this field. For

example, studies conducted with recombinant human collagen type III - 2-methacryloyloxyethyl phosphorylcholine (COL-MPC) hydrogel showed 87.4% transparency, 1.86 MPa tensile strength, and 11.42 MPa elastic modulus, compared to 78%, 3.81 MPa, and 3-13 MPa, respectively, in healthy human corneal tissue. After transplantation into mini pigs, COL-MPC hydrogel showed corneal epithelial and stromal regeneration, tear film and sensory nerve regeneration ^[79]. Similar results were observed in guinea pigs with COL-MPC hydrogel obtained using porcine collagen type I, demonstrating corneal cell and nerve regeneration, tear film layer formation, and the maintenance of optic clarity ^[80]. Another study showed that crosslinking collagen type I with N-isopropylacrylamide, acrylic acid, and acryloxysuccinimide produced an optically clear scaffold that facilitated epithelial outgrowth and the ingrowth of both keratocytes and nerves ^[81]. Additionally, using a 4D bioengineering method, a curved collagen-based hydrogel similar to natural corneal tissue was produced. *In vitro* studies demonstrated that corneal cells maintained their phenotypes within this structure, and the scaffold exhibited structural and mechanical properties highly similar to those of natural cornea ^[82]. Another study involved co-culturing CSSCs, HCECs, and dorsal root ganglion (DRG) cells in a scaffold created with collagen hydrogel and RGD-modified silk film. *In vitro* studies showed multi-layered epithelial cells, aligned CSSCs, and dense neuronal innervation within this scaffold ^[83].

The LiQD cornea, an FDA-approved cell-free artificial cornea developed for transplantation, comprises short collagen-like peptides (CLP) and a hydrogel formed with PEG and containing fibrinogen. This hydrogel undergoes gelation below 37°C and remains liquid above this temperature. *In vivo* studies conducted in pigs over 12 months have demonstrated an increase in regeneration capacity and a reduction in the risks of immune rejection and allergies (**Figure 6**) ^[84].

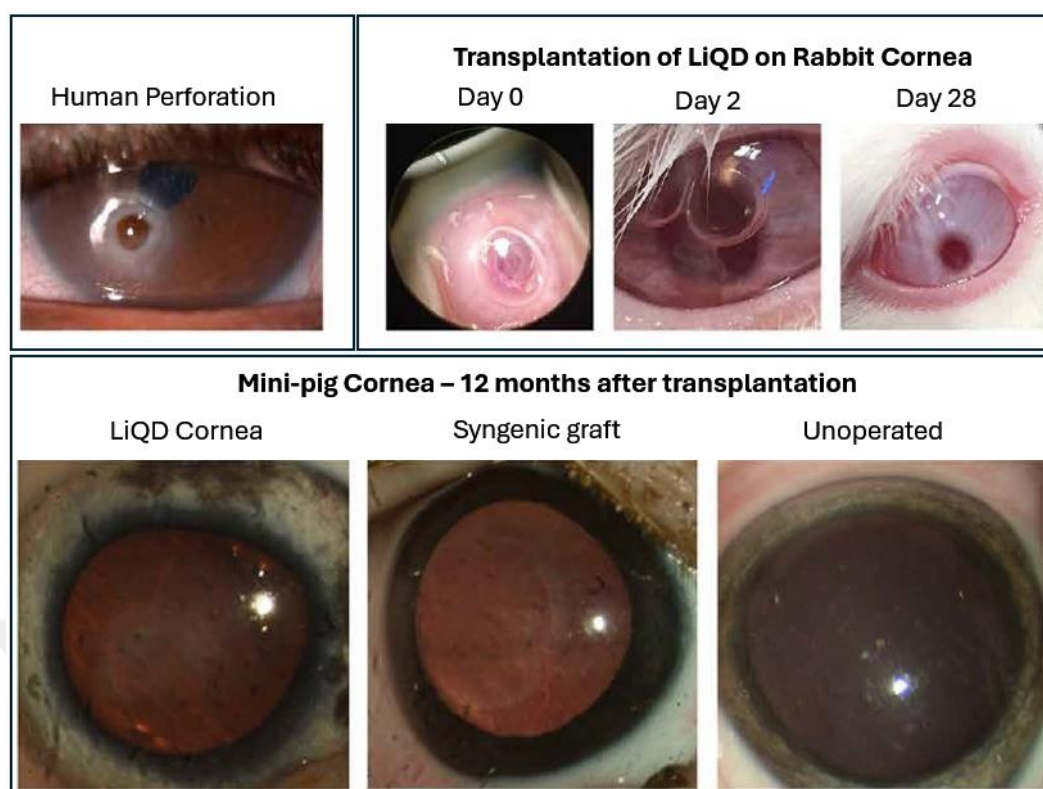


Figure 6. The results of *in vivo* studies conducted with LiQD artificial cornea in rabbits and mini-pigs (Reproduced from McTiernan et al. with permission from ©Science Advances) ^[84].

The examples described above demonstrate that various advancements have been made in CTE studies. However, the most effective treatment method remains keratoplasty, which relies on donor cornea. Therefore, efforts should continue to develop artificial corneas to reduce dependence on donor corneas. In line with this aim, corneal cells isolated from donor corneas were evaluated through phenotype and genotype analyses within the scope of this thesis. After isolating donor corneal cells, the aim was to determine their suitability for CTE use. Similarly, it was intended to mimic corneal tissues through 2D and 3D cell culture processes conducted using elastomer membranes and peptide hydrogels. This thesis provides a study that can serve as a resource in CTE literature.

2. Materials and Methods

2.1. General Cell Culture Techniques

In this study, the general cell culture techniques detailed below were utilized.

2.1.1. Throwing and Seeding of Corneal Cells

The cell freezing vial containing the cell sample, taken from the liquid nitrogen tank, is rapidly thawed in a water bath at 37°C until only a small amount of ice remains. This thawing process must not exceed 2 minutes to prevent cell loss. The sample, transferred to a Falcon tube, is diluted at least 10-fold with growth media to reduce the toxic effects of dimethyl sulfoxide (DMSO) at room temperature. After centrifugation at 300 x g for 5 minutes at 4°C, the pellet is resuspended in 1 mL of growth media. A 10 µL sample of the cell-media solution is mixed with Trypan Blue solution at a 1:1 ratio and loaded onto a hemocytometer for cell counting. Trypan Blue dye enters dead cells, making them appear blue, while live cells remain unstained and appear white. The total amount and viability percentage can be determined by counting the live and dead cells in the five squares of a hemocytometer. After determining the total viable cell count and viability percentage, 6600 cells/cm² are seeded into the cell culture plate, and an adequate amount of growth media is added. The culture is maintained in an incubator at 37°C with 5% CO₂, with the growth media being changed every two days.

2.1.2. Subculturing and Freezing of Corneal Cells

Once the cells reach 70% confluency, the growth media is collected, and the surface is washed with sterile phosphate-buffered saline (PBS) to remove any media residues. A

sufficient amount of 0.05% Trypsin-EDTA solution (Pan Biotech, P10-023100) is added to cover the surface of the culture plate, and it is incubated at 37°C for 5 minutes. At the end of this period, fetal bovine serum (FBS) (Pan Biotech, P30-3602) is added to inactivate the trypsin, and the solution is collected into a Falcon tube and centrifuged at 300xg for 5 minutes at 4°C. The pellet is resuspended in 1 mL of growth media, and the total viable cell count, and viability percentage are determined using the protocol described in Section 2.1.1.

The falcon tube containing the cells is gently tapped with a fingertip to distribute the cell pellet. Pre-cooled freezing media (4°C) is then slowly added to the tube while gently shaking it. The cell aggregates are separated by careful pipetting without creating bubbles, and the solution is transferred into a freezing vial. The freezing process is conducted with a maximum of 1×10^6 cells per 1 mL. The vials containing the cell samples are placed into a Mr. Frosty Freezing Container (Nalgene, 5100-0001) for gradual freezing, kept overnight at -80°C, and then transferred to a liquid nitrogen tank for long-term storage. The freezing media is composed of growth media, FBS, and DMSO in a 7:2:1 volume ratio.

2.1.3. SV40 Immortalization

The immortalization process was carried out on cell samples that had reached 80% confluency using Lipofectamine 3000 Transfection Reagent (Invitrogen, L3000001) and pSG5 Large T-SV40gp6 (SV40T) plasmid (pSG5 Large T was a gift from William Hahn (Addgene plasmid # 9053; <http://n2t.net/addgene:9053>; RRID: Addgene_9053). After collecting the growth media, the culture plate was washed twice with sterile PBS, and Opti-MEM (Gibco, 31985-047) medium was added. Two solutions were prepared in this protocol: the diluted lipofectamine solution and the diluted DNA solution. The diluted lipofectamine solution was prepared by mixing 125 μ L of Opti-MEM and 5 μ L of Lipofectamine 3000 Reagent, followed by 2-3 seconds of vortexing in two separate Eppendorf tubes. The diluted DNA solution was prepared by mixing 5 μ g of SV40 plasmid, 10 μ L of P3000 Reagent, and 250 μ L of Opti-MEM in an Eppendorf tube. 125 μ L was added to each diluted lipofectamine solution from the prepared diluted DNA

solution and incubated at room temperature for 15 minutes. After incubation, the prepared solutions were gently added to the cell samples, and the culture plate was placed in a cell culture incubator for 2-4 days. Cells that successfully underwent immortalization continued to thrive, while non-immortalized cells died and were removed through media changes (**Figure 7**).

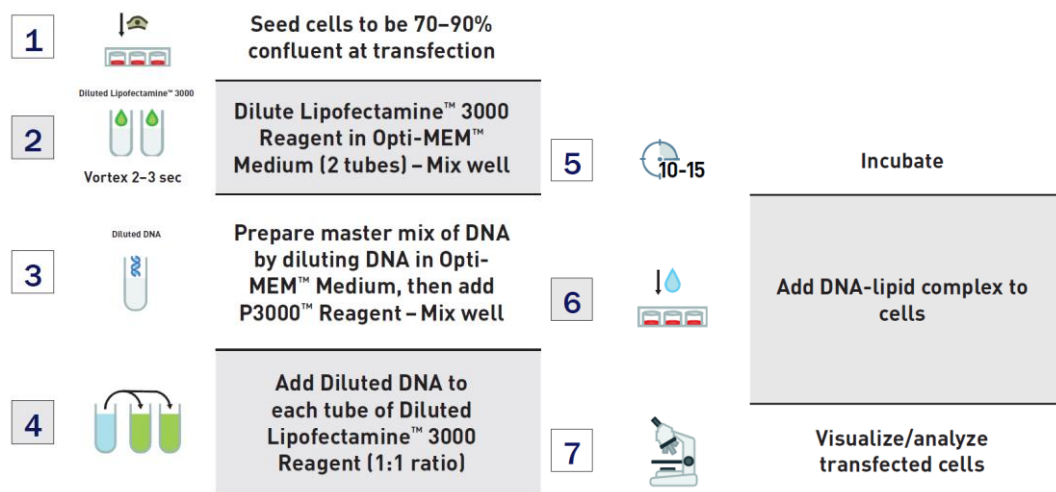


Figure 7. The immortalization process was conducted using Lipofectamine 3000 and the SV40T plasmid ^[85].

2.2. General Cell Characterization Techniques

To characterize the corneal and limbal cells used in this study, immunocytochemistry (ICC), gene expression analysis, trans hydro electrical resistance (TEER) measurement, and biocompatibility assays were conducted. These techniques are detailed below.

2.2.1. Immunocytochemistry (ICC)

When the confluency of cells seeded on glass slides or tissue scaffolds approaches 100%, the samples are washed twice with PBS to remove residual media. For fixation, 4%

paraformaldehyde is added, and the samples are incubated at room temperature for 15 minutes. After two 10-minute washes with PBS, a solution of 0.1% Triton X-100 (Sigma Aldrich, 9002-93-1) and 3% bovine serum albumin (BSA) (Sigma Aldrich, 10735078001) in PBS is added, followed by a 35-minute incubation at room temperature to perform permeabilization and blocking.

Following two 10-minute PBS washes, the diluted primary antibody solution is added and incubated at room temperature for 1 hour. According to the manufacturer's recommended ratio, the primary antibodies are diluted in a PBS solution containing 3% BSA. After the incubation period, the samples were washed twice for 10 minutes with PBS, and the diluted secondary antibody solution was added and incubated for 1 hour at room temperature in the dark. At the end of the incubation, the samples were washed twice with PBS for 10 minutes. Mounting Medium with DAPI (İbidi, 50011) is then applied and allowed to sit for 5 minutes.

Samples on glass slides are mounted with a coverslip and sealed around the edges with nail polish to prevent air exposure. Tissue scaffold samples are placed on a confocal dish (İbidi, 81156) with the cell-bearing surface facing the bottom of the dish, covered with a glass slide, and sealed with nail polish to prevent air contact. The prepared samples are observed under a confocal microscope (Zeiss, LSM 710). Detailed information about the antibodies used in the study is provided below (**Table 1**).

Table 1. The antibodies used in the study and their fundamental characteristics.

Antibody	Brand	Dilution Factor of Antibody	Related Corneal Layer
ZO-1	Thermo Fisher Scientific ZO-1 Polyclonal Antibody (18-7430)	1/250	CEC, CEnC
Na-K ATPase	Abcam Anti-alpha 1 Sodium Potassium ATPase antibody [464.6] (ab237969)	1/200	CEnC

Integrin β 1	Abcam Anti-integrin beta 1 antibody [12G19] (ab202643)	1/150	CEC
CK3	Abcam Anti-Cytokeratin 3/CK-3 antibody [AE5] (ab68260)	1/50	CEC
COL I	Abcam Anti-Collagen I antibody (ab90395)	1/2000	KC
KERA	Abcam Anti-Keratocan/KTN Antibody (ab230175)	1/100	KC
α -SMA	Abcam Anti-Alpha Smooth muscle actin antibody [E184] (ab196919)	1/100	KC
ALDH3A1	Abcam Anti-ALDH3A1 antibody (ab76976)	1/100	KC
Vim	Abcam Anti-Vimentin antibody [EPR3776] (ab92547)	1/500	Stem cells
Ki67	Abcam Anti-Ki67 antibody (ab15580)	1/100	All cell types (CEC, KC, CEnC) (proliferation)
Phalloidin	Abcam Cytopainter Phalloidin iFlour 647 (ab176759)	1/1000	All cell types (CEC, KC, CEnC) (actin filament)
Secondary antibody - rabbit	Abcam Goat Anti-Rabbit IgG H&L (ab150077)	1/500	

Secondary antibody - mouse	Abcam Goat Anti-Mouse IgG H&L (ab175473)	1/500	
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2.2.2. Gene Expression

Within the scope of this thesis study, a gene expression assay was applied for cell characterization. The cell samples are lifted from the surface of the culture plate by trypsinization to obtain a cell pellet. The following procedures are sequentially applied to the collected cells.

RNA Isolation

1 mL of TRIzol Reagent (Invitrogen, 15596026) is added to the cell pellet and incubated at room temperature for 5 minutes. Following this, 200µL of chloroform (Sigma Aldrich, 650498) is added, and the samples are vortexed for 2-3 minutes before being incubated at room temperature for 15 minutes. After centrifugation at 12000xg for 15 minutes at 4°C, the transparent supernatant remains at the top layer and is collected into new Eppendorf tubes. Subsequently, 500µL of isopropanol is added, and the mixture is incubated at room temperature for 10 minutes. The samples are then centrifuged at 12000xg for 10 minutes at 4°C, and the supernatant is removed. After adding 1 mL of 75% ethanol to the pellet and vortexing, the samples are centrifuged at 7500xg for 5 minutes at 4°C. After removing the supernatant, the pellet is air-dried for 30 minutes to ensure complete evaporation of ethanol. Finally, the pellet is resuspended in 20µL of RNase-free water, and the quantity is measured using a nanodrop (Thermo Scientific, Nanodrop 2000c).

Complementary Deoxyribonucleic Acid (cDNA) Synthesis

The SuperScript III First Strand Synthesis Kit (Invitrogen, 18080-051) was utilized for cDNA synthesis. 5µg of RNA isolated in the previous step is combined with 1µL of primer solution and 1µL of 10nM deoxynucleotide triphosphates (dNTP) mix, and the volume is adjusted to 10 µL. The prepared reaction mixture is incubated at 65°C for 5 minutes. After incubation, the samples are kept on ice for at least 1 minute. From the kit components, 2 µL of buffer, 4 µL of MgCl₂ solution, 2 µL of 0.1M DTT, 1 µL RNaseOUT, and SuperScript III are mixed with the 10 µL reaction mixture prepared in the previous step and gently mixed. The prepared mixture is subjected to polymerase chain reaction (PCR) for 10 minutes at 25°C, 50 minutes at 50°C, and the final 5 minutes at 85°C, using a PCR machine (Eppendorf, Mastercycler Gradient). The resulting cDNA samples are stored at -20°C.

Real-Time PCR (RT-PCR)

The PCR samples obtained in the previous step are diluted 1:5 with RNase-free water. A mixture is prepared with 5 µL of diluted cDNA sample, 0.5 µL of forward primer, 0.5 µL of reverse primer, 10 µL of LightCycler 480 SYBR Green Master Mix (Roche, 04707516001), and 4 µL of water, resulting in a total volume of 20 µL. The researcher designed the reverse and forward primers used in the study and synthesized by LetGen Biotechnology (Izmir, Turkey) (**Table 3**). The preparation of the mixture is carried out on ice and in the dark. After loading the mixture into a LightCycler 480 Multiwell Plate-96 (Roche, 0429692001) and sealing with a cover sealer, it is centrifuged at 1500 x g for 2 minutes. The RT-PCR steps are conducted on a LightCycler 480 (Roche) (**Table 2**).

Table 2. Main information about reaction cycles of RT-PCR.

Preincubation	1 cycle	95°C 10dk
Amplification	45 cycles	95°C 10sec
		60°C 30sec
		72°C 10sec
Melting Curve	1 cycle	95°C 5sec

		65°C 1dk
		97°C ∞
Cooling	1 cycle	40°C 10sec

Table 3. Primers were used in the study.

Gene Name	Description	Cell Source	Sequences of reverse and forward primers
HPRT1	Reference gene ^[86]	All layers	F-ACAGGACTGAACGTCTTGCT R-GCACACAGAGGGCTACAATG
Ki67	Proliferation gene ^[87]	All layers	F-CAGTCAATTTAGTACAGGCCAC R-ACTCTGTAGGGTCGAGCAG
KRT3	Main CEC markers ^[88]	CEC	F-GGGGAATTCAGGAAGTGACTA R-GCCTTTACTTGCCCAATCTG
KRT12			F-GTTCTAGTTCCGGCTTTGGG R-GAGAGAATACCTAGAGAGCC
ZO-1	Tight junction protein ^[89]	CEC, CEnC	F-CAGCCAAGCAATGGCAGTC R-TCCACTGTTTTAGGTGTGTGAT
B-catenin	Stratification marker ^[90]	CEC	F-TGTATGAGTGGGAACAGGGA R-CAAACGCTGGACATTAGTGG
COL1	The first main keratocyte marker for extracellular matrix formation ^[5]	KC	F-CAAGGTGTTGTGCGATGAC R-GGTGGTTTCTTGGTCCGGTG
KERA	The second main keratocyte marker for extracellular matrix formation ^[91]	KC	F-ACAACCTGATAGAAACCATTCC R-TACCTCCTCTAGCTCATTATCTTC
ALDH3A1	Crystallin protein, important for transparency ^[92]	KC	F-AGACCTGCACAAGAATGAATG R-GGAGCTTCTGGATCATGTACT
ACTA2	Myofibroblast marker ^[93]	KC	F-GAAGATCCTGACTGAGCGT R-TCCAGAGCTACATAACACAGTT

AQP1	Formation of water balance ^[94]	KC, CEnC	F-CTCTCTGTAGCCCTTGGACAC R-GTTGCTGAAGTTGTGTGTGAT
COL4A1	Descemet's membrane formation ^[95]	CEnC	F-CTGGATTGGTCGGTTTCCA R-CGTAGAAACCAAGTCCTCTGT
ATPA1	Formation of nutrient and water balance ^[96]	CEnC	F-GACAGGGACATGGATGAACT R-ACGAGCAGATGTTAATCCCC
KRT5	Limbal epithelial stem cell marker ^[95]	LESC	F-GAAATCAACAAGCGTACCAC R- CTCATCCATCAGTGCATCAA
KRT14			F-GACCATGCAGAACCTCAATG R-AGTCTTTGATCTCAGCAGGC
P63 α	Stem cell marker ^[97]	LESC, CSSC	F-TCCACCAGTCCCTATAACACA R-CTGGGGTAGTCGGTGTGGA
SOX2	Stem cell marker ^[97]		F-TGAAGGAGCACCCGGATTAT R-CGGGCAGCGTGTACTTATC
VIM	Stem cell marker ^[98]		F-GGAACAGCATGTCCAAATCG R-AGCCACACTTTCATATTGCTG
CD90	Mesenchymal stem cell marker ^[98]	CSSC	F-TAACGGCCTGCCTAGTG R-TTCTTTGTCTCACGGGTCA
CD34	Differentiation marker for limbal mesenchymal stem cells to keratocyte ^[99]	CSSC, KC	F-GCGCTTTGCTTGCTGAGT R-TGGGTAGGTAAGTCTGGGGTA

At the end of the RT-PCR reaction, Cp values were obtained for each sample. The Cp value of the HPRT1 reference gene was used as a control, and the Δ Cp value was calculated as $(Cp^{\text{control}} - Cp^{\text{interest}})$. Then, the results obtained by calculating the $2^{(-\Delta Cp)}$ value were evaluated to determine the expression level.

2.2.3. Trans Epithelial Electrical Resistance (TEER) Measurement

TEER measurements were conducted using a Digital Multimeter (UNI-T UT89X, China) and Chopsticks Electrode Set (Word Precision Instruments, Florida, USA) to observe membrane barrier integrity. The cells seeded on the cell culture insert (Thermo Fisher, 140620) were first washed with PBS to remove the residual medium. After PBS was added to both the top and bottom of the cell culture insert placed in a 24-well plate, the measurement electrode was inserted. One electrode arm was positioned inside the insert, while the other was submerged in the PBS solution in the well of the 24-well plate. A frequency of 60 mHz and a voltage of 6 V were applied, and the resulting resistance was recorded. Blank measurements were conducted using a cell culture insert without cells. The measurements of CECs were carried out for a 15-day culture period.

2.2.4. Collagen Staining

After fixation with 4% PFA, the cell samples to be stained are washed with PBS. They are then incubated with Direct Red 80 dye (Sigma Aldrich, 365548) prepared in saturated aqueous picric acid solution for 1 hour. The samples are washed twice with acidified water containing 5 ml of acetic acid per liter of water. Subsequently, the samples are washed three times with 100% ethanol and once with xylene.

2.2.5. Biocompatibility Assays

In the experiments conducted within the scope of this thesis, two types of cell viability tests were used, namely CCK8 (Abcam, ab228554) and MTS (Abcam, ab197010). The same procedure was applied for both kits. Initially, the growth media was collected, and the surface was washed once with PBS to remove cell debris. The cell viability reagent, diluted in growth media at a ratio of 1:10, was added to the samples of the tested cells, and the mixture was incubated in a cell culture incubator for 4 hours. After incubation, the solutions transferred to the 96-well plate were measured using the spectrophotometer (Tecan, Infinite 200 Pro). When CCK8 was used, the measurement was conducted at 450 nm, while for MTS, it was performed at 490 nm. In the study, a positive control consisted

of only cell samples without tissue scaffolds, while a negative control consisted of only tissue scaffold samples without cells. The maximum optical density (OD) value obtained from the cell control group was considered 100% viability, and the percentage viability values of all samples were calculated accordingly.

2.3. Dissection of Human Donor Cornea

Corneas unsuitable for transplantation were obtained from Haydarpaşa Numune Training and Research Hospital (under Ethical Approval 771/11/2019#20 and SUNUM-2019-82). Dissection was performed within 24 hours of procurement. 10 human donor corneas were acquired for this study. The corneas were washed several times with 50 µg/mL Gentamicin (Pan Biotech, P06-03050) in PBS and rinsed with sterile phosphate-buffered saline (PBS) before dissection. Dissection tools, pre-sterilized by autoclaving, were further sterilized with 70% ethanol and rinsed with sterile PBS at each stage of the procedure.

Under a stereo microscope (Leica M165 C), careful dissection of the limbal and corneal tissues was performed. Initially, the trabecular meshwork was completely removed. After cleaning the tissue with PBS, the epithelial layer was grasped and peeled off with forceps. To ensure complete removal of epithelial cells, the surface was scraped with the back of angled forceps. After washing with gentamicin solution and PBS, the endothelial layer was similarly collected, and the tissue was rewashed. Subsequently, the scleral tissue was entirely removed, leaving only the limbal and corneal tissues. Retaining the sclera until this step helped to stabilize the tissue during dissection, thereby preventing damage to the endothelial and epithelial layers. The remaining corneal stroma and limbus tissues were then separated using a scalpel. Each tissue was cut into small pieces. The dissected corneal and limbal tissues were used for cell isolation.

2.4. Isolation of Primary Corneal and Limbal Cells

Enzymatic and explant culture methods were employed to isolate primary cells, detailed below.

2.4.1. Corneal Epithelial Cells

The collected tissue strips were placed onto a culture plate in the primary corneal epithelial cell isolation using the explant culture technique. To allow the tissue strips to adhere to the surface, they were incubated in a cell culture incubator for approximately 15 minutes. Once the adhesion process was complete, the culture plate was gently supplemented with medium, initiating the cell culture process. Care was taken to add the medium slowly and to prevent the tissue strips from detaching from the surface. Three different media were used in the study as growth media, namely complete media, complete media with epithelial growth factor (EGF), and serum-free media (**Table 4**).

Table 4. Growth media used in the culture studies of primary corneal epithelial cells.

Media Name	Media Type	Supplements
Complete Media	DMEM-F12 (Pan Biotech P04-41250)	8% FBS 1% Penicillin-streptomycin solution (Pan Biotech, P06-07100)
Complete Media with EGF	DMEM-F12	8% FBS 1% Penicillin-streptomycin solution 10ng/mL EGF
Serum-Free Media	Keratinocyte Serum Free Media (KSFM) (Gibco, 17005042)	0,005 µg/mL EGF (Gibco, 37000015) 5 µg/mL bovine pituitary extract (BPE) (Gibco, 37000015) 1% Penicillin-streptomycin solution

For enzymatic isolation, the collected tissue strips were incubated in a solution containing 1.2 U/mL Dispase II (Sigma Aldrich, D4693) prepared in complete media, with shaking, for 1 hour at 37°C. Subsequently, they were centrifuged at 300xg for 5 minutes. The pellet was then seeded onto a culture plate, and the cell culture process was initiated by adding complete media, as described in Table 4. Additionally, the same procedure was conducted by isolating the tissue strips in the enzyme solution for 2 hours, followed by initiation of the culture process with serum-free media.

2.4.2. Corneal Keratocyte Cells

The corneal stromal layer, separated from other tissues, is initially fragmented into small pieces. The corneal tissues used for explant culture are placed onto a cell culture plate and allowed to adhere to the surface for 15 minutes in the incubator. Subsequently, the media is gently added, and the culture process is initiated. The addition of media is applied slowly to prevent the tissue pieces from detaching from the surface. During this process, DMEM-F12 supplemented with 5% FBS and 1% Penicillin-Streptomycin solution is used as the growth media. The explant culture procedure is applied to Corneas #1, #4, and #5. The obtained cells are subcultured, as described in Section 2.1.2.

The tissue fragments were cut into small pieces to carry out enzymatic isolation and placed in a Collagenase I (Merck, 9001-12-1) enzyme solution. For this process, Cornea #2 tissue was incubated in a 4mg/mL enzyme solution for 1 hour, Cornea tissue #7 in a 2mg/mL enzyme solution for 1 hour, and Cornea tissues #3, #6, #8, #9 and #10 in a 2mg/mL enzyme solution for 30 minutes. The incubation was carried out with agitation at 37°C. After incubation, the samples were centrifuged at 300xg for 5 minutes, resuspended in 1mL growth media, and seeded onto a cell culture plate. DMEM-12 supplemented with 5% FBS and 1% Penicillin-Streptomycin solution was used as the growth media.

Cells obtained from Cornea #6 were immortalized at passage 1, while cells obtained from Cornea #10 were immortalized at passage 0 using the protocol described in Section 2.1.3. The immortalized cells were subcultured to establish cell stocks.

Primary passage 2 and immortalized passages 9, 12, and 15 KCs obtained from Cornea #6 were characterized using the ICC and gene expression protocols described in Section 2.2.

2.4.3. Corneal Endothelial Cells

A surface-coated cell culture plate was used for both enzymatic and explant cultures of corneal endothelial cells. For coating, 0.2 mL/cm² of FNC Coating Mix (Athena Enzyme Systems, 0407) was applied to the plate, and after 30 seconds of incubation at room temperature, the excess solution was removed. Subsequently, cell culture procedures were carried out. Surface coating was freshly performed for each culture process.

Enzymatic and explant isolation techniques were applied to isolate primary CEnCs from donor corneas. In the explant culture method, endothelial strips collected with the DM layer of the donor tissue were placed onto culture plates coated with an FNC Coating Mix. After being held in the cell culture incubator for 15 minutes to allow the tissue strips to adhere to the surface, the cell culture process was initiated by gently adding the growth medium. Opti-MEM supplemented with 5% FBS, 1% Penicillin-Streptomycin Solution, 2.5 mM L-Glutamine (Pan biotech, P04-80100), 50 µg/ml Bovine Pituitary Extract, 0.08% chondroitin sulfate (Carbosynth, 31458), 200 µg/ml CaCl₂ (Sigma Aldrich, C5670), and 1.3 mM L-ascorbic acid (Sigma Aldrich, A4403) was used as the growth medium. The explant culture technique was applied for Corneas #1, #2, #3, #4, #5, #6, #7, #8, and #10.

Two different enzymatic culture techniques were applied to isolate CEnCs from donor corneas. In the method applied to Cornea #3, the collected tissue strips were incubated in 0.25% Trypsin / 0.02% EDTA Solution (Pan Biotech, P10-019100) for 5 minutes at 37°C under agitation. Subsequently, mechanical disruption was performed by pipetting for a few seconds to ensure cell dissociation. The cell pellet obtained by centrifugation at 300xg for 5 minutes was then seeded onto a cell culture plate coated with FNC Coating Mix. In the technique applied to Cornea #9, the collected tissue strips were incubated overnight in OptiMEM supplemented with 8% FBS for stabilization. After incubation,

the medium was removed by centrifugation at 300xg for 5 minutes, and the tissue strips were washed once with Hank's Balanced Salt Solution (HBSS) and centrifuged again. Subsequently, the tissue strips were incubated in 0.02% ethylenediaminetetraacetic acid (EDTA) solution at 37°C for 1 hour to weaken the junctions between the cells. Mechanical disruption was then performed by pipetting and centrifugation to obtain the cell pellet. The collected cells were seeded onto a cell culture plate coated with FNC Coating Mix. The same growth medium described for the explant culture was used in both techniques described above.

2.4.4. Limbal Cells

Studies have been conducted to isolate LESC and CSSC separately, as the limbal region contains LESC in the epithelial layer and CSSC in the stromal layer.

Cell Culture of LESC

Two different techniques were employed for the isolation of LESC. Initially, collected limbal and corneal epithelial layers were incubated in a 1.2 U/mL Dispase solution prepared in KSFM for 30 minutes at 37°C with shaking. Following centrifugation at 300xg for 5 minutes, the harvested cell pellet was seeded into culture plates and cultured in KSFM supplemented with 0.005 µg/mL EGF, 5 µg/mL BPE, and 1% Penicillin-Streptomycin solution. This technique was applied to Cornea #7. The isolated cells were immortalized at passage 0 and then subcultured.

In the other technique, the collected limbal layer was fragmented into small pieces and placed onto culture plates with the epithelium facing upwards. A drop of FBS was added onto the tissue pieces, then incubated overnight in a cell culture incubator at 37°C with 5% CO₂. After removing the FBS, growth media was added to initiate the culture process. In this study, the growth media consisted of DMEM-F12 supplemented with 5 ng/mL EGF, 5 mg/mL-5 mg/mL-5 ng/mL insulin-transferrin-sodium selenite (ITS) (Sigma

Aldrich, 11074547001), 0.5 mg/mL hydrocortisone (Sigma, 50-23-7), 0.5% DMSO, 1% Penicillin-Streptomycin Solution, and 5% FBS.

Cell Culture of CSSC

- *Isolation of primary CSSC*

For CSSC isolation, both explant and enzymatic culture techniques were employed. In the explant culture method, tissue pieces were placed onto culture plates and incubated in a cell culture incubator for 15 minutes to allow the tissue pieces to adhere to the surface. Following incubation, growth media was gently added to initiate the culture process. In this step, DMEM-F12 supplemented with 5% FBS and 1% Penicillin-Streptomycin solution was used as the growth media. This process was applied to Cornea #1, #4, and #5. The isolated primary cells were then subcultured.

In the enzymatic culture technique, tissue pieces were incubated in a Collagenase I enzyme solution prepared in growth media at 37°C with agitation. After incubation, the resulting cell pellet obtained by centrifugation at 300xg for 5 minutes was seeded onto culture plates to initiate the culture process. In this procedure, Cornea #2 underwent isolation for 1 hour in a 4mg/mL enzyme solution, Cornea #7 underwent isolation for 1 hour in a 2mg/mL enzyme solution, and Cornea #3, #6, #9, and #10 underwent isolation for 30 minutes in a 2mg/mL enzyme solution. DMEM-F12 supplemented with 5% FBS and 1% Penicillin-Streptomycin solution was used as the growth media.

- *Immortalization of CSSC*

The primary CSSCs obtained from the enzymatic isolation of Cornea 10 were subcultured to passage 1. These cells underwent the SV40 immortalization technique described in Section 2.1.3. The immortalized cells at passage 1 were subcultured up to passage 14.

Cells from different passage numbers were characterized using ICC and gene expression analysis with the protocol explained in Section 2.2.

- *The effect of the tissue transfer on CSSC morphology*

In the explant culture, tissue pieces showing cell outgrowth were transferred to initiate another cell outgrowth. In this study conducted on Cornea #4, when the cells from the tissues covered approximately 70% of the surface, a tissue piece was collected and transferred to a new cell culture plate. After being kept in the cell culture incubator for 15 minutes to ensure tissue adhesion, growth media were added to initiate the culture process. The morphologies of the cells obtained from the transfer of three tissue pieces were evaluated.

- *Differentiation of CSSC to corneal cells*

CSSCs isolated from Cornea #9 by incubating in 2mg/mL Collagenase I solution for 30 minutes were cultured in growth media until passage 2. DMEM-F12 supplemented with 5% FBS and 1% Penicillin-Streptomycin solution was used as the growth media. In passage 2, the cells were gradually subjected to media change. For this purpose, three different groups were formed, each containing CSSCs at passage 2 cultured in three different media. In the first group, culture was continued with the initial growth media, which was serum-supplemented. In the second group, KSFM supplemented with 0.005 µg/mL EGF, 5 µg/mL BPE, and 1% Penicillin-Streptomycin solution, referred to as epithelial serum-free media, was used as the culture media. In the third group, KSFM supplemented with 0.005 µg/mL EGF, 5 µg/mL BPE, 1% Penicillin-Streptomycin, 500 ng/mL hydrocortisone, and 0.005 mg/mL insulin was used as the culture media, termed as keratocyte serum-free media. Cells at passage 4 cultured in the media mentioned above were characterized. ICC and gene expression assays described in Section 2.2 were performed for characterization.

2.5. Creation of Cell Stocks and Characterization of Corneal Cell Lines

2.5.1. hCEC

The hCEC cell line was obtained from Afsun Şahin's Lab at Koç Hospital, Istanbul, Turkey. For the cell culture of hCECs, KSFM supplemented with 0.005 µg/mL EGF, 5 µg/mL BPE, and 1% Penicillin-Streptomycin solution was used as the growth media. After establishing a cell stock through subculturing, characterization studies were conducted. The mechanobiological effects of air-liquid interphase (ALI) culture conditions on cells were observed by culturing them in serum-free and complete media under submerged and ALI conditions. This process was carried out for one month in serum-free and complete media. Serum-free media contains KSFM supplemented with 0.005 µg/mL EGF, 5 µg/mL BPE, and 1% Penicillin-Streptomycin solution. Complete media contains DMEM-F12 supplemented with 10% FBS, 10 ng/mL EGF, and 1% Penicillin-Streptomycin solution. At the end of the culture process, ICC, gene expression, and TEER measurement assays were conducted. ALI studies were performed using cell culture inserts compatible with 24-well plates. While the bottom of the insert contained media, the surface containing the cells was exposed to air. The applied cell culture techniques are detailed in Section 2.1, while the characterization techniques are described in Section 2.2.

2.5.2. hKC

The immortalized human KC (hKC) (T0579) cell line was purchased from Applied Biological Materials Inc. (Richmond, Canada). Prigrow X Series Medium (Abm, TM0579) supplemented with 20µg/mL Geneticin (Abm, G418) was used as the growth media. The surface of the cell culture plate intended for KC cell line culture was coated

with Applied Cell Extracellular Matrix (Abm, D422) to facilitate the procedures. After establishing a cell stock through subculturing, characterization was performed using ICC and gene expression assays. The applied cell culture techniques are detailed in Section 2.1, while the characterization techniques are described in Section 2.2.

2.5.3. hCEnC

The human CEnC (hCEnC) cell line (HCEC-B4G12) was purchased from DSMZ GmbH (Leibniz, Germany). Human Endothelial SFM (Gibco, 11111044) supplemented with 10 ng/mL recombinant human fibroblast growth factor 2 (FGF2) (Peprotech, 100-18B) and 1% Penicillin-streptomycin solution was used as the growth medium during the cell culture process. The surface of the cell culture plate intended for cell culture was coated with FNC Coating Mix. After establishing a cell stock through subculturing, characterization was performed using ICC and gene expression assays. The applied cell culture techniques are detailed in Section 2.1, while the characterization techniques are described in Section 2.2.

2.6. Co-culture of Corneal Cells

Co-culture studies were conducted using the three corneal cell lines mentioned above: hCEC, hKC, and hCEnC. The conducted studies are detailed below.

2.6.1. Selection of Optimum Culture Media for Co-culture

Corneal cells were seeded onto 24-well plate-compatible cell culture inserts at 6600 cells/cm² density. After the seeded cells adhered to the surface, inserts containing three

different corneal cell types were placed together into a single well of a 6-well plate and cultured together in the same medium. The cells were cultured for 15 days with medium changes every two days. Because of the 0.4 μ m pore size of the cell culture inserts, there was no cell migration between the cultured cells, preventing cross-contamination. However, this pore size allowed for the passage of secreted materials between the cells, facilitating communication between cells. For comparison of the effectiveness of co-culture, corneal cells used in the study were also cultured alone on 24-well plates at a density of 6600 cells/cm², and these groups were evaluated as control groups.

For the co-culture of corneal cells, the optimal growth media was determined by culturing each of the three corneal cell types individually and in mixtures at 1:1 and 1:1:1 ratios for 15 days (**Table 5**). At the end of the culture period, the MTS cell viability assay, as outlined in Section 2.2.5, was applied to the samples to select the growth media that provided the highest cell viability.

Table 5. The details of the culture medias used in the co-culture study.

Media Number	Media Content	Description
M1	KSFM+EGF+BPE	Corneal Epithelial Growth Media
M2	EndoSFm+ FGF	Corneal Endothelial Growth Media
M3	Prigrow	Corneal Keratocyte Growth Media
M4	M1 : M2 : M3	(1:1:1)
M5	M1 : M3	(1:1)
M6	M2 : M3	(1:1)
M7	M1 : M2	(1:1)

2.6.2. Determination of the Effect of Co-culture on Corneal Cells

The effects of the co-culture system were determined by applying characterization assays to cell samples cultured for 15 days in the selected optimal medium, as described in

Section 2.6.1. In this context, the cell-specific phenotypes of corneal cells after co-culture were evaluated by comparing them with a single culture.

Characterization of Co-cultured hCEC

They were cultured under single and co-culture conditions to determine the co-culture effect on hCEC cells. While the M2 medium was used for the co-culture process, M2 and M1 media were used for single culture. After a 15-day culture period, ICC and gene expression assays, as outlined in Section 2.2, were applied to assess CK3 and ZO1 markers. Additionally, TEER measurements, as described in Section 2.2.3, were conducted on all hCEC samples throughout the 15-day culture process.

Characterization of Co-cultured hCEnC

The hCEnC cells were cultured under single and co-culture conditions to determine the co-culture effect. Both co-culture and single cultures of hCEnC cells were conducted in the M2 medium. After a 15-day culture period, ICC and gene expression assays, as outlined in Section 2.2, were applied to assess Na-K ATPase and ZO1 markers. Additionally, TEER measurements, as described in Section 2.2.3, were conducted on all hCEC samples throughout the 15-day culture process.

Characterization of Co-cultured hKC

The hKC cells were cultured under single and co-culture conditions to determine the co-culture effect. Co-culture was conducted using M2 medium, while single cultures were

performed using M2 and M3 media. After a 15-day culture period, ICC and gene expression assays, as outlined in Section 2.2, were applied to assess COL I and KERA markers.

2.7. Cornea Tissue Engineering Studies

CTE studies were conducted using the aforementioned corneal cell lines. The culture process and media used were implemented according to the specifications outlined in Section 2.5.

2.7.1. Corneal Epithelium Tissue Engineering

Corneal epithelial tissue engineering studies were conducted on poly(glycerol sebacate) (PGS) membranes produced as part of project member Kamal Asadipakdel's master thesis project. Three transparent PGS membranes were prepared with Dr. Blade using different pre-polymers synthesized in 50, 75, and 105 minutes, followed by crosslinking at 130°C for 48 hours. After Kamal Asadipakdel determined the transparency and mechanical properties of the produced PGS membranes, cell culture assays were initiated. These membranes were sterilized with 70% ethanol for 1 hour and exposed to UV light for 1 hour on each side. A 6600 hCEC/cm² cell density was seeded onto the sterilized PGS membranes. After a 14-day culture period, the cells were subjected to the MTS biocompatibility assay outlined in Section 2.2.5. Characterization studies for CK-3 and ZO1 markers were conducted after a 15-day culture period using the ICC protocol described in Section 2.2.1.

2.7.2. Corneal Stroma

3D cell culture studies with hKC were conducted using hydrogels prepared by project member Ebru Demir. Hydrogels were created using RADA peptide hydrogel, crosslinked and uncrosslinked PGS nanoparticles (PGS-NP). Crosslinking was carried out by EDC/NHS. A cell density of 6600 hKC/cm² was seeded onto the hydrogel, and the cultures were maintained for 15 days. At the end of the culture period, characterization studies were conducted using the CCK8 viability assay and ICC assay, as outlined in Section 2.2.

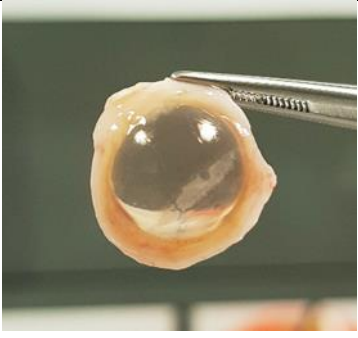
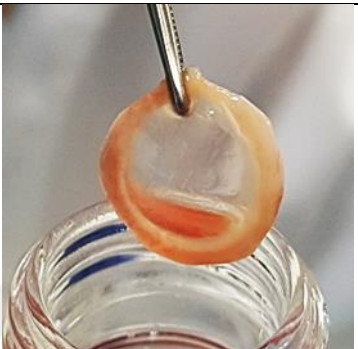
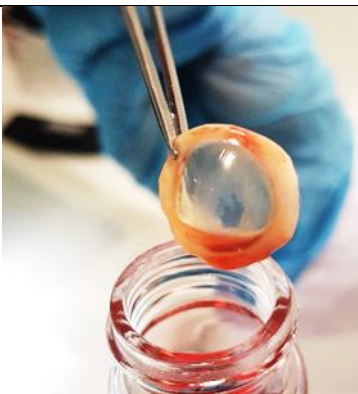
3. Results and Discussions

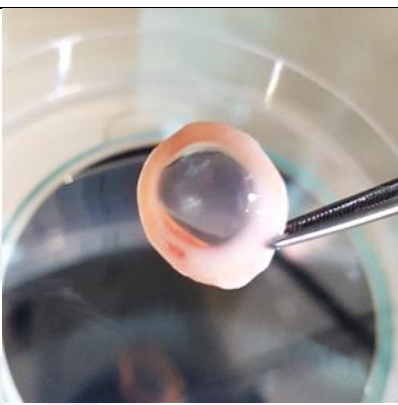
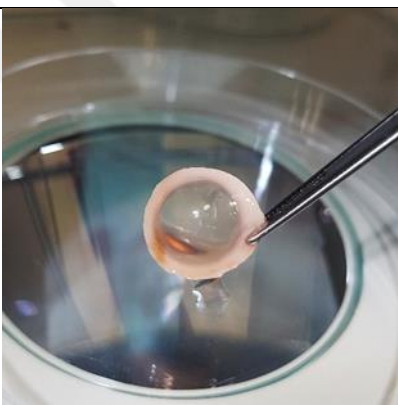
3.1. Dissection of Human Donor Cornea

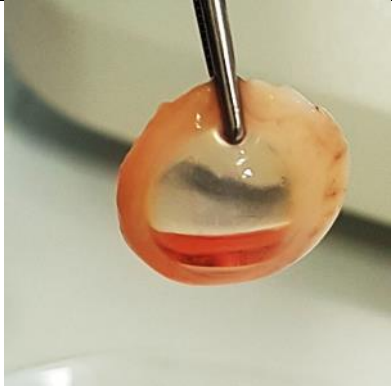
For this study, 10 human donor corneas were obtained from Haydarpaşa Numune and Research Hospital (**Table 6**).

Table 6. The main information on human donor corneas includes donor age and gender, endothelial cell count, and tissue pictures.

Cornea #	Donor Age	Gender Female (F) or Male (M)	Endothelial Cell # per mm ²	
1	74	M	1400	ND

2	57	M	3054	
3	55	M	1000	
4	55	M	1300	
5	72	F	2700	

6	72	F	2700	
7	69	M	ND	
8	69	M	ND	
9	57	M	4288	

10	57	M	4635	
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The donor corneas detailed in Table 6 underwent dissection to obtain the cell layers separately (**Figure 8**).

In the cornea dissection process, sterilization is crucial to prevent contamination from the environment and during tissue transfer. All equipment was sterilized, and the dissection tools were autoclaved before the procedure. Tissue sterilization was performed using 50 µg/mL Gentamicin, an antibiotic commonly used in corneal cell culture studies ^[100]. Initially, the trabecular meshwork, which is not evaluated in this study, was completely removed to prevent its cells from contaminating the study. Subsequently, the epithelial and endothelial layers, the topmost and bottommost layers of the cornea, were carefully grasped and peeled off with forceps. During this process, the surrounding scleral tissue was used as a holding area to stabilize the tissue, preventing damage to the epithelial and endothelial cells. At the end of the procedure, the scleral tissue was entirely removed, leaving only the limbus and corneal stroma tissues. The limbus and corneal layers were carefully separated and cut into small pieces. Cell culture procedures were initiated for primary cell isolation using the tissue groups collected during dissection.

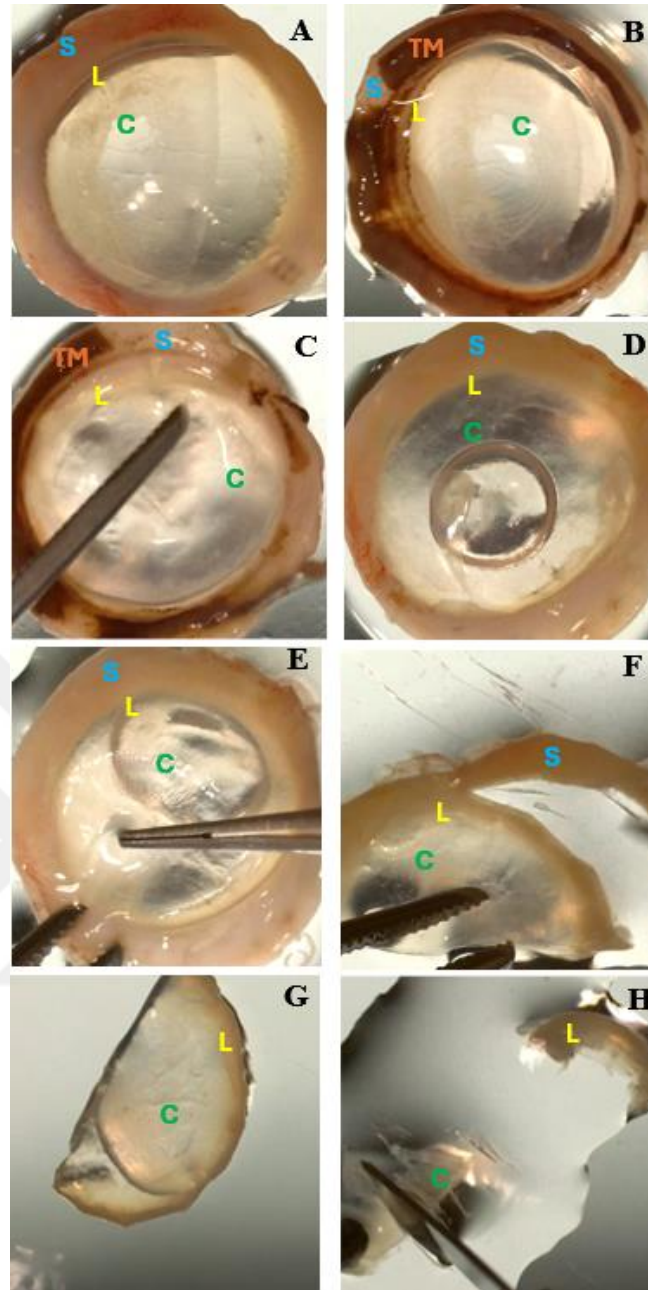


Figure 8. Dissection processes of corneal and limbal tissues. (A) Anterior surface of the cornea, (B) Posterior surface of the cornea, (C) Posterior surface of the cornea before collecting the trabecular meshwork, (D) Posterior surface of the cornea after collecting the trabecular meshwork, (E) Collection of corneal epithelial and endothelial layers, (F) Removal of scleral tissue, (G) Limbal and corneal stroma tissues, (H) Separation and cutting of limbal and corneal stroma into small pieces (C: cornea, L: limbus, S: sclera, TM: trabecular meshwork).

3.2. Isolation of Primary Corneal and Limbal Cells

Enzymatic and explant culture methods have been applied for primary cell isolation, detailed below.

3.2.1. Corneal Epithelial Cells

Primary hCECs isolated using the explant culture technique were cultured in three different media: complete media, complete media with EGF, and serum-free media.

In Cornea #1, #2, #4, #6, and #8 samples cultured with complete media, cell outgrowth was observed at the end of the 15-day culture period (**Figure 9**). The complete media used in this study was selected as a general growth medium, as it is preferred in media comparisons in the literature^[101]. The addition of EGF as a supplement to the medium in epithelial cell culture is a well-documented practice^[102]. KSFM, used as the serum-free medium in this study, has been shown to support the proliferation and viability of CECs^[103]. Cell outgrowth from tissue strips was observed in this study in all three media. The emerging cells exhibited a circular-like morphology consistent with CEC cell morphology^[104].

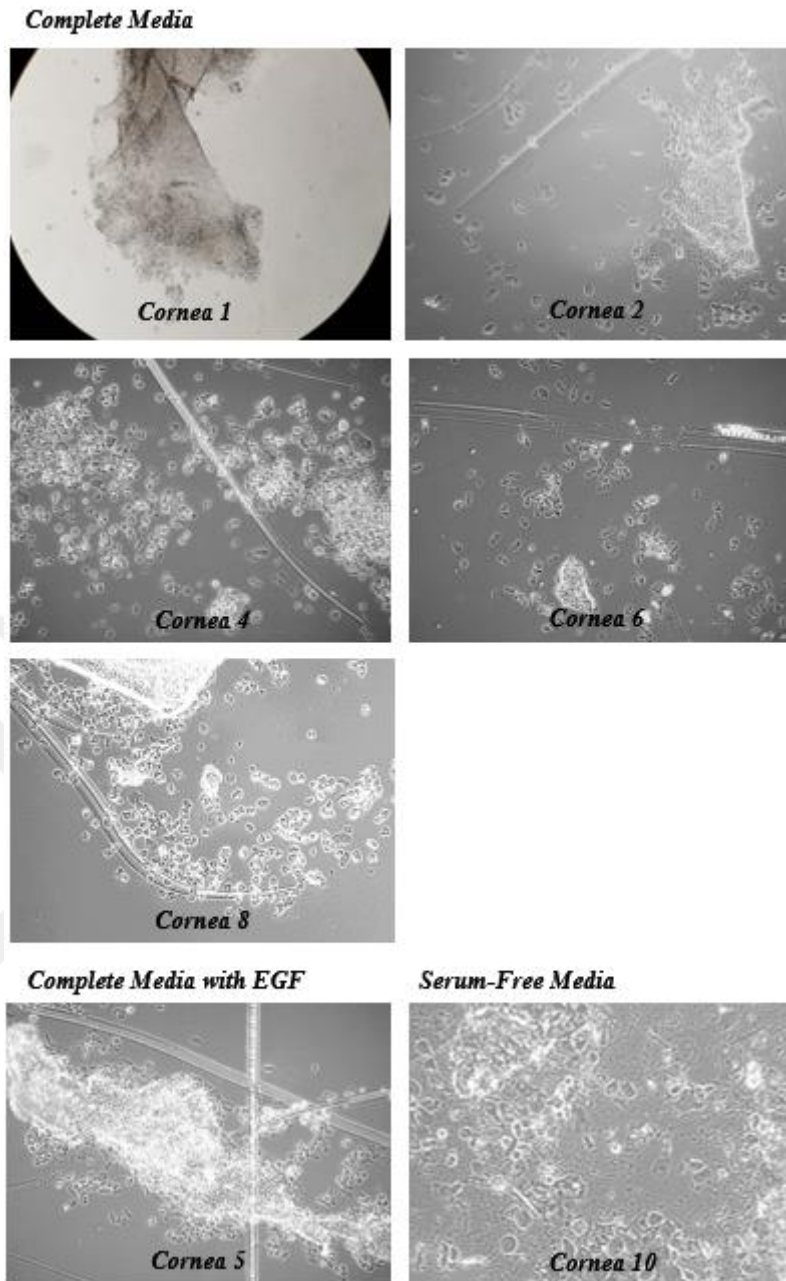


Figure 9. Morphological results of primary hCECs cultured for 15 days in three different media using the explant culture technique (10X magnification).

When these cells reached confluence on the surface of the culture plate, they were removed by trypsinization for cell counting. The culture processes for Cornea #1, #2, #4, #5, #6, and #8 were terminated at passage 0 due to 0% viability. The Cornea 10 sample was immortalized at passage 0. However, upon removal, 0% viability was obtained, indicating that the immortalization process was unsuccessful, and thus, the culture process was concluded.

In Cornea #3 and #9, subjected to the enzymatic culture process, the culture was terminated because of the absence of adherent and proliferative cells. The primary reason for the failure of enzymatic isolation could be insufficient enzymatic digestion. However, according to literature sources, incubation with 1.2 U/mL dispase for 1 hour is considered suitable for CEC isolation ^[105]. Additionally, CEC isolation has been achieved with increased enzyme concentration and incubation duration ^[106]. The primary hCECs isolated in this study could not maintain viability during subculture. This may be related to the viability of hCECs on the tissue and, consequently the initial cell counts and viability ^[107]. The donor's health status is another influential parameter. Factors such as donor age, smoking, and diabetes affect corneal tissue health and, thus, primary cell isolation ^[108]. However, the health status of the donors in this study is unknown. Cornea #5, #6, and #8 used in the explant culture exhibited opacity (**Table 6**), which may have contributed to the failure of the culture process. Mechanical damage might have occurred while collecting the donor epithelial tissue strips, which could negatively impact subculture success. Additionally, the size of the tissue strips is a factor that affects cell outgrowth from the tissue. This study collected small tissue strips by grasping and pulling the epithelial layer with the basement membrane from the relatively small cornea. Since adjusting the size of these strips during dissection was not possible, this could be another contributing factor.

3.2.2. Corneal Keratocyte

The explant culture technique for primary KC isolation was applied to Cornea #1, #4, and #5. Tissues from Cornea #4 and #5 were discarded because of the absence of cell outgrowth. Approximately 15 days after starting the culture, cell outgrowth was observed from Cornea #1 (**Figure 10 A-B**). Within about a month, the cells that emerged from the tissue proliferated and spread across the surface of the culture plate (**Figure 10 C-D**). These cells were then removed by trypsinization and subcultured. Cells in passage 1, cultured for 2 weeks, exhibited a dendritic cell morphology (**Figure 10 E-F**).

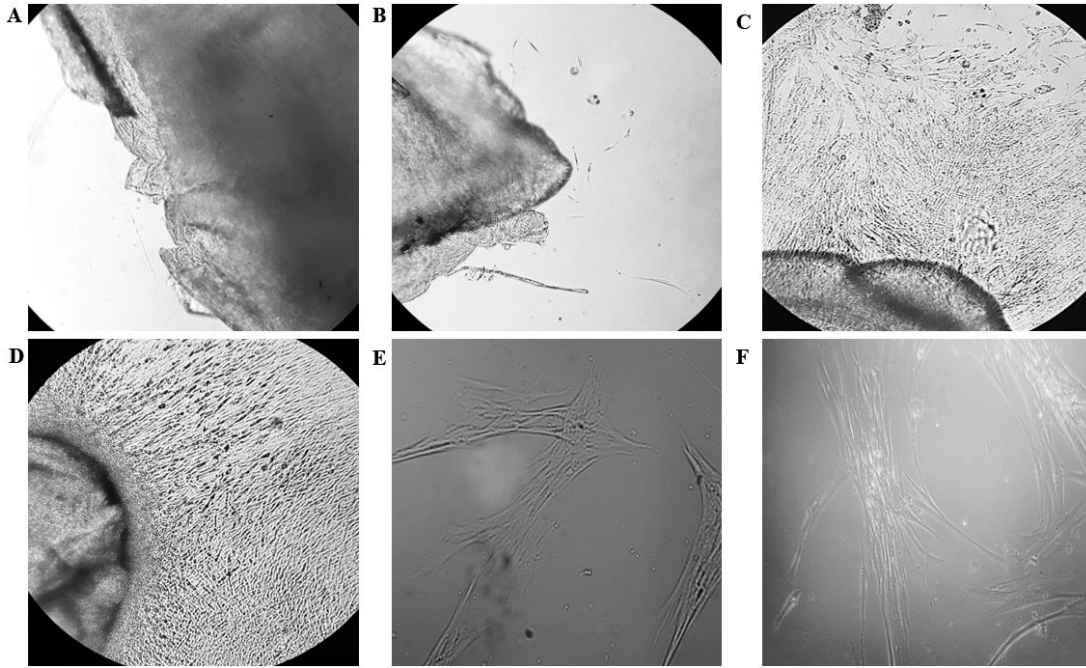


Figure 10. KC cells isolated from Cornea 1 using the explant culture technique. Passage 0 cells at 12 days (A), 18 days (B), 26 days (C), and 32 days (D) after culture initiation, and passage 1 cells after 1 week (E) and 2 weeks (F) of culture.

At the end of the explant culture process applied to three corneas, cell isolation was successful in only one cornea. The donor ages for Cornea #4 and #5 were 55, while for Cornea #1, it was 74. This indicates that the results obtained are not caused by donor age. Donor cornea health is one of the most influential parameters in primary cell isolation. As shown in the donor cornea images in Table 6, Cornea #4 and #5 exhibit opacification. The negative results may be because of the health condition of the donor corneas.

In the explant culture of KCs, different enzyme concentrations and incubation times were tested. A 4 mg/mL Collagenase I solution was applied for 1 hour of isolation process to Cornea #2. However, cell outgrowth was not observed after this application. Considering that the result might be because of high enzyme concentration, the enzyme amount was reduced by half, and the same process was repeated on Cornea #7 with a 2 mg/mL enzyme solution. The isolated cells exhibited a dendritic cell morphology and maintained this morphology upon passage (**Figure 11 A-B**). However, it was observed that the cells in passage 1 contained a high amount of stress vesicles (**Figure 11-B**). To minimize this stress condition, the enzymatic isolation time was halved. Enzymatic isolation was performed for 30 minutes with a 2 mg/mL enzyme solution on Cornea #3, #6, #8, #9, and

#10. Cells were obtained from all samples except Cornea #8. As seen in the visual shared in Table 6, opacity is present in Cornea #8, possibly because of donor cornea health and donor age (69 years). The isolation process was successful in other corneal tissues, and cells with dendritic KC morphology were isolated from each tissue (**Figure 11 C-F**). When reviewing the literature, it is observed that enzymatic isolation is applied at different concentrations and durations. For example, in the study by Nagymihály et al., human KC isolation was conducted with a 3 mg/mL Collagenase solution for 3 hours ^[90]. However, cell isolation was unsuccessful in the present study with a 4 mg/mL enzyme solution for 1 hour of incubation. Therefore, when the enzyme concentration was halved in the isolation process, successful isolation was achieved in Cornea #7. However, the isolation time was halved due to the presence of a significant amount of stress vesicles in the isolated cells. It is evident in the literature that isolation performed with a 2 mg/mL Collagenase solution for 30 minutes of incubation provides successful results. For instance, in the study by Słoniecka et al., human KCs were isolated by incubating corneal stromal tissue in a 2 mg/mL Collagenase solution for 30 minutes ^[109].

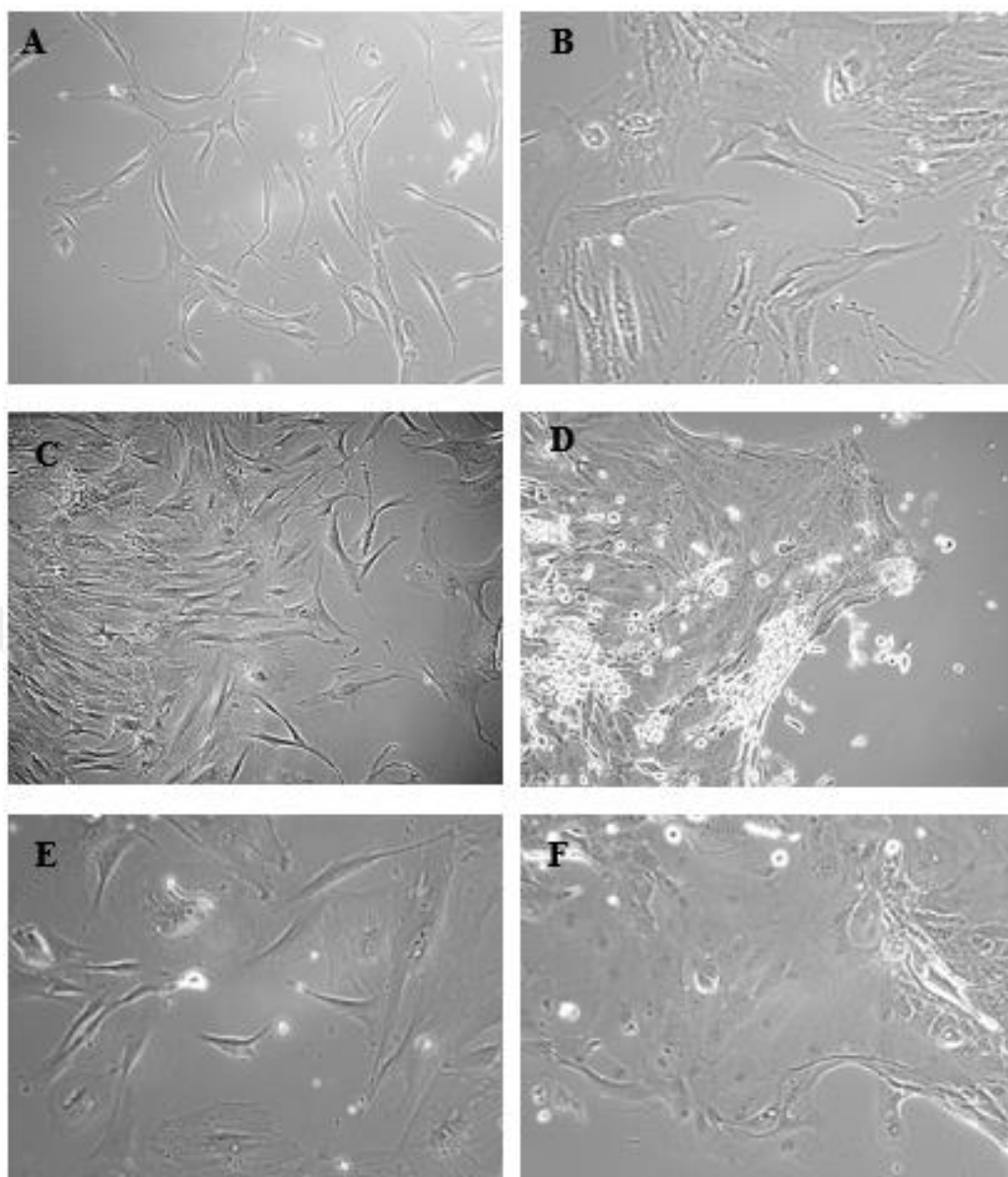


Figure 11. The primary KCs obtained from different donor corneas and their cell morphologies. Morphologies of passage 0 (A) and passage 1 (B) KCs obtained with 1-hour isolation in a 2 mg/mL Collagenase I solution after 15 days of culture. Morphologies of passage 0 KCs obtained from Cornea 3 (C), Cornea 6 (D), Cornea 9 (E), and Cornea 10 (F) with 30 minutes of isolation in a 2 mg/mL Collagenase I solution after 15 days of culture (10X Magnification).

After primary KCs were isolated, they were immortalized. Both primary and immortalized KCs were characterized and evaluated through ICC and gene expression assays.

Immortalization of Primary hKC

Primary KCs isolated from Cornea #6 and #10 by incubation with a 2 mg/mL enzyme solution for 30 minutes were immortalized at different passage numbers. Cells isolated from Cornea #6 were immortalized at passage 1, while cells from Cornea #10 were immortalized at passage 0 using SV40 immortalization. When the Cornea #10 cells immortalized at passage 0 were subcultured to passage 1, their proliferation capacity ceased. However, the Cornea #6 cells immortalized at passage 1 underwent a successful subculture process and were subcultured up to passage 17, where their proliferation capacity ceased, thus concluding the subculture process (**Figure 12**). The SV40 immortalization technique is the most commonly used method for immortalization.

This technique inhibits oncogenes such as p53 and pRB, preventing cell cycle arrest. p53 is a tumor suppressor gene and functions as a sequence-specific DNA binding transcription factor. When double-stranded DNA breaks occur, p53 switches to its active form and increases the expression of p53-responsive proteins, one of which is p21. p21 prevents the cell from transitioning from the G1 phase to the S phase of the cell cycle, leading to cell cycle arrest in cells with damaged DNA. Similarly, if the Bax protein is overexpressed by p53, apoptosis occurs. The large tumor antigen of simian virus 40 (SV40) forms a complex with p53, inhibiting its activity and preventing p53 from binding to DNA and functioning as a transcription factor^[110]. As a result, the cell does not undergo cell cycle arrest or apoptosis and continues to divide. Normally, a cell enters a state called senescence after dividing for a while, meaning its ability to divide stops. In immortalized cells, however, the cell continues to divide for a while without entering senescence but eventually enters a crisis phase and loses its proliferation capacity^[111]. In this thesis study, primary KCs were immortalized at passage 1, overcoming the stress of immortalization and undergoing subculture for 17 passages. However, subculturing was stopped because of a significant decrease in proliferation capacity starting from passage 17.

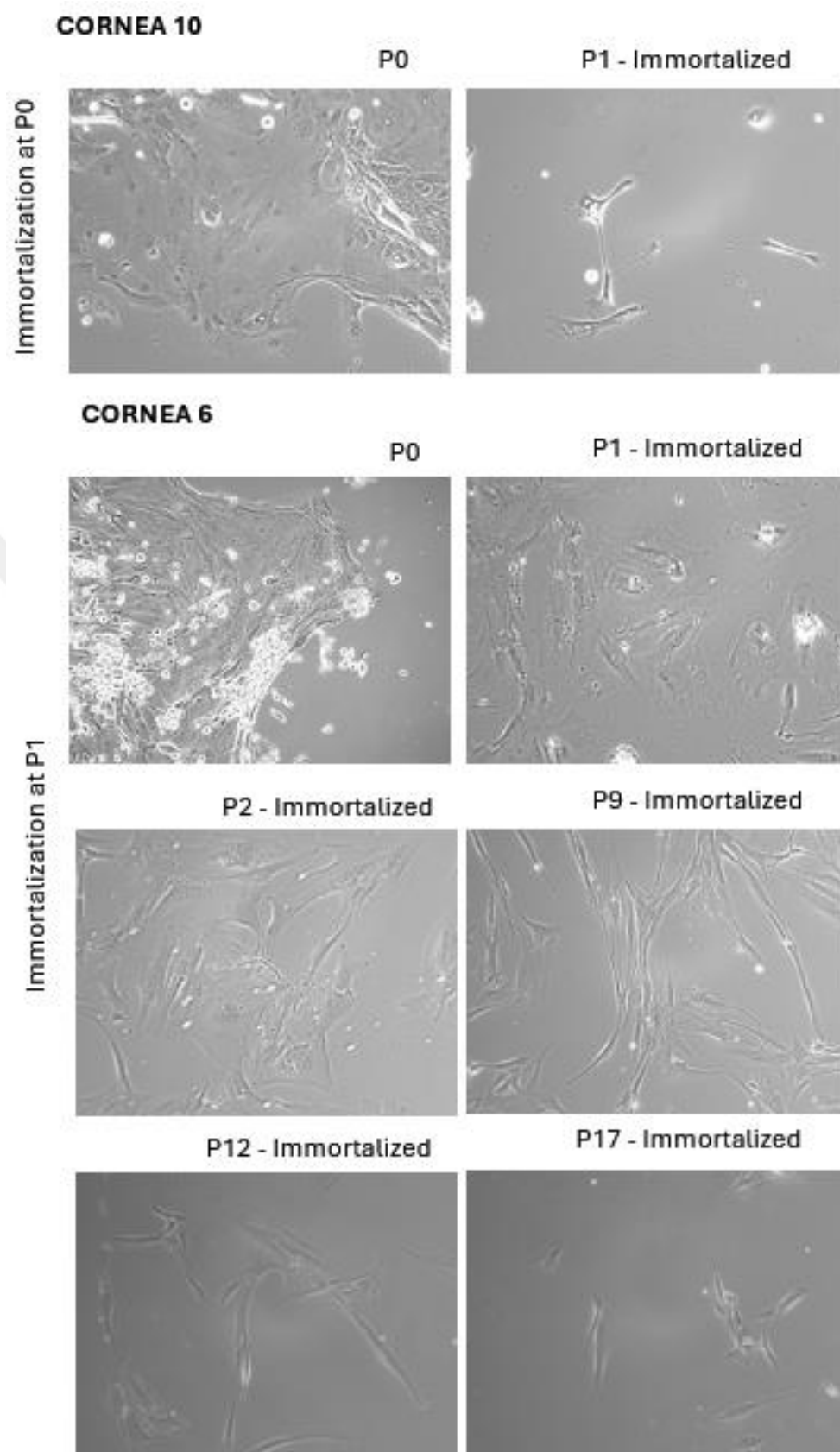


Figure 12. Immortalization and subculturing of primary KCs at different passages (10X Magnification).

Characterization of Primary and Immortalized hKCs

The cells isolated from Cornea #6 were utilized to characterize human KCs. For this study, ICC and gene expression assays were conducted on primary passage 2 and immortalized passages #9, #12, and #15 KC cells. It is observed that the main KC markers, COL I and KERA, are expressed in both primary and immortalized cells. However, cell density and the expressions of COL I and KERA decreased in increasing immortalized cell passages. The expression of ALDH protein, associated with transparency, is observed in all cell groups, but it decreases in high immortalization passages. The α -SMA marker, indicating the transformation of KCs into myofibroblast cells, was low in primary passage 2 KCs but expressed in immortalized cells (**Figure 13**).

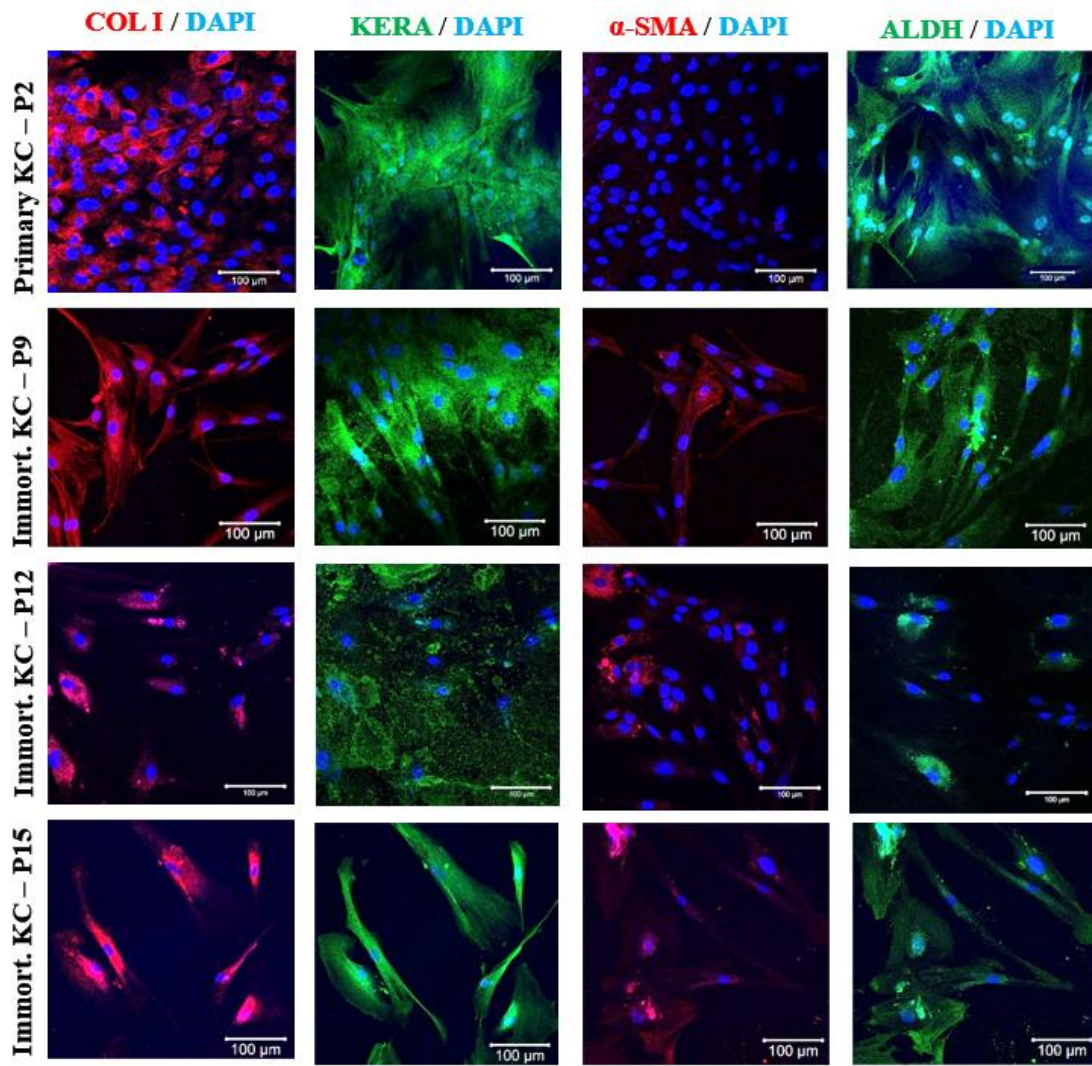


Figure 13. The ICC results of primary passage 2 and immortalized passages 9, 12, and 15 KCs with main KC markers (P: passage).

The ICC results presented above aimed to obtain definitive data by conducting gene expression studies on the cell groups. Accordingly, key KC and myofibroblast marker expressions were examined (**Figure 14**). The expression of the ECM molecule, COL I, was highest in the immortalized KC passage 12 sample (2- Δ Cp: 47.57), surpassing that of primary KC (2- Δ Cp: 31.68) (**Figure 14-A**). KCs with high COL I expression are crucial for ECM regeneration. The highly organized and oriented collagen fibrils in the corneal stroma ensure corneal transparency, facilitating light transmission to the lens and contributing to good vision ^[12]. In light of this information, primary passage 2 and immortalized passage 12 cells exhibiting high COL I expression are suitable for CTE studies. Another corneal stroma ECM molecule, KERA, stabilizes and orients COL I

fibrils ^[112]. In this study, it was observed that primary passage 2, immortalized passages 9 and 12 KCs exhibited similar KERA expression levels (**Figure 14-B**). However, immortalized passage 15 KCs showed significantly higher KERA expression. Based on sources from the literature, it can be inferred that high KERA expression is associated with fibrillogenesis, which plays a role in the development of keratoconus ^[113]. Therefore, the low COL I expression and extreme increase in KERA expression in immortalized passage 15 KCs may be because of their differentiation into stromal fibroblasts.

The sample with the highest expressions of ALDH3A1 and AQP1 is immortalized passage 12 KC (**Figure 14-C**). ALDH is an enzyme that metabolizes aldehydes and is found in the corneal stroma as crystallin proteins. The presence of these crystallin proteins in KC cells helps maintain corneal transparency by facilitating the transmission of light to the lens ^[18]. The high ALDH3A1 expression in the immortalized passage 12 KC sample makes this cell group suitable for CTE studies. AQP is a transmembrane water channel that maintains corneal stromal hydration, thereby preserving transparency ^[114]. The high expression of AQP1 in immortalized passage 12 KCs makes this cell group suitable for CTE studies.

When there is an injury in the corneal stroma, KCs differentiate into myofibroblast cells and contribute to wound healing. Myofibroblast cells exhibit characteristics of smooth muscle cells and contain stress fibers, with α -SMA being the gene that expresses this, also known as ACTA2. During myofibroblast transformation, the expression of proteoglycans and crystallin proteins decreases while collagen expression increases ^[115]. This phenomenon is observed in primary passage 2 KCs, where this sample has the highest ACTA2 expression (2- Δ Cp: 164.82) (**Figure 14-D**). The reason for this result could be the mechanical damage incurred during the dissection process.

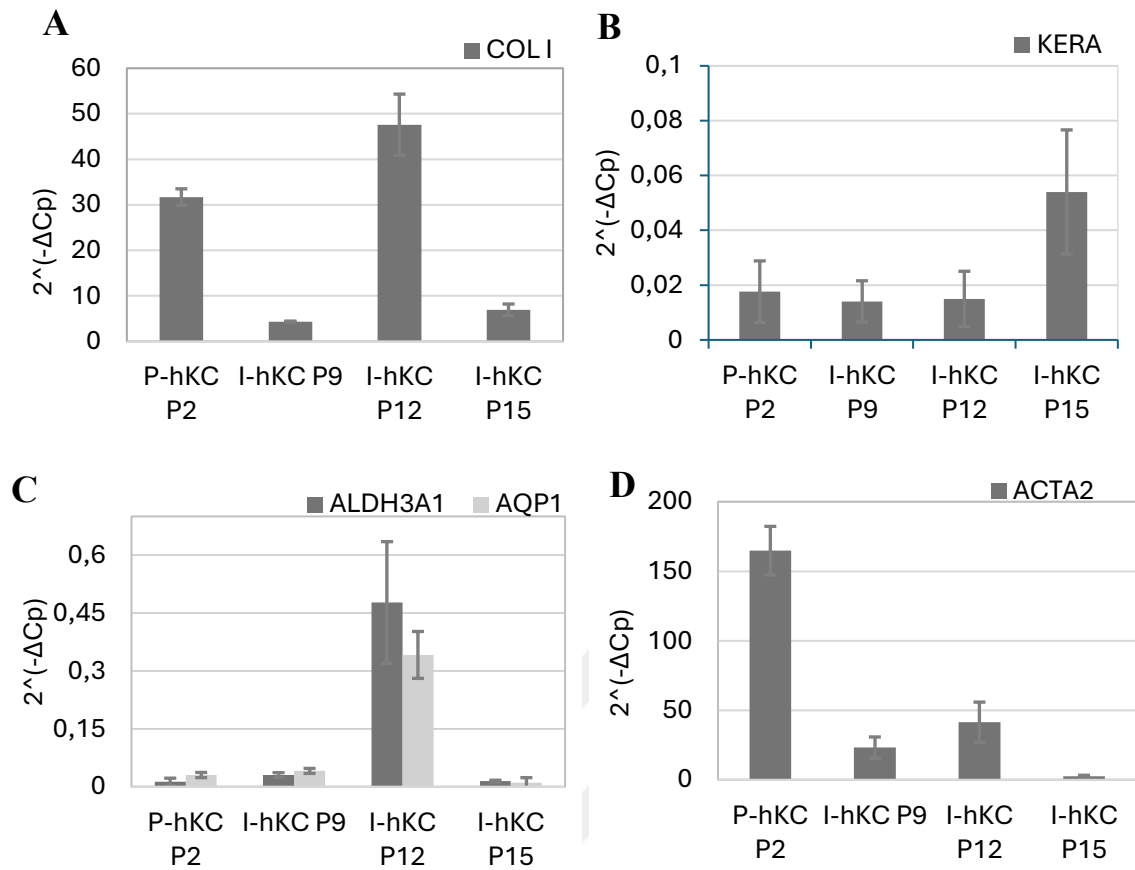


Figure 14. Gene expression results for main corneal keratocyte markers and myofibroblast marker of primary and immortalized KCs (n=2) (Relative Quantification, Mean $\pm$ SEM).

Based on the results described above, it was determined that immortalized KCs at passage 12, which have high COL I, ALDH3A1 and AQP1 expression, are suitable for CTE studies.

The expression profiles of genes belonging to other corneal and limbal cells were evaluated within the scope of this study. It is known that both LESC and MSC are present in the limbus region. When the expression of specific genes associated with these cells was tested, the highest expressions were observed in VIM and CD90 (**Figure 15**). In contrast, the expression of other markers was notably low (**Table 3**). The expression of the VIM gene was detected in all cell samples, with the highest expression observed in immortalized passage 12 keratocytes (KC). Vimentin, an intermediate filament protein, provides mechanical support to cells, maintains cellular structural integrity, and facilitates cell movement ^[116]. Vimentin is mainly utilized as a stem cell marker, especially in MSCs

[117]. Literature sources suggest that vimentin can be considered a marker for KCs and CSSCs because of its high expression in these cells [118]. Based on this information, it can be inferred that the high vimentin expression in both primary and immortalized KCs evaluated in this study indicates their keratocytic nature, with immortalized passage 15 KCs undergoing the lowest vimentin expression, indicative of fibroblastic cell transformation (**Figure 15-A**).

CSSCs are CD90+/CD34-, similar to other MSCs, whereas, in KCs, this profile is reversed, being CD90-/CD34+ [119]. Therefore, CD90 and CD34 serve as parameters in the differentiation of limbal stem cells into KCs. As described above, immortalized passage 12 KCs exhibited high expression levels of all KC markers. Similarly, the expression of CD90 in this cell group appears to be significantly higher compared to other samples (**Figure 15-B**). Based on literature sources, it can be suggested that CD90 expression increases in immortalized cells [119b]. Thus, the obtained result is consistent with the literature.

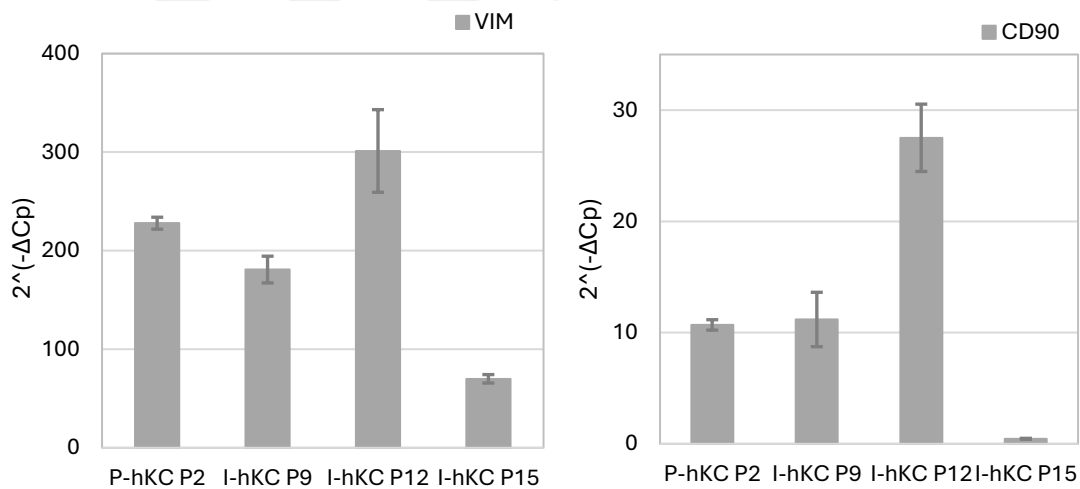


Figure 15. The expression results of the stem cell marker VIM and the MSC marker CD90 in primary and immortalized KCs (n=2) (Relative Quantification, Mean ± SEM).

In this study, it was observed that the expression levels of the p63, SOX2, and CD34 genes were significantly low in both the primary and immortalized KC cell groups (**Table 7**). Additionally, the expression of the KRT-5 gene, a marker for LESC, also exhibited low expression across all cell groups. However, in immortalized KCs, the expression of

the KRT-14 LESC marker gene was slightly higher compared to other genes (**Table 7**). CECs and KCs possess distinct intermediate filament structures, with KCs expressing vimentin while CECs express cytokeratin. Based on this information, the expression of KRT-14 was expected in the KC samples tested in this study. However, literature sources indicate that while KCs do not exhibit cytokeratin expression in vivo, expression is observed in in vitro studies [120].

Table 7. Gene expression results of primary and immortalized hKCs using main limbal cell markers (n=2) (Relative Quantification, Mean $\pm$ SEM).

	$2^{(-\Delta C_p)} \pm \text{std}$			
	P-hKC P2	I-hKC P9	I-hKC p12	I-hKC P15
<i>P63</i>	0,005 $\pm$ 0,0001	0,045 $\pm$ 0,013	0,022 $\pm$ 0,007	0,027 $\pm$ 0,003
<i>SOX2</i>	0,005 $\pm$ 0,003	0,003 $\pm$ 0,005	0,006 $\pm$ 0,001	0,013 $\pm$ 0,001
<i>CD34</i>	0,0006 $\pm$ 0,001	0,005 $\pm$ 0,001	0,001 $\pm$ 0,006	0,017 $\pm$ 0,02
<i>KRT5</i>	0,0004 $\pm$ 0,0003	0,0007 $\pm$ 0,0009	0,0001 $\pm$ 0,0001	0,013 $\pm$ 0,017
<i>KRT14</i>	0,0006 $\pm$ 0,0001	0,013 $\pm$ 0,006	0,237 $\pm$ 0,04	0,220 $\pm$ 0,08

The expression of CEC-specific markers, KRT-3 and -12, ZO-1, and β -catenin, was evaluated within this context (**Table 8**). The expression of KRT-3 and -12 genes showed negligibly low expression levels across all tested cell groups. KRT-3 and -12 are specific markers for CECs and are not expressed by KCs, similar to other corneal and limbal cells [121]. Similarly, low expression of the tight junction protein ZO-1 was observed (**Table 8**). Unlike other corneal cells, KCs do not form intercellular connections and do not express ZO-1 [122]. Another CEC marker, β -catenin expression, was present in all samples except for immortalized passage 15 KCs, attributed to the differentiation of immortalized passage 15 cells. The expression of β -catenin by KCs is expected, as β -catenin is a transcription factor that plays a role in the Wnt signaling pathway, which regulates functions such as cell growth, survival, and differentiation. The Wnt/ β -catenin signaling pathway in the cornea is involved in CEC stratification and LESC proliferation. This pathway also plays a role in the interaction between CECs and KCs, thus facilitating CEC stratification [123]. Based on this information, it can be inferred that the observation of β -catenin expression in the KC cell groups tested in this study is an expected result.

Within the scope of the study, the COL4A1 and ATPA1 genes, which are specific markers for CEnCs, were evaluated (**Table 8**). COL4 is the fundamental collagen protein that forms the DM structure and is expressed by CEnCs ^[124]. It is known that posterior KCs also express COL4 ^[124b]. In the KC cell groups tested in this study, COL4 gene expression was detected. However, maximum COL4 expression was observed in primary KCs, which is attributed to the high myofibroblast character in primary KCs. This is because COL4 plays a role in preventing the myofibroblast transformation of KCs, thereby increasing the KC character in the cell population in later passages. As a result, in immortalized cells, especially in immortalized passage 12 KCs, a high KC character was achieved. Another essential marker for CEnCs, Na-K ATPase, encoded by the ATPA1 gene, showed low expression in all sample groups. Na-K ATPase is a membrane protein found in CEnCs and facilitates the removal of excess water from the corneal stroma into the aqueous humor [Alaminos, 2008]. In light of this information, it is not expected for KCs to exhibit high ATPA1 expression, which is consistent with the results obtained from this study.

Table 8. The gene expression profiles of primary and immortalized KCs for CEC and CEnC specific genes (n=2) (Relative Quantification, Mean $\pm$ SEM).

Genes	$2^{(-\Delta C_p)} \pm \text{std}$			
	P-hKC P2	I-hKC P9	I-hKC p12	I-hKC P15
<i>KRT3</i>	0,001 $\pm$ 0,001	0,002 $\pm$ 0,003	0,005 $\pm$ 0,008	0,051 $\pm$ 0,025
<i>KRT12</i>	0,0008 $\pm$ 6,8 E-05	0,015 $\pm$ 0,008	0,095 $\pm$ 0,020	0,573 $\pm$ 0,047
<i>β-catenin</i>	11,34 $\pm$ 1,92	3,1 $\pm$ 0,77	6,5 $\pm$ 1,24	0,821 $\pm$ 0,031
<i>ZO1</i>	1,37 $\pm$ 0,26	1,16 $\pm$ 0,703	1,99 $\pm$ 0,891	0,176 $\pm$ 0,004
<i>COL4A2</i>	17,45 $\pm$ 0,715	1,20 $\pm$ 0,161	2,22 $\pm$ 0,49	0,223 $\pm$ 0,055
<i>ATPA1</i>	2,85 $\pm$ 0,968	1,96 $\pm$ 0,864	4,39 $\pm$ 1,49	0,197 $\pm$ 0,019

When all the results described above are compiled, it is evident that immortalized passage 12 KC is the most suitable cell group for use in CTE studies. However, in later passages, immortalized KC undergoes fibroblastic differentiation, which makes the use of high-passage immortalized KC inappropriate for CTE studies. Primary KC cells have a myofibroblastic character derived from the dissection process. Nonetheless, this condition can be eliminated through subculturing, as these cells also express markers such as COL4 that inhibit myofibroblast transformation. The evidence for this is that

immortalized KC at passage 12 exhibits a high KC character, indicating that the myofibroblast character has been eliminated during the subculture process. Consequently, it has been found appropriate to immortalize primary KC isolated from donor tissue and subsequently use immortalized passage 12 KC in CTE studies.

3.2.3. Corneal Endothelial Cells

Within the scope of this study, various efforts were made to isolate endothelial cells from human donor corneal tissues obtained from the eye bank. The first critical aspect to consider is surface coating. Surface coating is essential for the adhesion of endothelial cells to the culture surface. Surface coating facilitates cell attachment to the culture plate surface and supports cell viability. The surface coating process uses ECM proteins, with proteins commonly found in the DM being preferred. The DM ECM molecules used in surface coating that have shown positive effects in CEnC culture include fibronectin, laminin, COL-I, -IV, -VI, -VIII proteins, and heparan sulfate, dermatan sulfate, and keratan sulfate proteoglycans ^[125]. In this study, the FNC Coating Mix solution was used as the ECM protein, containing bovine fibronectin, bovine collagen type I, and bovine serum albumin ^[126]. The positive effects of FNC Coating Mix on CEnC culture have been documented in the literature ^[125]. In this study, surface coating was applied in both enzymatic and explant cultures.

The explant culture technique is commonly applied in the literature for CEnC isolation. In this thesis, the explant culture technique was used to isolate primary CEnC from Corneas #1, # 2, #4, #5, #6, #7, #8, and #10. The technique and growth media used in this process were applied exactly as described in Walshe et al.'s study ^[127]. Polygonal CEnC outgrowth was observed from the tissue strips (**Figure 16**). However, when the cells were harvested using trypsinization and counted, no viability was observed, and subculturing was not possible. A dilute trypsin solution (0.05%) was used during cell harvesting to minimize enzymatic damage. A quick and practical approach was employed to prevent cell loss during subculturing. The negative results may be attributed to the health of the donor tissue. In the literature, CEnC isolation has been successfully achieved using the technique applied in this study ^[127-128].

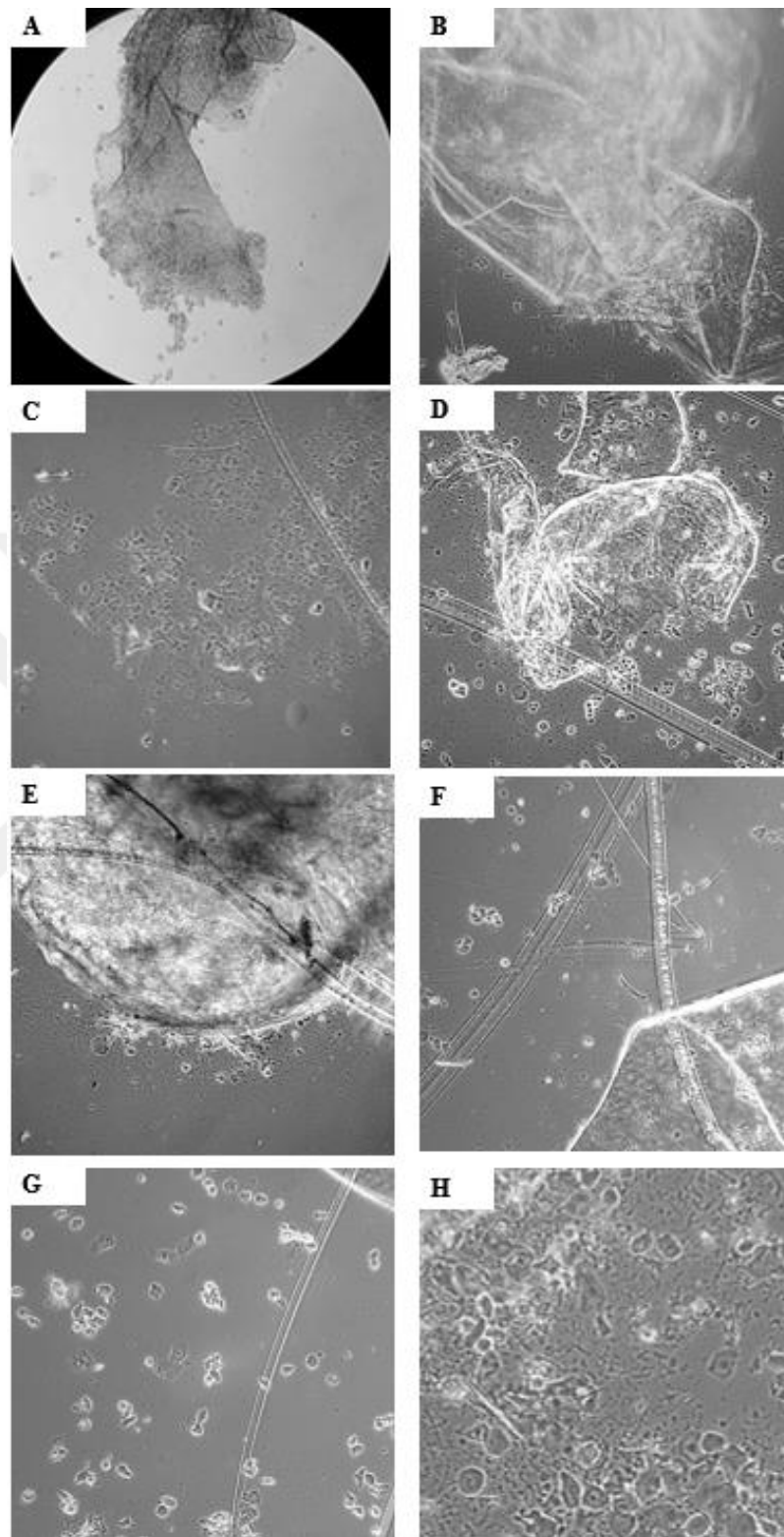


Figure 16. Morphologies of CEnCs isolated using the explant culture technique. (A) CEnCs isolated from Cornea #1, (B) Cornea #2, (C) Cornea #4, (D) Cornea #5, (E)

Cornea #6, (F) Cornea #7, (G) Cornea #8, and (H) Cornea #10 after 2 weeks of culture (10X magnification).

Collagenase, dispase, and trypsin enzymes are commonly used for the enzymatic isolation of CEnCs [129]. In this study, the tissue strips collected for enzymatic isolation were incubated in a 0.25% trypsin solution. However, no cell outgrowth was observed. Li et al. successfully isolated CEnCs by incubating tissue strips with 0.25% trypsin for 3 hours [129]. However, this study did not perform extended enzymatic isolation because of concerns that prolonged enzymatic isolation could damage cell viability. For Cornea #9, the collected tissue strips were stabilized by overnight incubation in Opti-MEM supplemented with 8% FBS. Following stabilization, the tissue was washed with HBSS and incubated with 0.02% EDTA to disrupt intercellular junctions. Despite these steps, no cell isolation was achieved from the cultured sample. Joyce et al. successfully used the same procedure to isolate CEnCs [100a]. The donor cornea used in this study had a cell density of 4288 cells/mm² and the donor was 57 years old, indicating that the negative result was not because of donor cornea cell density. However, since no additional information about the donor's health was available, conditions such as diabetes may have affected CEnC health and consequently the success of CEnC isolation.

3.2.4. Limbal Cells

In the limbal tissue, there are two types of stem cells. LESC are present in the limbal epithelial region, and these cells differentiate into CECs, thereby facilitating the regeneration of the corneal epithelium. In the limbal stroma, there are CSSCs that possess MSC characteristics. Because of their MSC characteristics, CSSCs have the potential to differentiate into various cell types, including KCs [130]. The studies conducted to isolate LESC and CSSCs within the scope of this thesis are detailed below.

Isolation of LESC

For the isolation of LESC, the technique applied by Robertson et al. was used ^[131]. Limbal epithelial tissue strips collected from the tissue were isolated using 1.2 U/mL dispase enzyme and the cells were cultured. The cells, which were immortalized at passage 0, were subcultured to continue the culture process. Cells cultured for 15 days at passage 0 proliferated densely on the surface (**Figure 17-A**). One week after SV40 immortalization, the cells were subcultured to passage 1, but the cells lost their viability (**Figure 17 B-C**). This indicates that the primary LESC were not immortalized. In the applied method, cells that fail to become immortalized die and separate from the immortalized cell population. This result suggests that modifying the immortalization technique could resolve the issue. In the study by Robertson et al., the isolated cells were immortalized using a retroviral vector encoding human telomerase reverse transcriptase (hTERT). The hTERT technique activates telomerase, preventing telomere erosion. Immortalization of human cells with hTERT ensures genetic stability while preventing telomere-dependent senescence ^[131]. Based on this information, it can be suggested that using the hTERT technique for LESC immortalization in this thesis study could have impacted the results obtained.

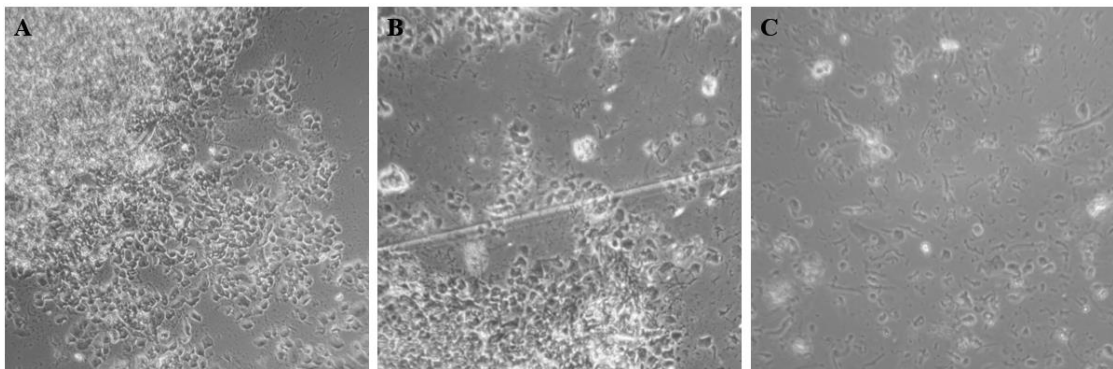


Figure 17. Morphological appearances of LESC cells isolated from Cornea 7 via enzymatic culture: (A) at passage 0 after 2 weeks of culture, (B) at passage 0 one week after immortalization, and (C) at passage 1 after one week of culture with immortalized cells (10X magnification).

LESC isolation was attempted from Cornea #8 using the explant culture technique. The culture process was carried out by placing limbal tissue fragments on the culture plate with the limbal epithelial layer facing upwards, as described in the method outlined by Kim et al. ^[105]. Cells that began to proliferate at passage 0 lost their viability when subcultured to passage 1 (**Figure 18**). Cornea #8 was obtained from a 69-year-old donor, and the endothelial cell count was zero upon receipt from the eye bank, indicating a potential issue with the donor's eye health. The negative outcome observed could be attributed to the poor health of the donor cornea.

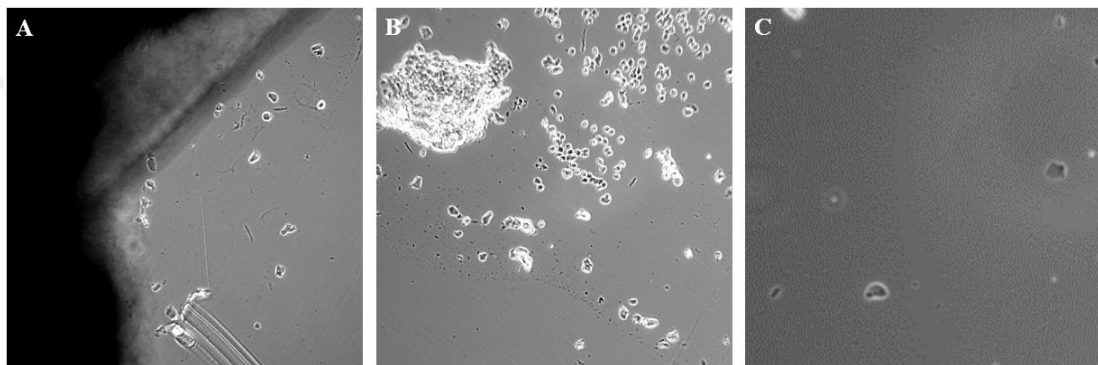


Figure 18. Morphologies of LSCs isolated from Cornea 8 via explant culture: (A) after 1 week of culture at passage 0, (B) after 2 weeks of culture at passage 0, and (C) after 1 week of culture at passage 1 (10X magnification).

Isolation of CSSC

CSSC isolation was attempted using both explant and enzymatic culture techniques. The explant culture technique was applied to Cornea #1, #4, #5, and #6, resulting in cell outgrowth from each tissue (**Figure 19**). After proliferation on the culture plate surface, cells were subcultured. However, cells isolated from Corneas #1, #4, and #5 lost their proliferation capacity during the subculture process. Cells isolated from Cornea #6 maintained their proliferation ability upon subculture, yielding cells with MSC-like, fibroblast-like morphology (**Figure 19-E**). Corneas #1 (from a 74-year-old donor with 1400 endothelial cells/mm²), #4 (from a 55-year-old donor with 1300 endothelial

cells/mm²), and #5 and #6 (both from a 72-year-old donor with 1400 endothelial cells/mm²) were obtained. Based on this information, the negative outcome is not caused by donor age. Therefore, it can be inferred that the initial endothelial cell count is associated with donor cornea health and affects the culture process.

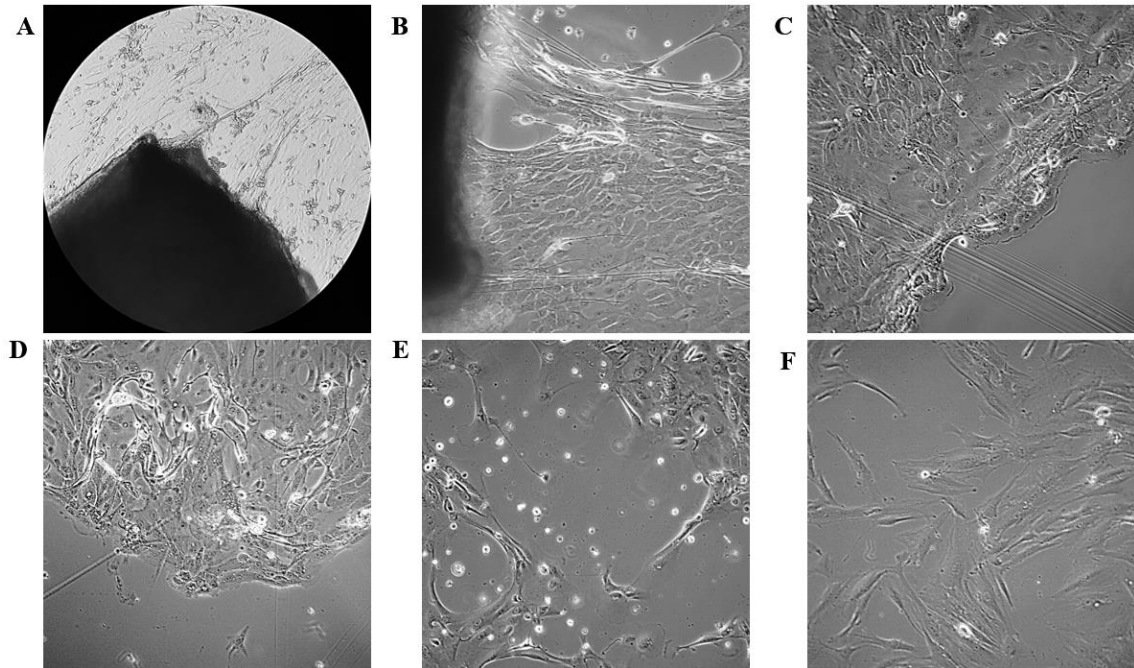


Figure 19. CSSC isolation via explant culture. Morphologies of CSSCs at passage 0 after 15 days of culture: (A) Cornea-1, (B) Cornea-4, (C) Cornea-5, (D) Cornea-6. Morphologies of CSSCs at passage 1: (E) Cornea-5 and (F) Cornea-6 (10X magnification).

Different enzyme concentrations and durations were tested for enzymatic isolation of CSSCs. In Cornea #2, enzymatic isolation was performed with a 4 mg/mL enzyme solution for 1 hour, while in Cornea #7, a 2 mg/mL enzyme solution was used for 1 hour. In Cornea #3, #9, and #10, enzymatic isolation was carried out with a 2 mg/mL enzyme solution for 30 minutes. CSSCs were obtained from all tissues subjected to isolation (**Figure 20**). However, it can be inferred from the results that Cornea #7 (0 CEnC count) and Cornea #3 (1000 CEnC count), which had low initial endothelial cell counts, yielded a small number of cells compared to other tissues.

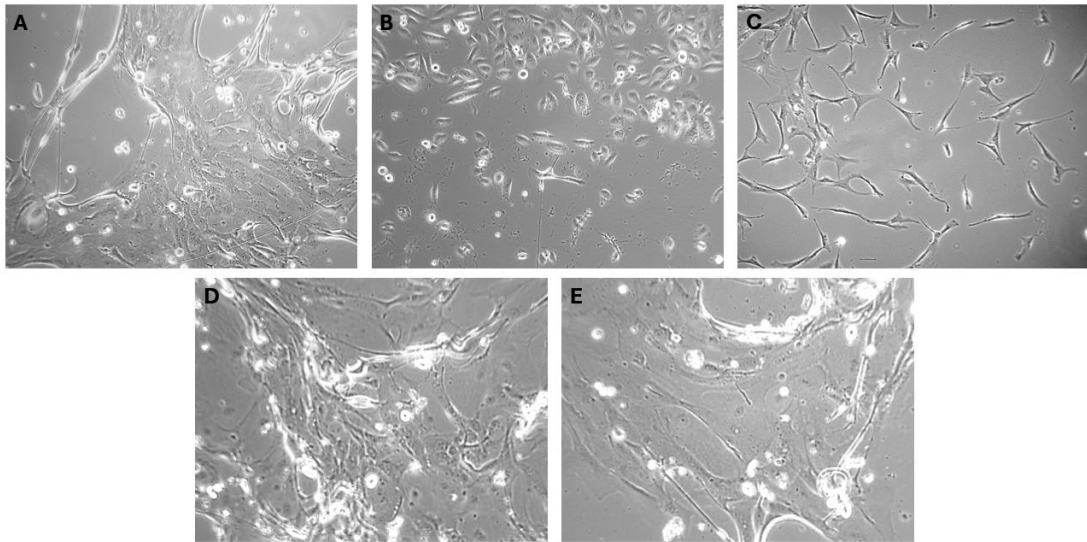


Figure 20. Morphologies of CSSCs isolated from Cornea-2 (A), Cornea-7 (B), Cornea-3 (C), Cornea-9 (D), and Cornea-10 (E) after 15 days of enzymatic culture (10X Magnification).

- *Immortalization of CSSCs*

The primary CSSCs obtained from enzymatic isolation of Cornea #10 were subcultured to passage 1. The SV40 immortalization technique was applied to these cells. The immortalized cells at passage 1 were further subcultured up to passage 14 (**Figure 21**).

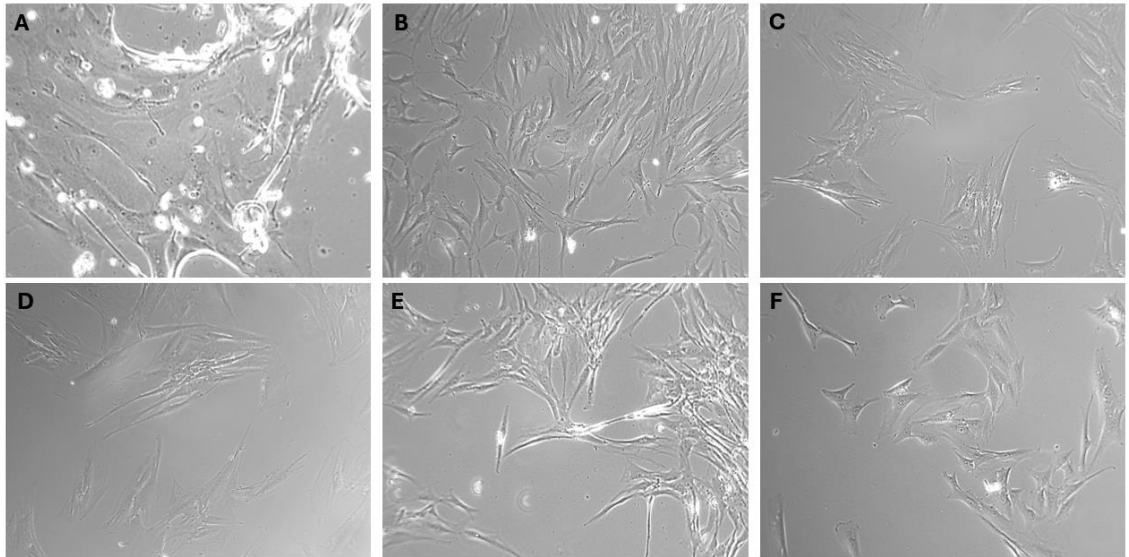


Figure 21. Morphologies of Cornea-10 CSSCs at primary passage 2 (A) and immortalized passages 2 (B), 7 (C), 9 (D), 12 (E), and 14 (F) (10X Magnification).

The isolated immortalized CSSCs were characterized using ICC and gene expression techniques for basic KC markers and stem cell markers. Based on the ICC results, it was observed that immortalized CSSCs exhibit dendritic morphology (**Figure 22**). CSSCs, being MSCs, typically display a fibroblast-like morphology ^[132]. The dendritic morphology observed in the tested cells in this study indicates their differentiation towards KCs. The expression of KC markers such as COL I and KERA in immortalized CSSCs further supports this conclusion (**Figure 22**). However, the presence of the stem cell marker vimentin suggests that differentiation is not complete. In immortalized passage 12, significantly lower Ki67 expression was observed compared to passage 9, where Ki67 is a proliferation marker ^[87]. Because of the eventual loss of proliferation capacity in later passages, the subculturing of immortalized CSSCs was sustained only up to passage 14. Immortalized cells typically enter crisis and cease proliferation after a certain number of subculture cycles ^[111].

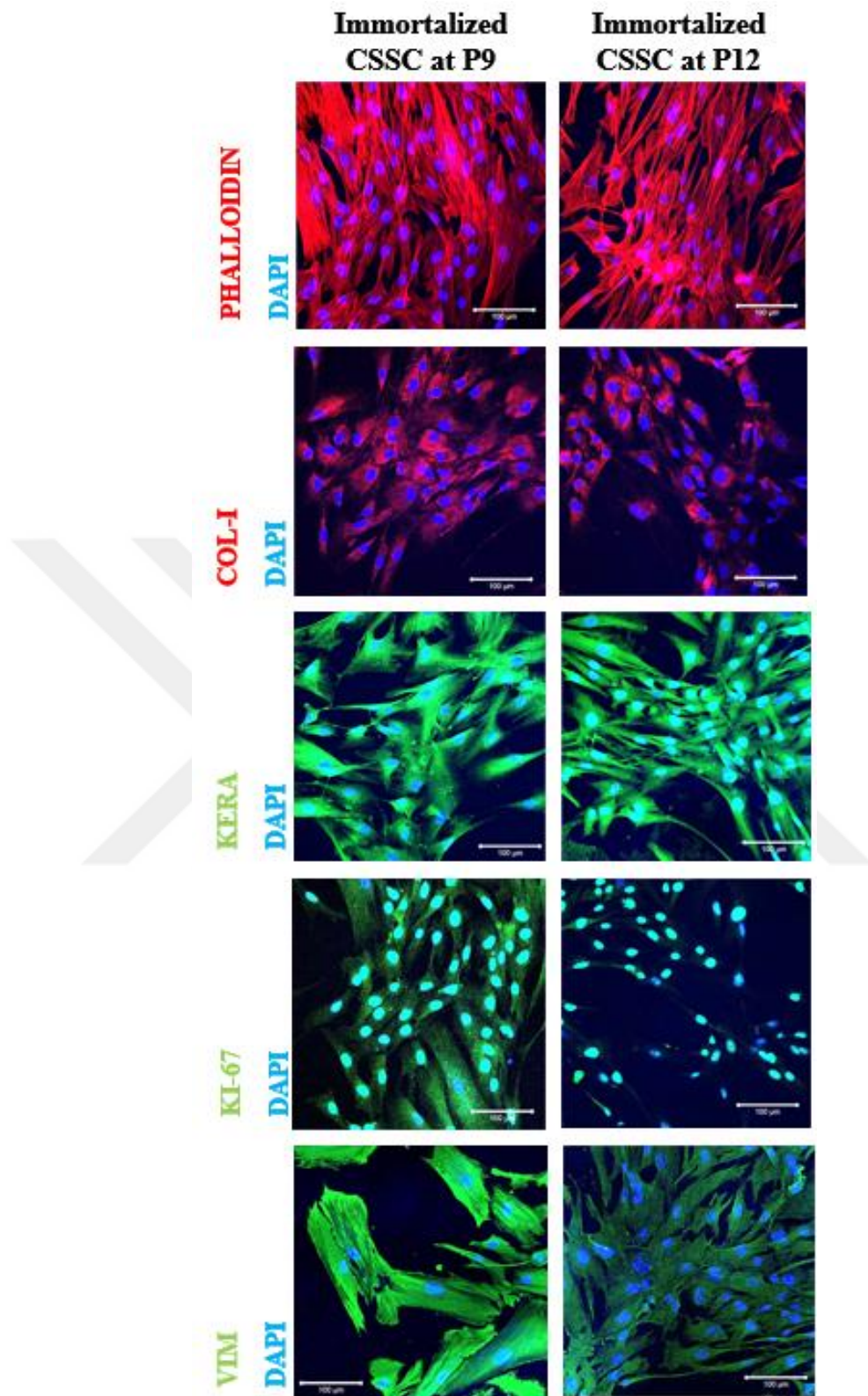
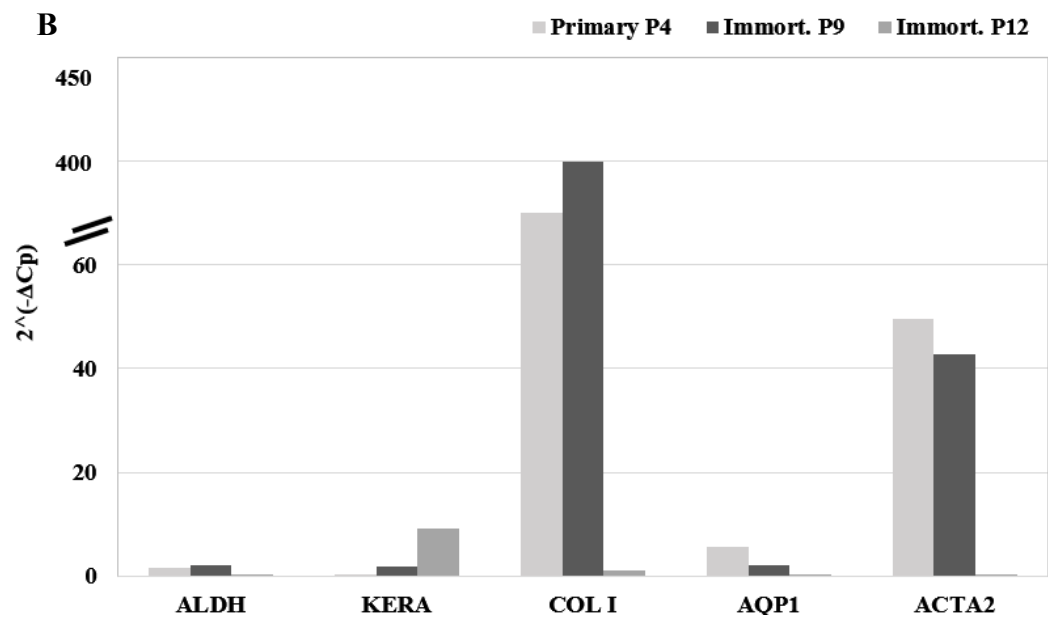
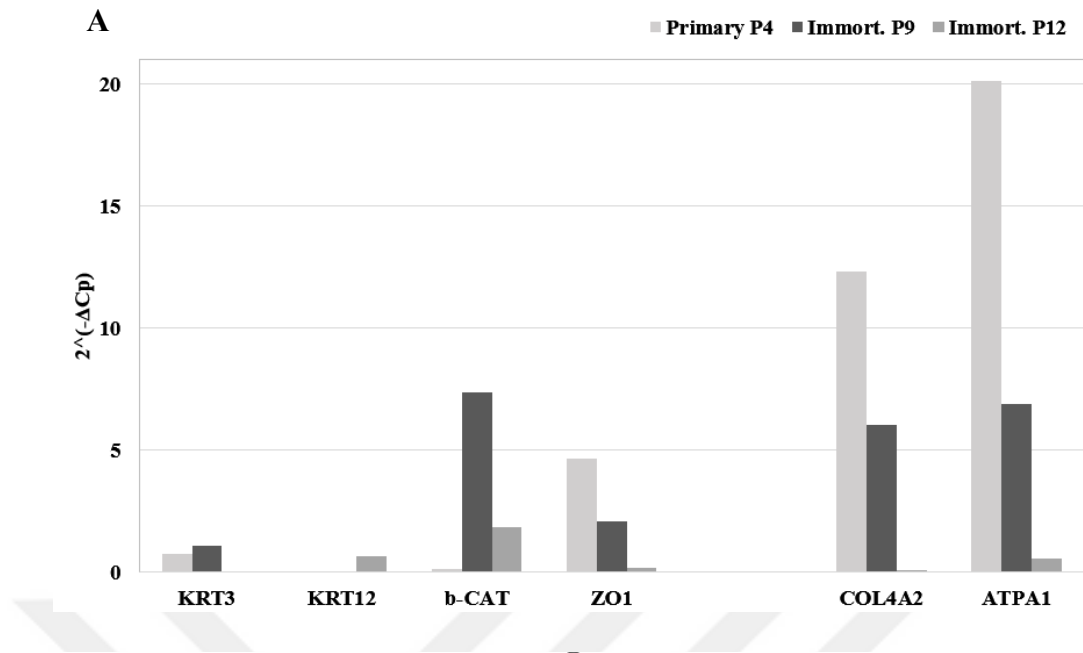


Figure 22. ICC results of immortalized KCs in passages 9 and 12.

For a comprehensive evaluation of the results obtained from ICC, gene expression studies were conducted using primary passage 4 CSSCs isolated from Cornea #9 as a reference (**Figure 23**). The expression of CEC markers was found to be negligible across all samples. The CEC-specific gene β -catenin had the highest expression, particularly in immortalized CSSC passage 9 (**Figure 23-A**). β -catenin is expressed in CECs as well as KCs, facilitating interaction between the epithelium and stroma layers ^[123]. Based on this finding, it can be inferred that immortalized passage 9 CSSCs possess the potential to differentiate into KCs. Furthermore, high CEC-specific genes COL4A2 and ATPA1 expression were observed in primary passage 4 and immortalized passage 9 CSSCs (**Figure 23-A**). COL4 is expressed by CECs and posterior KCs ^[124b], while ATPA1, which encodes Na-K ATPase, plays a role in stromal hydration balance and is expressed by CECs ^[133]. These results suggest that both primary passage 4 and immortalized passage 9 CSSCs retain the potential to differentiate into CECs and KCs.

When examining the expression profiles of key KC genes, ALDH, KERA, and AQP1 genes showed negligible expression levels (**Figure 23-B**). Both primary passage 4 and immortalized passage 9 CSSCs exhibited very high expression of COL I. Similarly, ACTA2, a marker for myofibroblasts, was also expressed but at lower levels than COL I. These results and the genes described above further confirm the potential of CSSCs to differentiate into KCs.

In the scope of the study, stem cell-specific genes were also tested, with the highest expression observed in the vimentin gene (**Figure 23-C**). Secondly, CD90, an MSC marker, showed high expression as well. The highest expression for these genes was observed in primary CSSCs, followed by immortalized passage 9, and the lowest expression in immortalized passage 12 CSSCs. Based on these findings, it can be stated that the CSSCs tested in the study possess the capacity to differentiate into other corneal cells and exhibit stem cell characteristics. Furthermore, other stem cell genes showed negligible expression levels. Evaluation of both primary and immortalized CSSCs revealed that they did not differentiate into KCs, as indicated by the insignificant expression of the CD34 gene. KCs are typically CD34⁺, whereas CSSCs were found to be CD34⁻. This aligns with findings that the isolated cells exhibited reduced proliferation capacity during subculturing, supported by the low Ki67 gene expression levels.



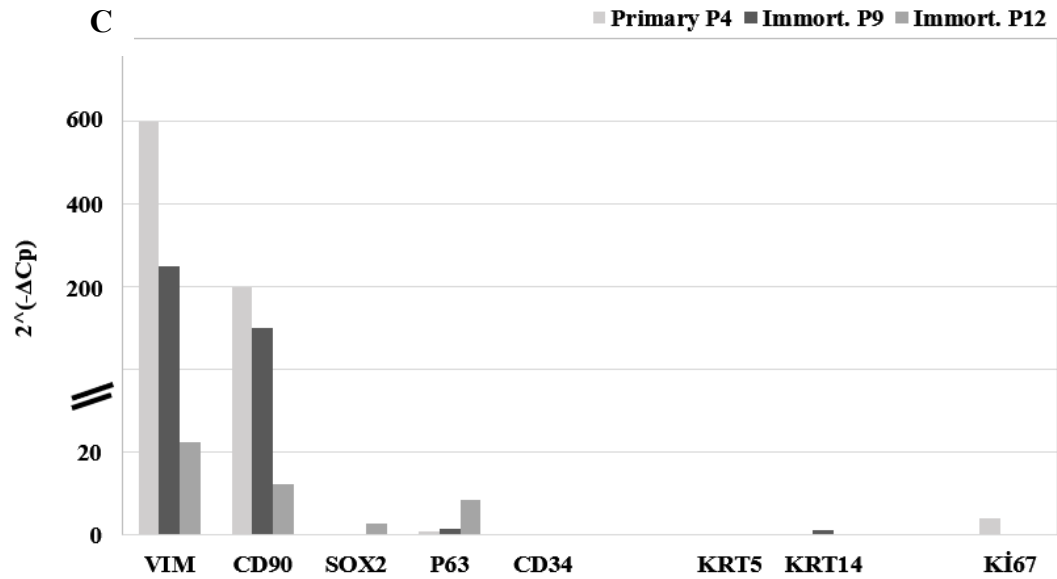


Figure 23. Gene expression results of primary passage 4, immortalized passages 9 and 12 CSSCs (n=1) (Relative Quantification, Mean $\pm$ SEM).

The results indicate that even after immortalization, CSSCs still hold the potential to be used as a source of stem cells for the cornea. The findings demonstrate that CSSCs have the capacity to differentiate into CEnC and KC.

- *Differentiation of CSSC to corneal cells*

CSSCs isolated by incubating in 2mg/mL enzyme solution for 30 minutes were cultured in serum-complemented media. Cells reaching passage 2 underwent gradual medium changes, resulting in three distinct cell groups cultured in different media: serum-supplemented, epithelial serum-free, and keratocyte serum-free media. The keratocyte serum-free medium, KSFM, supplemented with EGF, BPE, insulin, and hydrocortisone, is typically used for keratocyte cell culture ^[134]. However, in this study, CSSCs did not thrive and died in the keratocyte serum-free media. The epithelial serum-free media consisted of KSFM supplemented with EGF and BPE, supporting cell growth ^[135]. The difference between these two media lies in their insulin and hydrocortisone content.

Insulin is known to support KC proliferation ^[136], while hydrocortisone induces the release of specific growth factors in KCs ^[137]. Therefore, the negative outcome of CSSC culture in keratocyte serum-free media could be attributed to quantity-related factors.

Cell proliferation and subculturing were achieved in cultures conducted in serum-supplemented and epithelial serum-free media, and cells were characterized using the ICC technique (**Figure 24**). The results show that both cell groups exhibit basic CEC markers CK-3 and ZO-1 proteins. CSSCs cultured in epithelial serum-free media show higher CK-3 expression, which is consistent with this medium being used for CEC cultures and containing EGF ^[135]. This finding is thus consistent with the literature. While the main KC markers COL I and KERA are expressed in both groups, higher COL I expression is observed in cells cultured in serum-supplemented media. As observed in the previous section, CSSCs exhibit high COL I expression, indicating the potential for differentiation into KCs. Additionally, both cell groups show Ki67 expression, a proliferation marker, and display dendritic cell morphology.

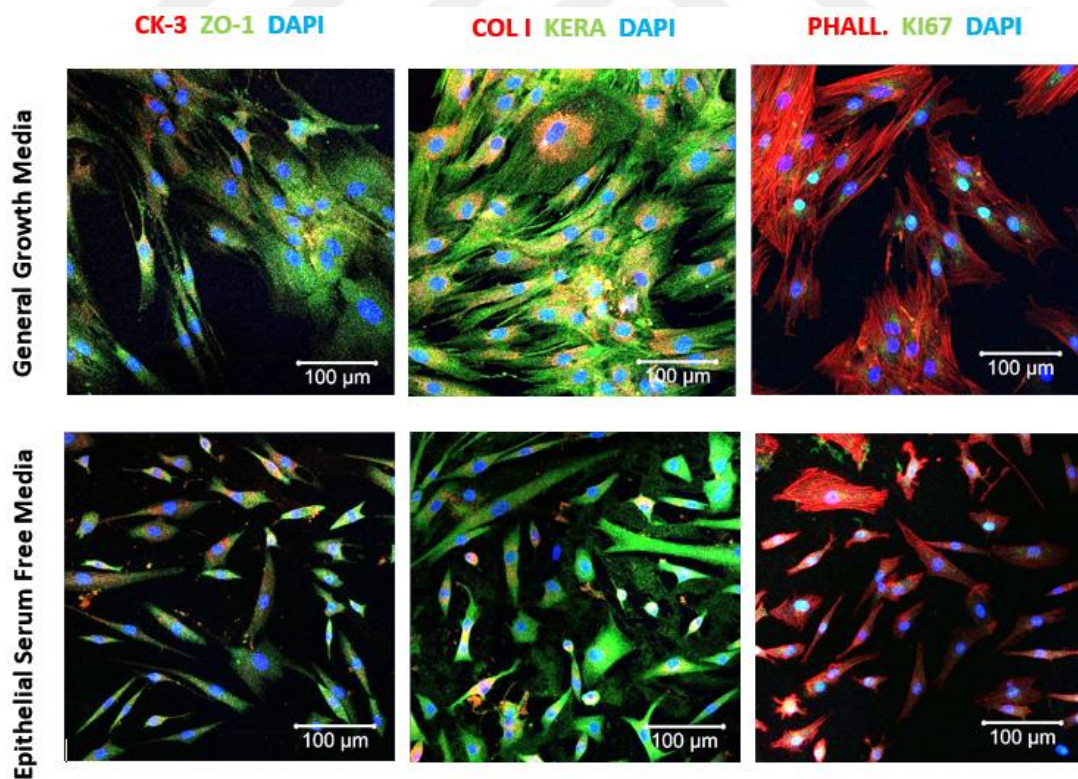


Figure 24. ICC results of CSSCs cultured in serum supplemented and epithelial serum free medias.

- *The effect of tissue transfer on CSSC morphology*

Cornea #4's limbal tissue was subjected to explant culture, where cell outgrowth was observed. Cell outgrowth was observed again after transferring the tissue to a new surface. To assess the potential for obtaining a high cell yield from a single tissue, limbal tissues with cell outgrowth were transferred three times, and the morphology of the resulting cells was evaluated. It was observed that as the tissue piece was transferred, it diminished in size, and the emerged cells underwent morphological changes, ultimately compromising cell viability (**Figure 25**). Based on these findings, it can be concluded that transferring tissue with cell outgrowth to a new culture plate is not suitable for achieving a high cell yield.

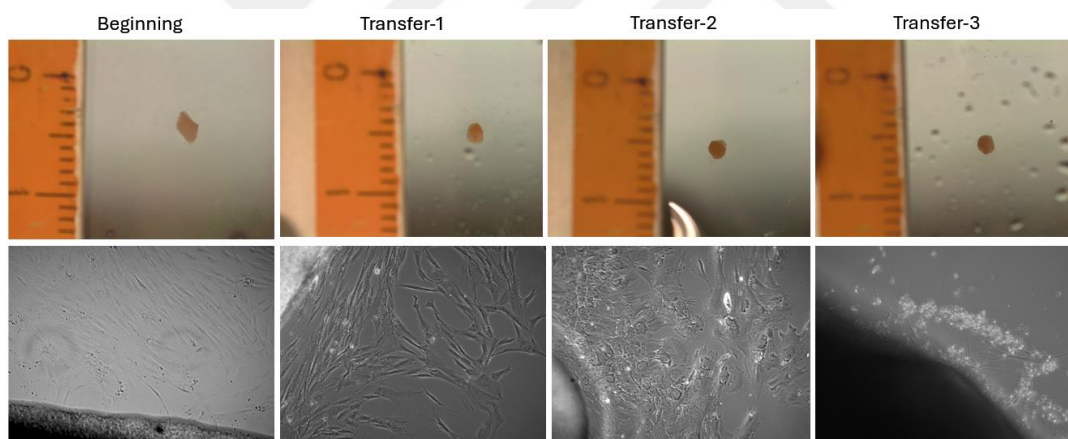


Figure 25. The effect of the tissue transfers on CSSC morphology (10X Magnification).

3.3. Creation of the Cell Stocks and Characterization of Cell Line Corneal Cells

3.3.1. hCEC

CECs are located on the outermost surface of the cornea and are in contact with tear fluid and air. Based on this information, studies were conducted within the scope of this research to observe the mechanobiological effects of the air-liquid interface (ALI) condition. Characterization studies were performed in four different experimental groups: control and ALI, both in complete and serum-free media (**Figure 26**).

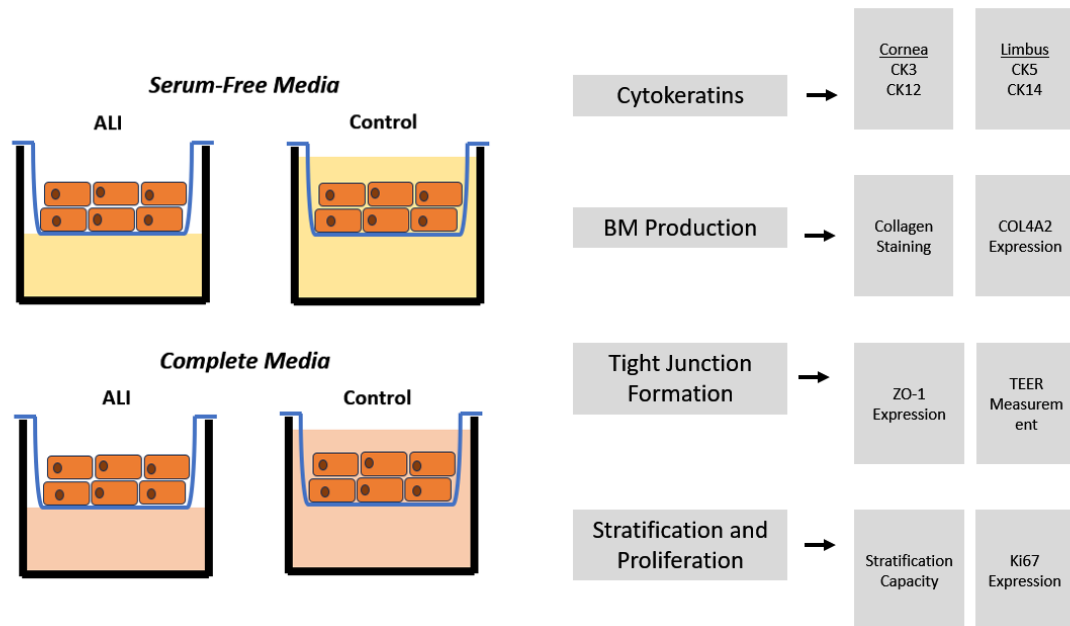
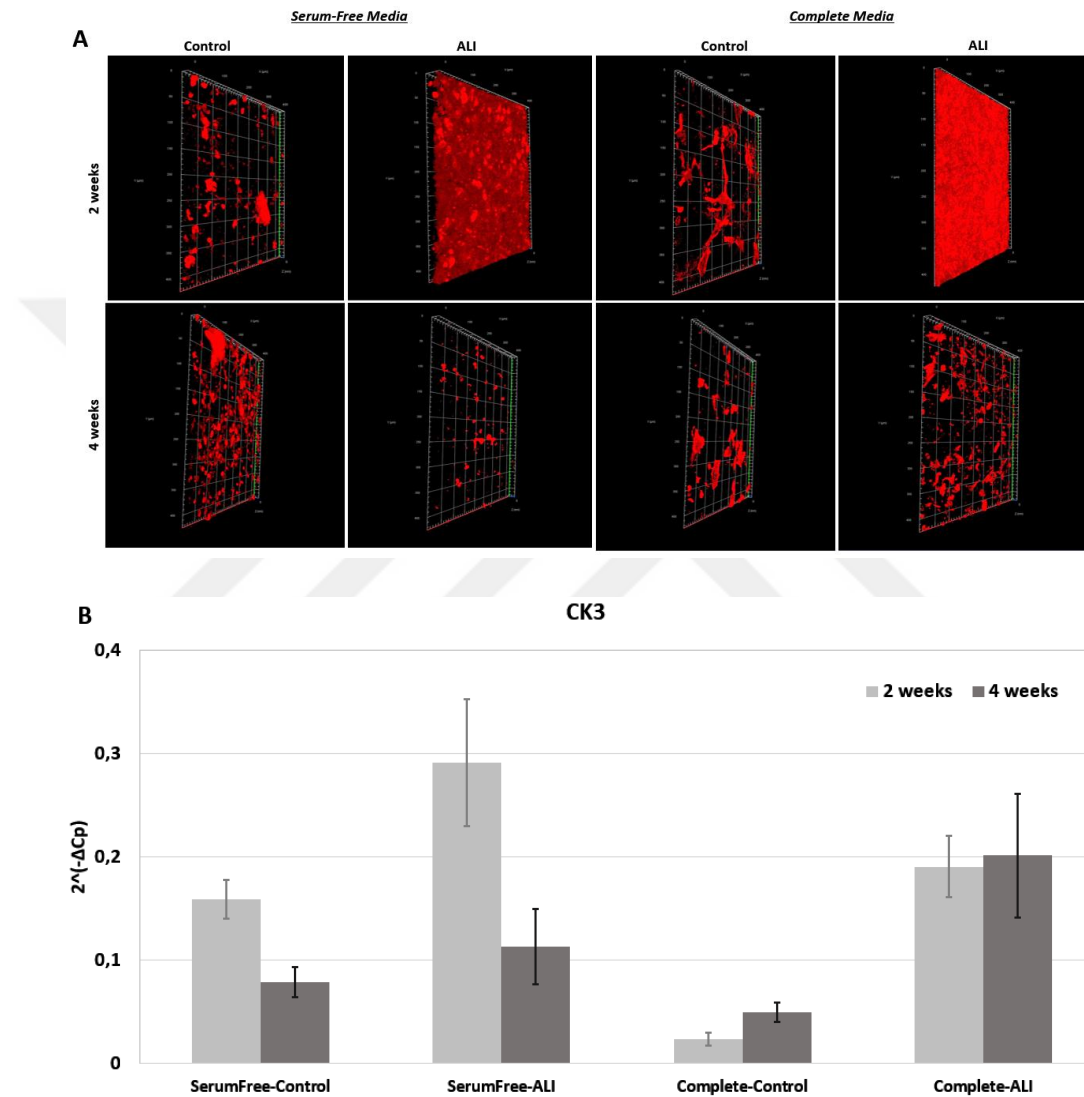


Figure 26. Summary of the study: Investigations were conducted to characterize the fundamental properties of human corneal epithelial cells (hCECs) cultured under control and ALI conditions in both serum-free and complete media.

After 2 weeks of culture, the CK expression of cell groups was evaluated, revealing that CK-3 expression was significantly higher under ALI conditions (**Figure 27-A**). However, this result showed a drastic decrease during the 4-week culture period. When examining the gene expressions of CK-3 and -12, specific to CECs, it was observed that expressions increased under ALI conditions in both media (**Figure 27 B-C**). Regarding the expression of CK-14, a LESC marker, it was found that all cell groups exhibited extremely high CK-14 expression (**Figure 27-D**). The isolation process involved collecting limbal and corneal epithelium for culture ^[131, 138]. Thus, the high CK-14 expression observed might be due to the remaining LSCs in the culture. Additionally, results indicated that under

ALI conditions in complete media, CK-14 expression decreased over the 4-week culture period, while CK-3 and -12 expressions increased. According to the literature, isolated and immortalized CECs show lower CK expression than natural corneal tissue and primary CECs [131, 139].



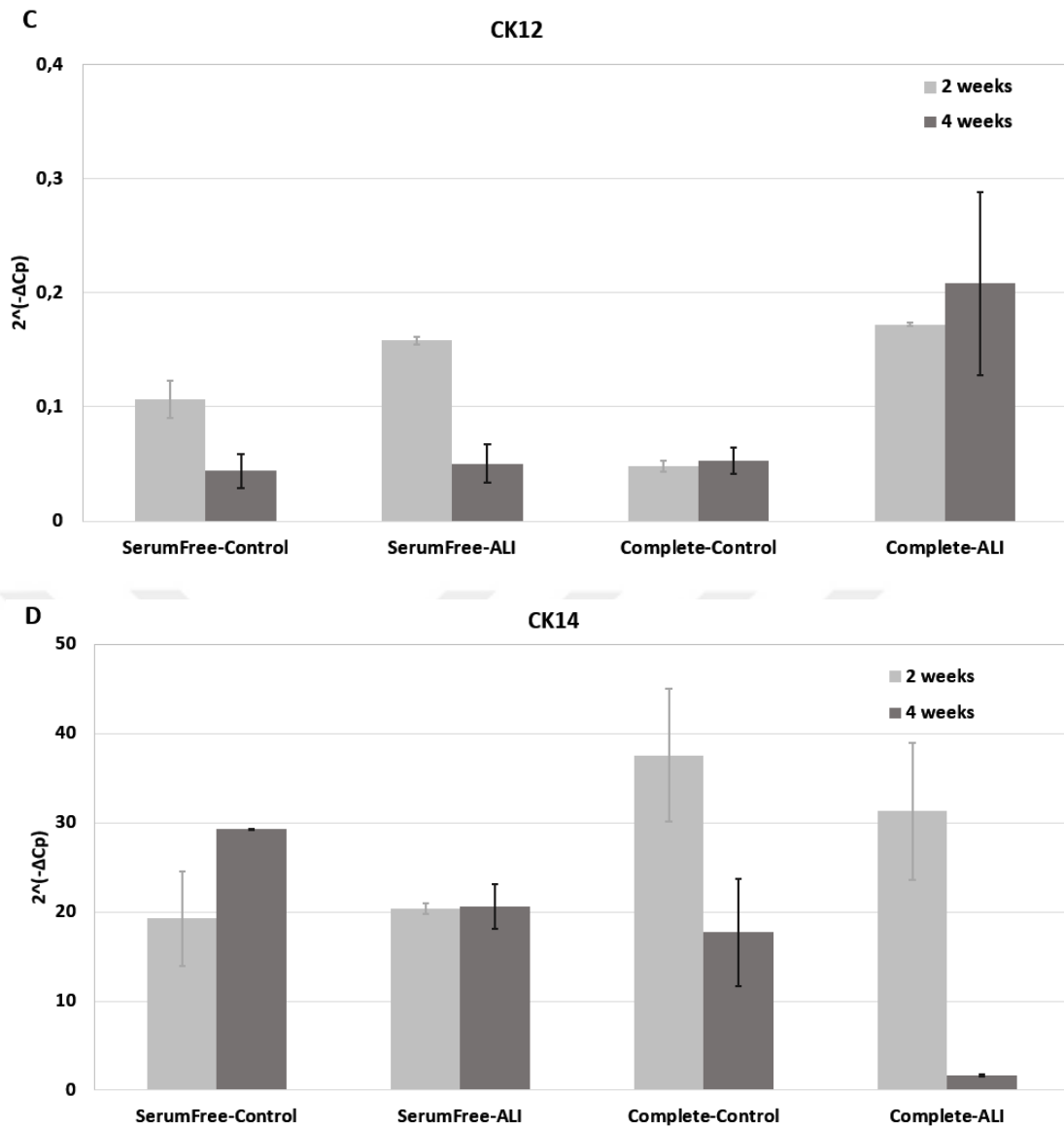
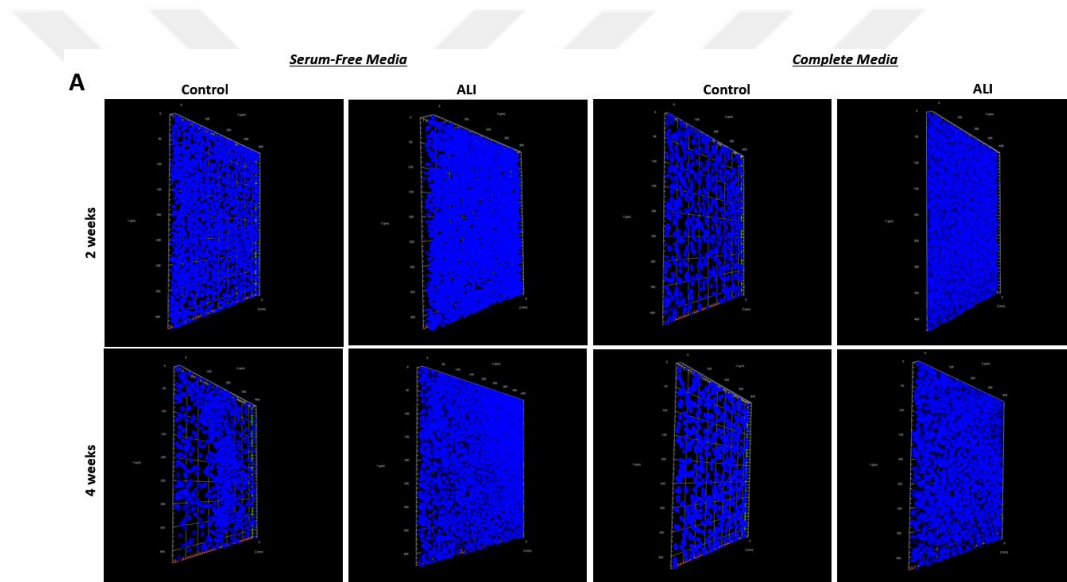


Figure 27. Evaluation of CK expression during the 2 and 4-week culture periods. (A) Z-stack images obtained from ICC assay (Red: CK3, x and y axis: 400 μ m). (B) CK3, (C) CK12, (D) CK14 gene expression profiles (n=2) (Relative Quantification, Mean $\pm$ SEM).

In natural tissue, CECs form a stratified cell layer of 5-7 layers with a thickness of approximately 36 μ m^[5, 140]. In this study, ICC was performed to obtain z-stack images and determine the stratification potential of the cell groups. To evaluate the stratification potential of the cell groups, thickness values were determined (**Figure 28**). The highest stratification was achieved under ALI conditions in serum-free media after 2 weeks of culture, measuring 32.25 μ m (**Figure 28-B**). When the culture period was extended to 4 weeks, the highest stratification was again observed in this sample, with a thickness of

15.3 μm . Various studies exist in the literature regarding the ALI culture of CECs. In a study by Robertson et al., CECs were cultured under submerged and ALI conditions and compared with natural cornea. The results indicated that while natural cornea has a 5-layer CEC structure, only a single layer was achieved under submerged culture and a 2-layer stratification was obtained under ALI culture ^[131]. In the study by Postnikoff et al., a 3–5-layer stratification was obtained for CECs cultured under ALI conditions in complete media ^[141]. The media used for CEC stratification or differentiation in the literature was employed as complete media in this study ^[141-142]. The results of this study indicate that the maximum stratification was achieved under ALI conditions in serum-free media after 2 weeks of culture.



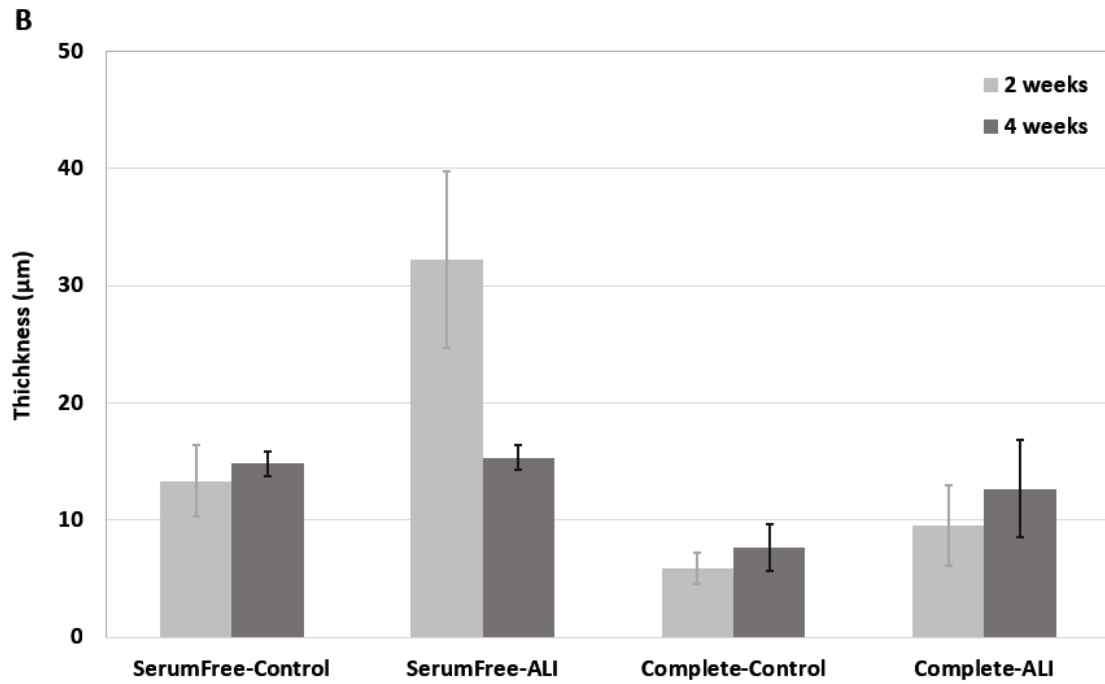


Figure 28. Stratification capacities of hCECs cultured under control and ALI conditions in two different media after 4 and 2 weeks of culture. (A) Z-stack images obtained from ICC application of DAPI-stained samples (Blue: nucleus). (B) Thickness data obtained from measurements of z-stack images of cell groups (n=6).

Another parameter evaluated in this study is proliferation capacity (**Figure 29**). Ki67 is a nuclear protein found in the nuclei of proliferating cells. Ki67 expression is not observed in non-proliferative cells or cells in the early G1 phase ^[143]. In this study, the maximum Ki67 gene expression was obtained under control conditions in complete media after 4 weeks of culture (**Figure 29-B**). The second highest expression was observed under control conditions in serum-free media after 4 weeks of culture. There are limited studies in the literature on the proliferation of stratified CECs. In the study by Silva et al., epidermal keratinocyte cells were cultured in serum-free media and then cultured in serum-containing media under ALI conditions on a collagen I matrix. The results showed that stratified epithelial cells exhibited higher Ki67 expression compared to natural tissue ^[144]. This study demonstrates that serum is an effective parameter in increasing proliferation capacity.

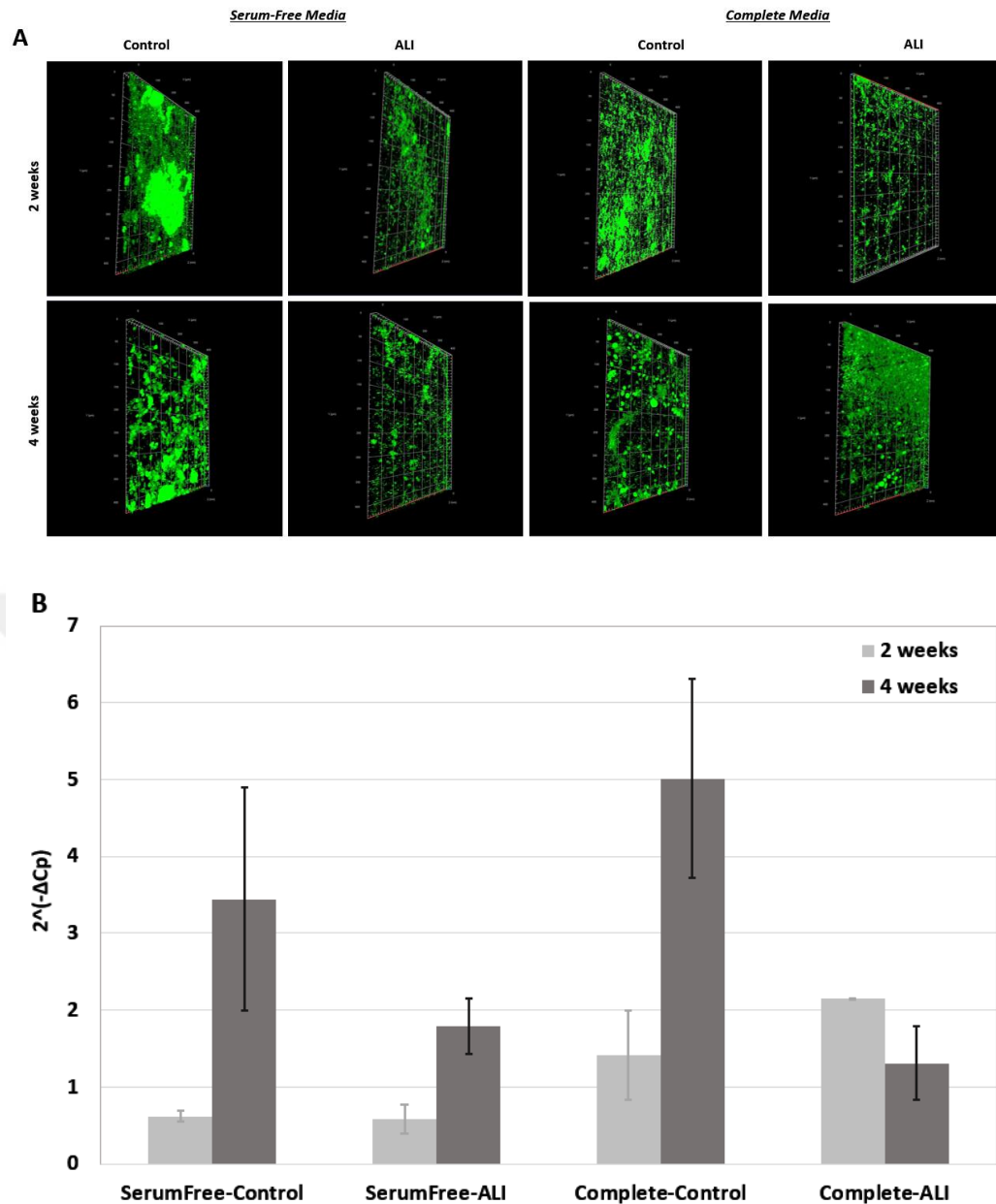
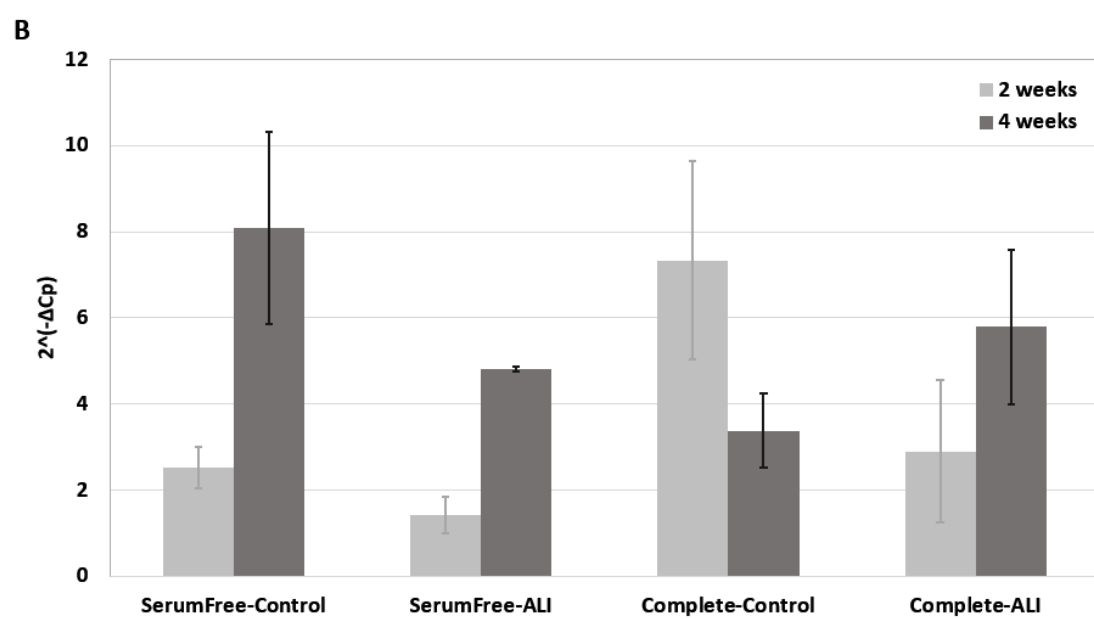
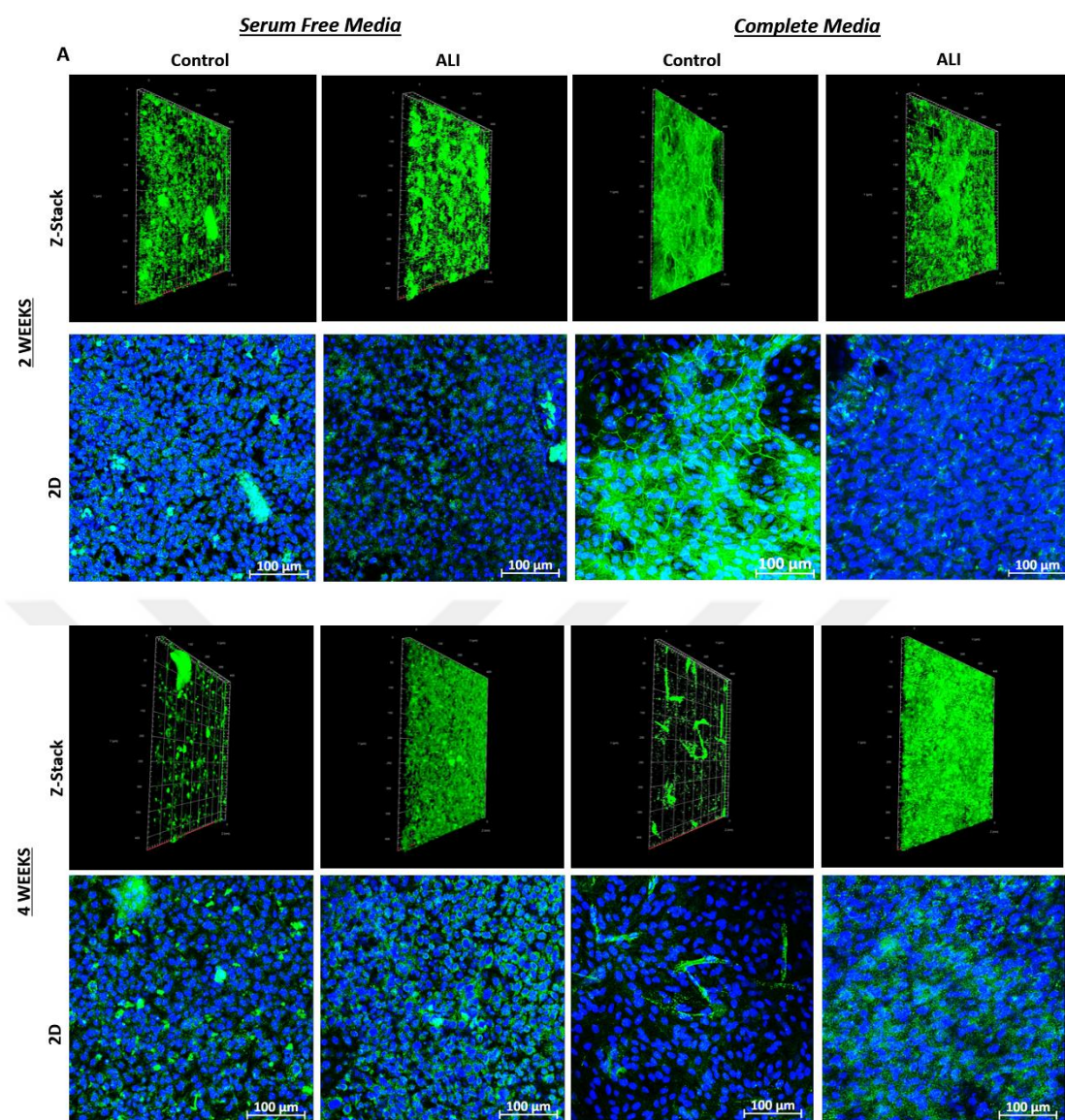


Figure 29. Ki67 proliferation marker results for hCECs cultured under ALI and control conditions in serum-free and complete media for 2 and 4 weeks: (A) ICC z-stack images (green: Ki67) and (B) gene expression results (n=2) (Relative Quantification, Mean $\pm$ SEM).

In the corneal epithelium layer, tight junctions between cells ensure controlled diffusion of fluids, electrolytes, macromolecules, and water. Claudins, occludins, and ZO proteins are present between CECs. ZO-1 is a membrane-associated protein that binds both to occludins and claudins and to the actin cytoskeleton ^[145]. To determine the integrity of

tight junctions, another parameter evaluated in this study, ICC and gene expression assays for the ZO-1 tight junction protein were conducted for 2 and 4 weeks of culture. After 2 weeks of culture, it was observed that tight junction structures were present in the cell membranes of samples cultured under control conditions in complete media (**Figure 30-A**). This result is corroborated by gene expression data, with the highest expression observed in samples cultured under control conditions in complete media after 2 weeks (**Figure 30-B**). TEER measurements taken over the 2-week culture period showed that the highest resistance was obtained under ALI conditions in complete media (**Figure 30-C**). TEER measurement is a technique to determine the integrity of barriers formed by junctions between epithelial cells ^[146]. As the density of epithelial cells in culture increases, the number of tight junctions between them also increases, resulting in higher TEER values. After a certain period, the TEER value decreases and stabilizes as the stratification process stabilizes ^[147]. In this study, TEER measurements taken over the 2-week culture period showed that the highest resistance was obtained under control conditions in complete media (**Figure 30-C**). Based on these results, it can be said that the presence of serum positively affects tight junction integrity. Continuous resistance increase was observed in cells cultured under control conditions in complete media, reaching a maximum on the 6th day of culture ($338.6 \text{ } \Omega/\text{cm}^2$). This suggests that the culture process in serum-containing media under submerged conditions positively impacts tight junction integrity. In the study by Seeber et al., TEER values were determined by culturing CECs in different media and under ALI conditions ^[147b]. The results showed that the maximum TEER value was reached in serum-free media in 3 days ($600 \text{ } \Omega \times \text{cm}^2$) and in complete media in 9 days ($300 \text{ } \Omega \times \text{cm}^2$) ^[147b]. In this study, the maximum TEER value in the complete media control group was reached on the 7th-8th day of culture, after which the TEER value stabilized, and the stratification process began. The literature indicates that the maximum TEER value obtained from CECs with proper tight junction integrity is approximately $400 \text{ } \Omega \times \text{cm}^2$ ^[148]. Based on this result, it can be concluded that culturing stratified CECs in complete media under submerged conditions offers a significant advantage.



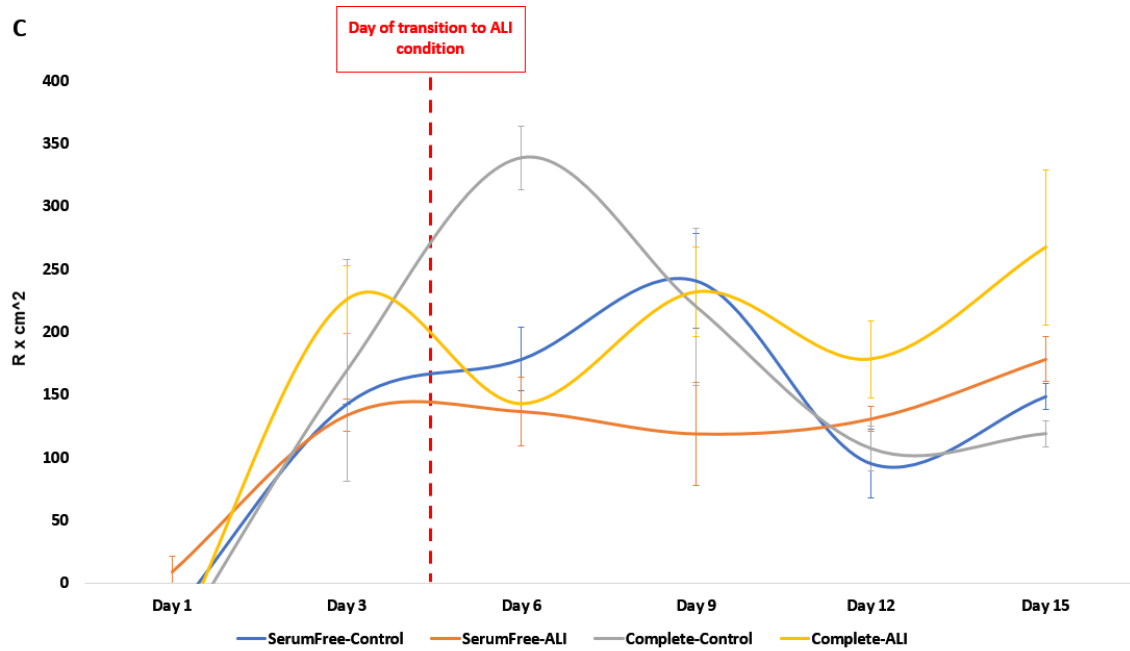


Figure 30. Tight junction integrity profiles of hCECs cultured under control and ALI conditions in serum-free and complete media for 2 and 4 weeks of culture: (A) ICC images (blue: nucleus, green: ZO-1), (B) ZO-1 gene expression profile (n=2) (Relative Quantification, Mean $\pm$ SEM), (C) TEER measurement results (n=3).

The capacity of hCECs cultured for 2 and 4 weeks to form BM was evaluated using collagen staining. BM contains collagens, laminins, heparan sulfate proteoglycans, and nidogens ^[149]. COL IV is a fundamental collagen type found in the BM ^[10, 150]. In healthy corneal BM, COL IV expression is higher compared to keratoconus cornea ^[151]. In this study, it was observed that cells produced collagen during the 4-week culture period in the tested cell groups (**Figure 31 A-B**). Samples cultured under control conditions in complete media for 4 weeks exhibited maximum COL IV expression and homogeneous distribution of collagen (**Figure 31-C**). The presence of a homogeneous collagen-containing BM layer is crucial for maintaining corneal permeability and regeneration capacity ^[149, 152]. Based on this information, it can be concluded that submerged culture of CECs in complete media is suitable for BM production in studies related to corneal tissue engineering.

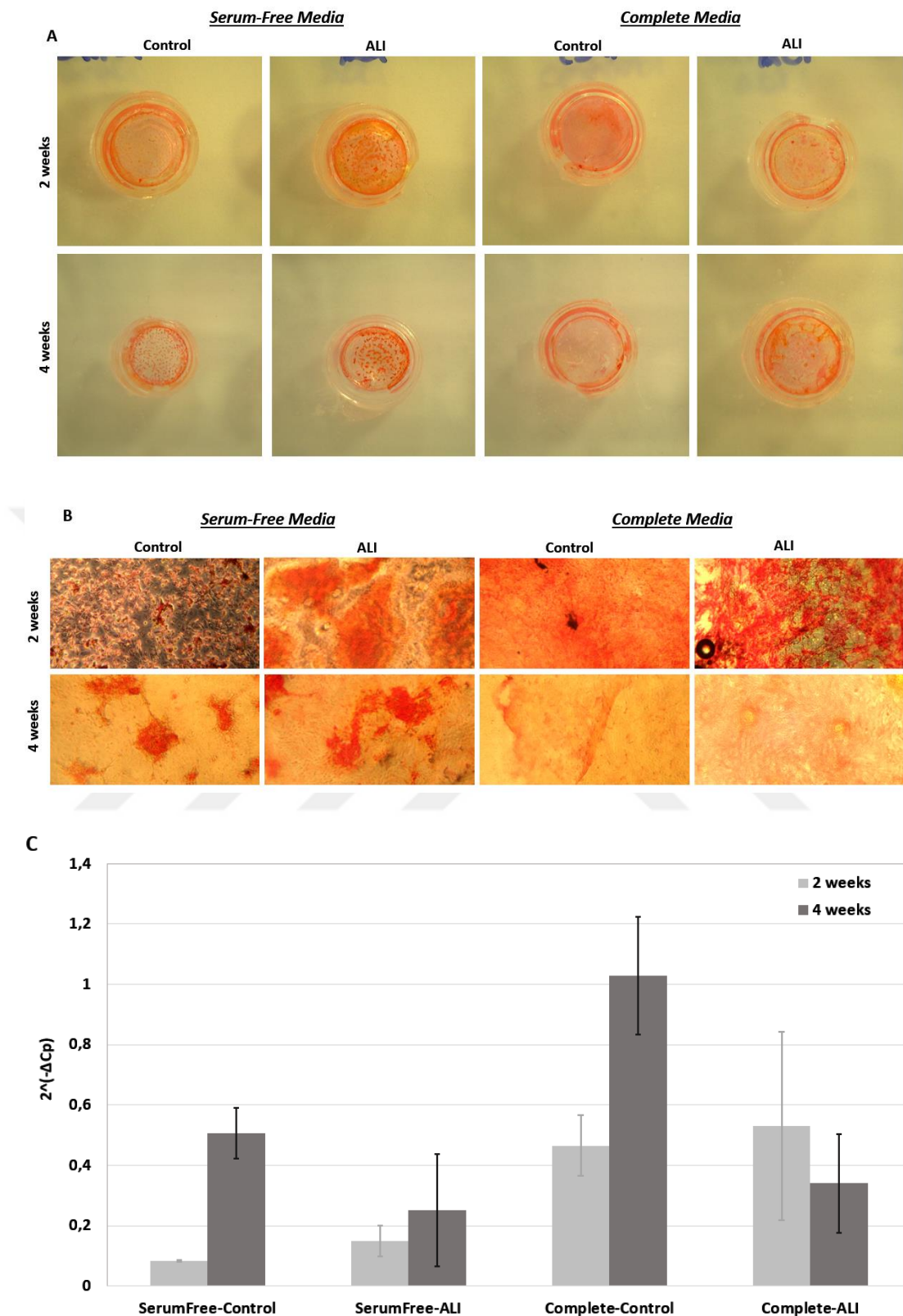


Figure 31. The BM production capacity of hCECs was assessed using collagen-stained samples. Photographs of collagen-stained samples (A) and microscope images (10X Magnification) (B) are shown. Gene expression results for COL4A2 (C) are presented (n=2) (Relative Quantification, Mean ± SEM).

Based on the results, it can be summarized that culturing hCECs for 2 weeks under serum-free media conditions in an ALI setup is suitable for obtaining a stratified hCEC layer. This approach achieves a thickness very close to that of the natural corneal epithelium layer. The use of serum in the culture medium enhances tight junction integrity and BM production capacity (**Table 9**). Based on the obtained results, it can be said that in the culture process, starting with submerged culture and then transitioning to ALI condition, the advantages obtained from both cultures can be combined. This experimental work within the scope of this thesis will be conducted using tissue scaffolds.

Table 9. The summary of the ALI culture and media effect on the hCECs.

		Serum -Free Media - Contr ol	Serum -Free Media -ALI	Complete Media- Control	Complete Media- ALI	Description
CK3 – CK12	Differences in expression level from 2 to 4 weeks	↓	↓	↑	↑	Complete Media-ALI has maximum CK3 and CK12 expression after 4 weeks culture.
CK14	Differences in expression level from 2 to 4 weeks	↑	↑	↓	↓	Complete Media-ALI has minimum expression after 4 weeks culture.
Stratification	Differences in stratification level from 2 to 4 weeks	↑	↓	↑	↑	The maximum stratification thickness is obtained from the Serum-Free Media-ALI sample after 2 weeks culture period.
	The thickness of stratified HCECs after 2 weeks (µm)	13,3	32,25	5,8	9,5	
	The thickness of stratified HCECs after 4 weeks (µm)	14,8	15,3	7,6	12,6	

Proliferation	Differences in Ki67 expression level from 2 to 4 weeks	↑	↑	↑	↓	Complete Media-control has maximum Ki67 expression after 4 weeks culture period.
Tight Junction	Differences in ZO1 expression level from 2 to 4 weeks	↑	↑	↓	↑	Tight junction formation and integrity are observed on the Complete Media-Control sample after a 2-week culture period.
	Maximum TEER value	79,4 Ω at day 9	58,8 Ω on day 15	111,7 Ω at day 6	90,1 Ω on day 14	
BM formation	Differences in COL4A2 expression level from 2 to 4 weeks	↑	↑	↑	↓	Complete Media-Control has maximum COL4A2 expression after 4 weeks culture and homogenous collagen distribution.

3.3.2. hKC

ICC and gene expression studies were conducted to characterize hKC cell lines. In passage 2 hKC cells, expressions of key KC markers such as COL I, KERA, and ALDH were observed (**Figure 32**).

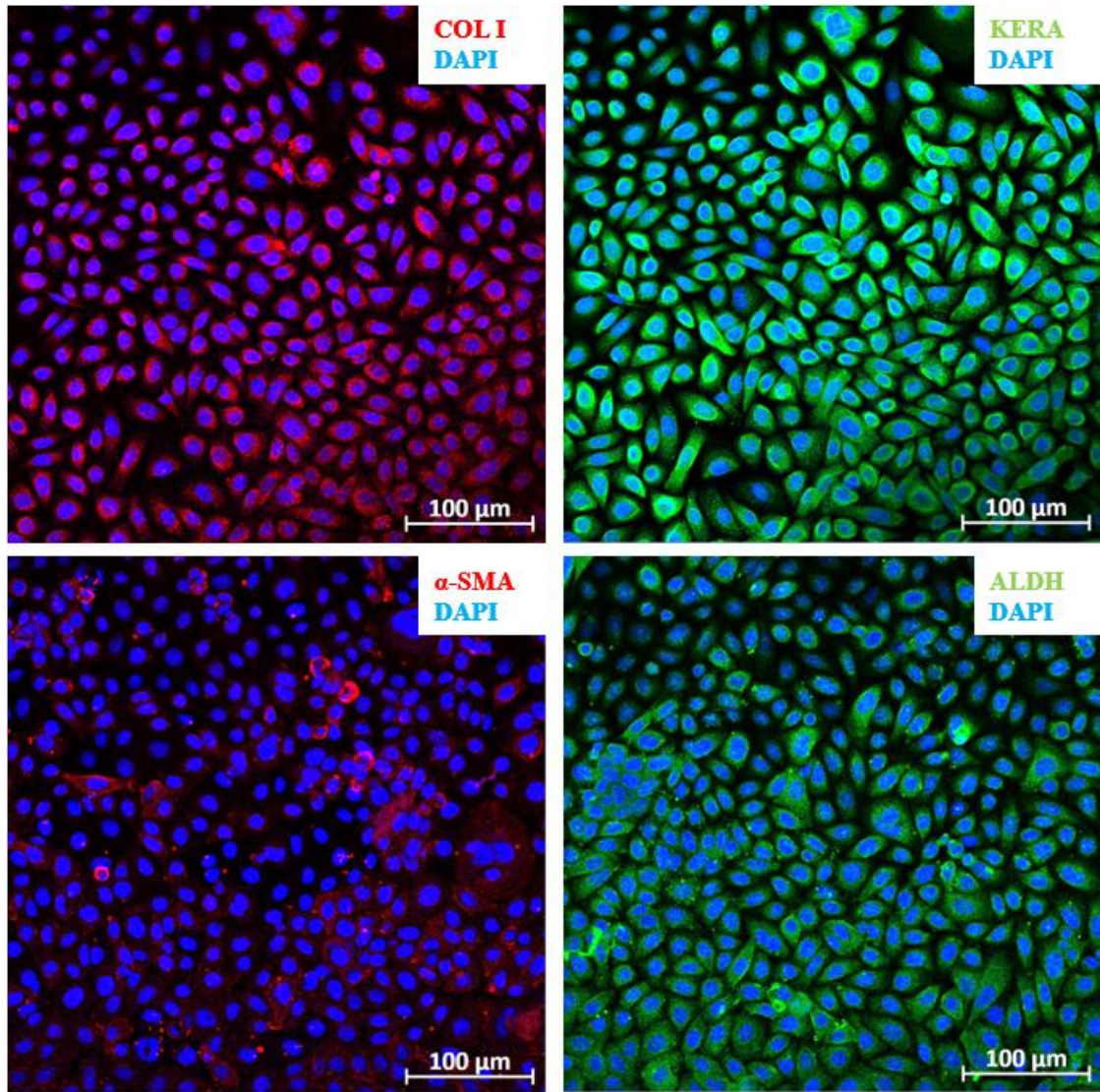
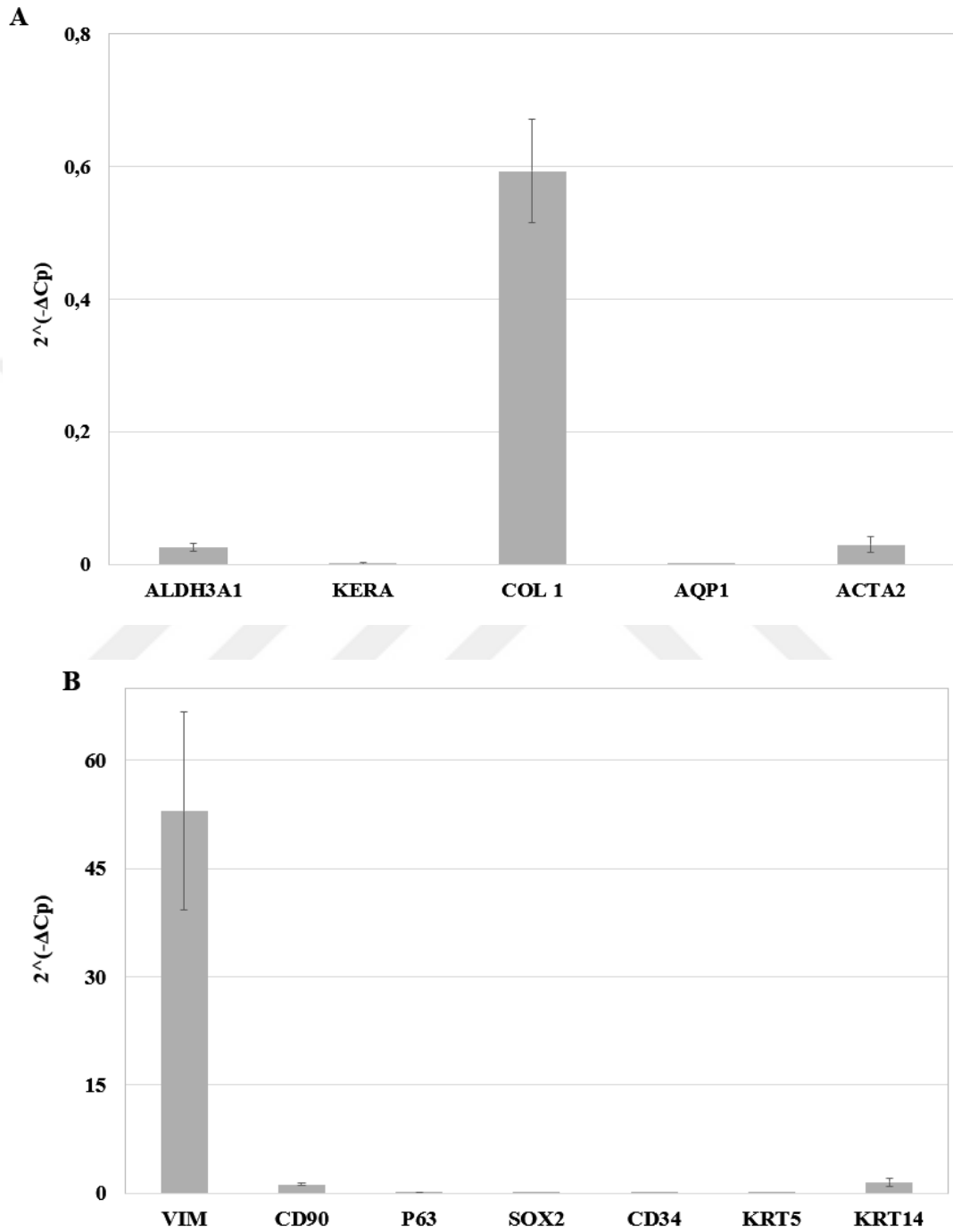


Figure 32. ICC results of hKCs at passage 2 using main KC and myofibroblast markers.

Gene expression studies were conducted in the characterization process of hKC cell line cells at passage 2 (**Figure 33**). Expression profiles of KC markers ALDH3A1, KERA, COL I, AQP1, and the myofibroblast marker ACTA2 were determined, with the highest expression observed in COL I (**Figure 33-A**). This high expression of COL I, a key marker of KCs, was expected for this cell group. Additionally, the highest expression was observed in vimentin (**Figure 33-B**). Vimentin, an intermediate filament type, provides mechanical support and maintains cell integrity ^[116]. Vimentin is a marker for MSCs ^[117], and high vimentin expression is observed in KCs and CSSCs ^[118]. High β -catenin expression was observed in hKC cells, consistent with the literature (**Figure 33-C**). The

interaction between CECs and KCs occurs through Wnt/ β -catenin signaling ^[123]. Another highly expressed gene, COL4A2, is expressed by posterior KCs ^[124b].



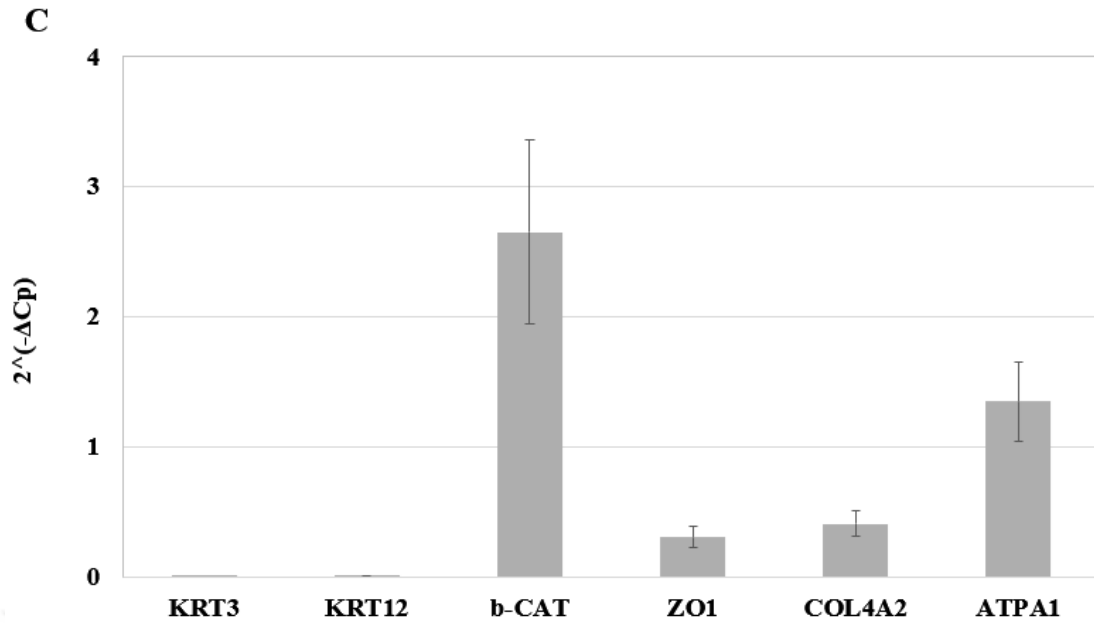


Figure 33. Gene expression results of hKC at passage 2 using main corneal and limbal marker genes (n=2) (Relative Quantification, Mean ± SEM).

Based on the results obtained, it can be concluded that the tested hKC cell line in this study is suitable for use in CTE studies.

3.3.3. hCEnC

For the ICC characterization of the hCEnC cell line, endothelial markers Na-K ATPase and ZO1, the proliferation marker Ki67, and the actin filament marker phalloidin were used (**Figure 34**). The results indicate that hCEnC cells express Na-K ATPase and ZO1 up to passage 20, showing high expression in later passages. Cells from different passages express the Ki67 proliferation protein while maintaining cell morphology.

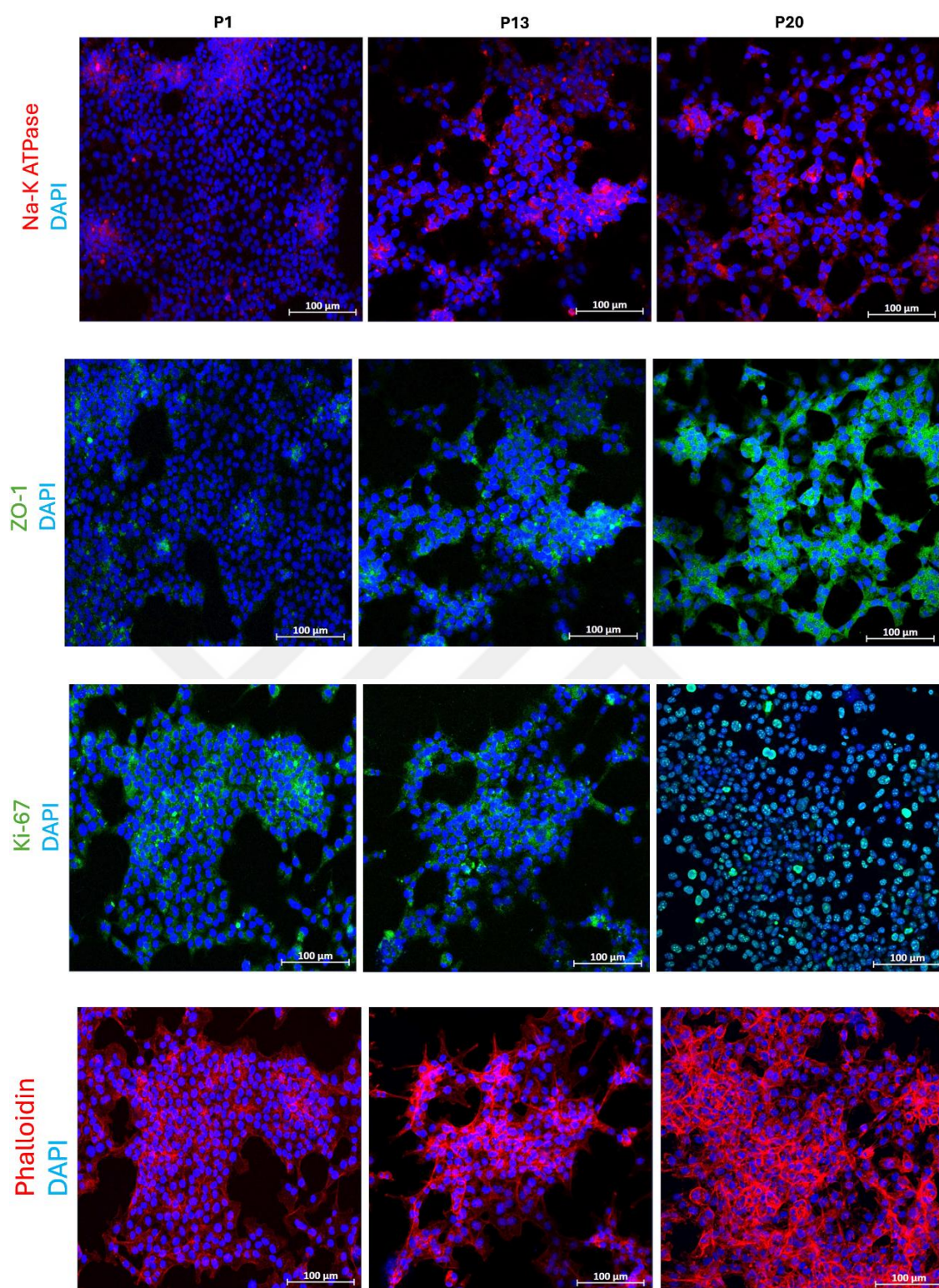


Figure 34. ICC results of hCEnCs at different passages using main endothelial markers, Na-K ATPase and ZO1, proliferation marker Ki67, and actin filament stain phalloidin.

Based on the results described above, it can be concluded that the hCEnC cell line exhibits endothelial characteristics and is suitable for use in CTE studies.

3.4. Co-culture of Corneal Cells

"Co-culture" in cell culture refers to the practice of cultivating different types of cells together in the same environment. This approach is utilized to investigate complex cellular interactions and to gain insights into specific cellular functions ^[153]. It is commonly employed to study communication, signaling pathways, and interactions between cells, allowing different cell types to grow together to mimic natural interactions. For example, one cell type may respond to substances secreted by another cell type ^[153]. Co-culture also supports cell differentiation and the formation of organoids, facilitating the development of more intricate and organ-like structures. This method enhances cell interactions, thereby replicating the architecture and function of specific tissues or organs. In immunological research, co-culturing different types of immune cells enhances our understanding of the immune system and interactions between immune cells. Co-culture techniques are valuable in tissue engineering and regenerative medicine for promoting specific cellular functions through targeted cell interactions. Widely used across various scientific disciplines in cell culture studies, co-culture serves as a crucial tool for comprehending intercellular interactions ^[153].

In the co-culture study, three types of corneal cells (hCEC, hKC, and HCEnC) were cultured both single and in co-culture. As previously explained, the cornea contains epithelial, keratocyte, and endothelial cells in different layers. The aim of this co-culture study was to elucidate the effects of corneal cells on each other. To this end, cells cultured under single culture conditions were compared with those cultured under co-culture conditions to determine the effects of co-culture (**Figure 35**). Initially, the study determined the most suitable medium for co-culture. Subsequently, the cells were characterized in co-culture and single-culture configurations in the selected medium.

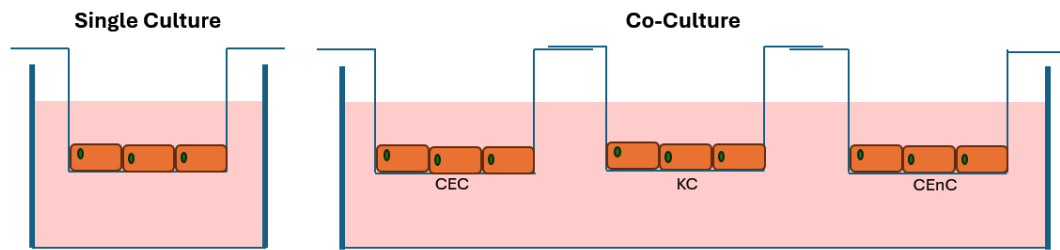


Figure 35. The culture set-up of the study. Single and co-culture were applied to determine the effect of co-culture on corneal cells.

3.4.1. Selection of Optimum Cell Culture Media for Co-culture of Corneal Cells

Corneal cell line cells were cultured in single- and co-culture setups using 7 different media formulations, as described in Table 5. After a 2-week culture period, an MTS viability assay was conducted to determine the optimal medium for co-culture (**Figure 35**).

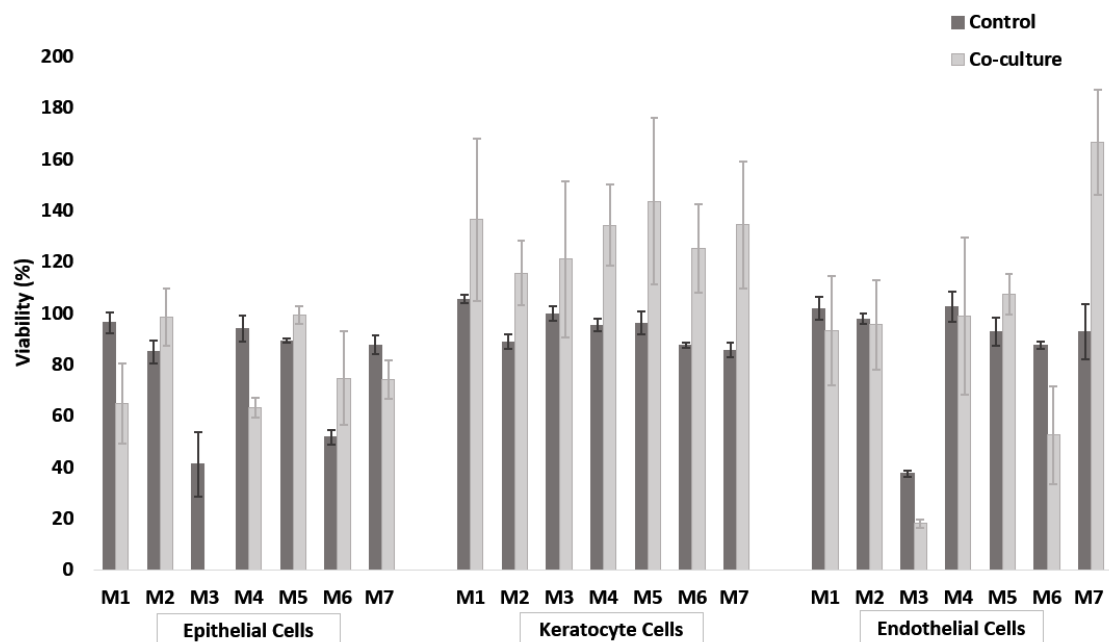


Figure 36. The graph illustrates the viability percentages of corneal cells cultured in different media. The dark gray bars indicate the control group, consisting of cells cultured

singly, while the light gray bars represent cells co-cultured. The media formulations tested include: M1: Keratinocyte Serum-Free Medium (KSFM), M2: Endothelial-SFM (Endo-SFM), M3: Prigrow Medium, M4: M1-M2-M3 at a 1:1:1 ratio, M5: M1-M2 at a 1:1 ratio, M6: M2-M3 at a 1:1 ratio, M7: M1-M2 at a 1:1 ratio (n=6).

Based on these results, M2 was selected as the most suitable medium for co-culturing all three cell types, and subsequent studies were conducted using this medium.

3.4.2. Determination of the Effect of Co-culture Process on Corneal Cells

Below are the detailed results of the characterization process conducted at the end of a 2-week culture period for corneal cells in M2 medium and their respective growth media, both in single- and co-culture processes.

Epithelial Cells

M1 medium is the growth medium for CECs. Therefore, CEC single-culture in the M1 medium was used as the control group. Additionally, single- and co-culture were performed in the M2 medium to investigate the effect of co-culture on cell characteristics. The cell groups were evaluated using ICC studies with fundamental CEC markers CK3 and ZO1 (**Figure 36**).

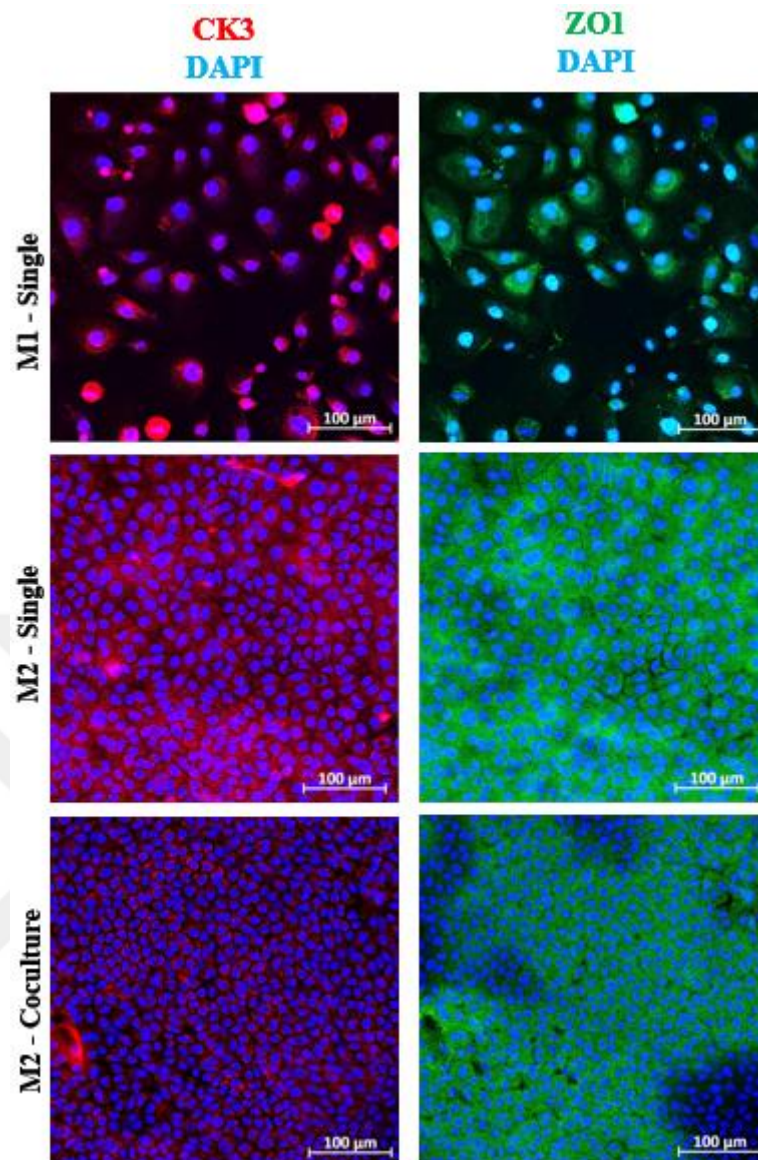


Figure 37. The results of ICC staining using corneal epithelial-specific markers CK3 and ZO1 for hCECs are presented. hCECs were cultured under the following conditions after a two-week culture period: single-culture in M1 and M2 media and co-culture in M2 medium.

Initially, an increase in hCEC cell density and basic marker expressions was noted in the M2 medium compared to M1. Specifically, a decrease in basic marker expressions was observed in the co-culture group when comparing hCECs cultured under co-culture conditions in M2 with those in single-culture in the same medium. While direct comparisons with similar studies are lacking, previous characterization studies of CECs co-cultured with embryonic stem cells (ESCs) have reported decreased expression of the

basic corneal epithelial marker CK-3. These findings suggested that ESCs supported the stem/progenitor phenotype of CECs ^[154]. Similarly, in this study, a decrease in CK-3 expression was observed when comparing single- and co-cultured hCECs in the M2 medium. Therefore, it can be inferred that the co-culture condition in the M2 medium supports the stem/progenitor phenotype of hCECs.

TEER measurements were conducted over a 2-week period in the CEC cell groups described above (**Figure 37**) to evaluate tight junction integrity. The results indicate that the M2 medium supports the tight junction integrity of hCECs. TEER measurements suggest stable or improved barrier function, indicating strong tight junctions between cells cultured in the M2 medium. Moreover, the co-culture condition also enhances the tight junction integrity of hCECs in the M2 medium. This suggests that co-culture with other cell types may improve or maintain tight junctions among hCECs. These findings demonstrate the supportive role of M2 medium and co-culture conditions in preserving or enhancing tight junction integrity in hCECs, which is crucial for their barrier function and overall cellular integrity.

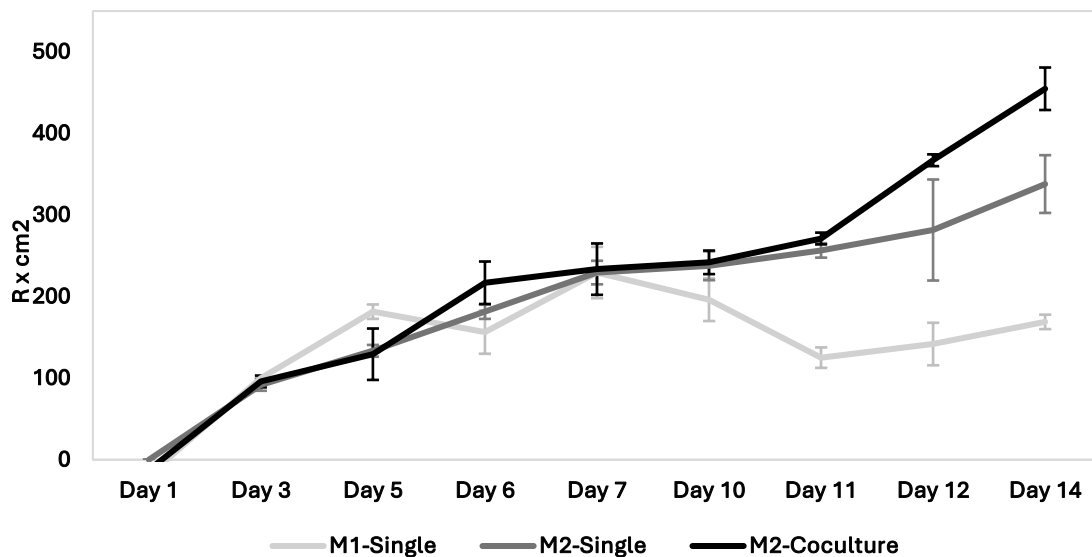


Figure 38. TEER measurements were conducted for HCECs cultured for two weeks under single-culture conditions in M1 and M2 media and under co-culture conditions in M2 medium (n=3).

Endothelial Cells

The M2 medium is the growth medium for CEnCs and has been selected as the optimal medium for co-culture. Accordingly, CEnCs cultured under single- and co-culture conditions in an M2 medium were subjected to ICC assays using the fundamental markers Na-K ATPase and ZO1 (Figure 38).

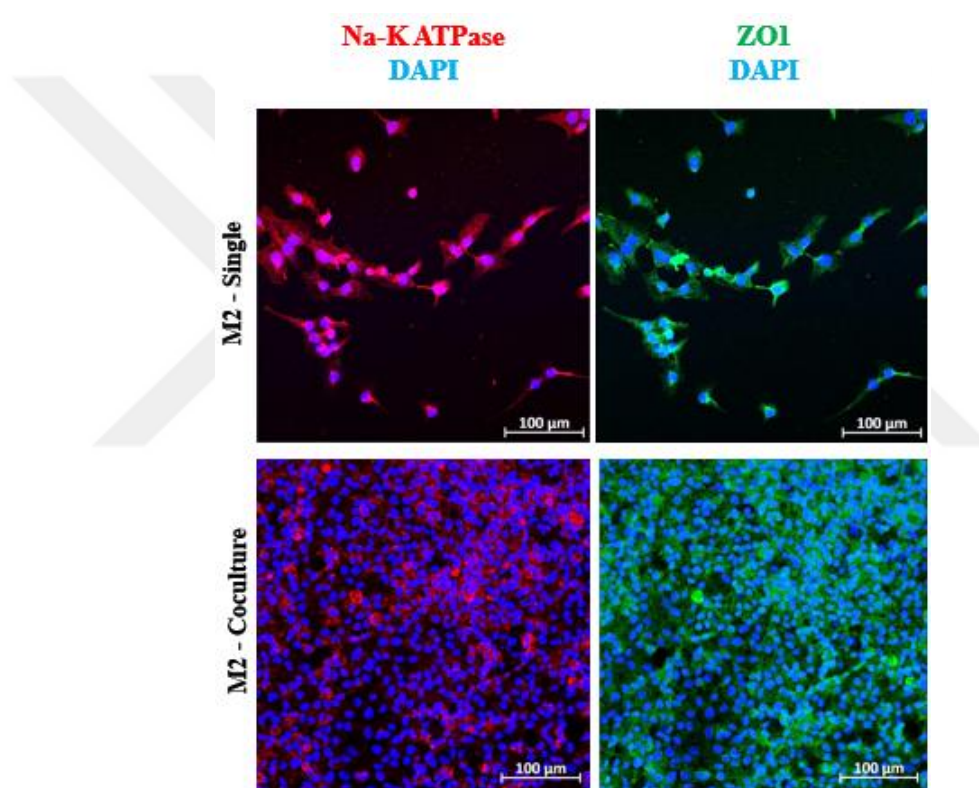


Figure 39. The results of ICC performed using corneal endothelial-specific markers Na-K ATPase and ZO1 for hCEnCs are presented. The hCEnCs were cultured under both single- and co-culture conditions in M2 media after a two-week culture period.

Based on the results obtained, it can be concluded that the co-culture condition significantly enhanced the proliferation capacity of hCEnCs. Additionally, there is clear evidence that the basic marker expressions of hCEnCs are notably elevated under the co-culture condition.

TEER measurements for hCEnCs were carried out on M2 media for 2 weeks in both single and co-culture conditions (Figure 39).

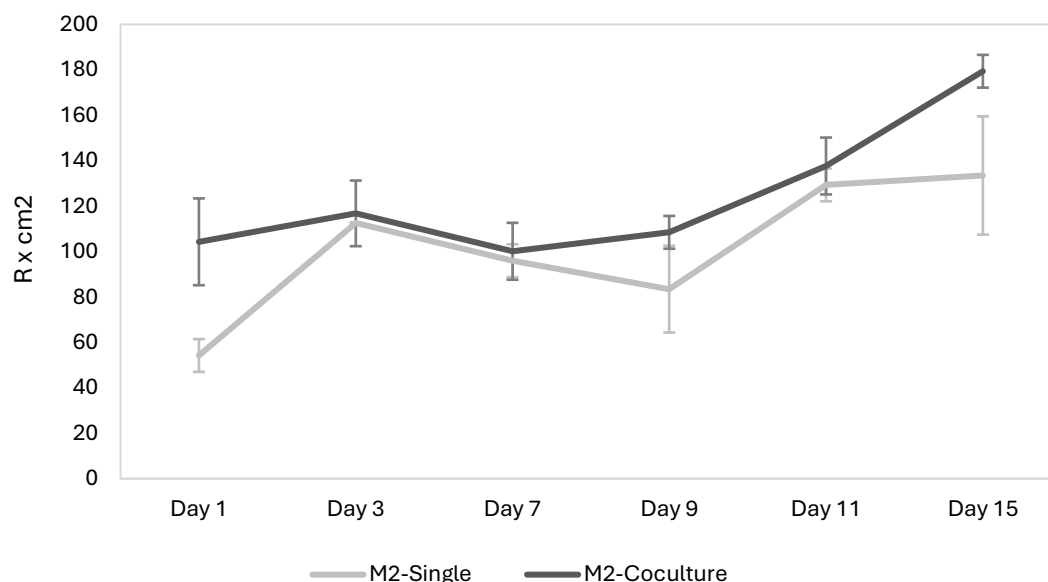


Figure 40. TEER measurements were conducted for hCEnCs cultured for two weeks under both single-culture and co-culture conditions in M2 media (n=3).

Weaker junctions are observed among hCEnCs compared to HCECs, which is crucial for maintaining material passage and transparency from the endothelium to the stroma ^[155]. Consequently, the TEER values obtained for hCEnCs are expected to be lower than those associated with HCECs. Upon examination of the TEER results obtained with corneal endothelium, values in the range of 40-80 Ωcm^2 have been achieved ^[156]. This study concludes that the co-culture condition enhances the tight junction integrity of hCEnCs, rendering them more stable.

Keratocyte Cells

The growth medium for hCKCs is M3 medium; therefore, the cells were single-cultured in this medium as the control group. M2 medium was chosen as the optimal medium for co-culture. This study aimed to examine the influence of the co-culture condition on the cell characteristics of hCKCs cultured in the M2 medium. This was achieved by performing ICC using main corneal keratocyte markers (Figure 40).

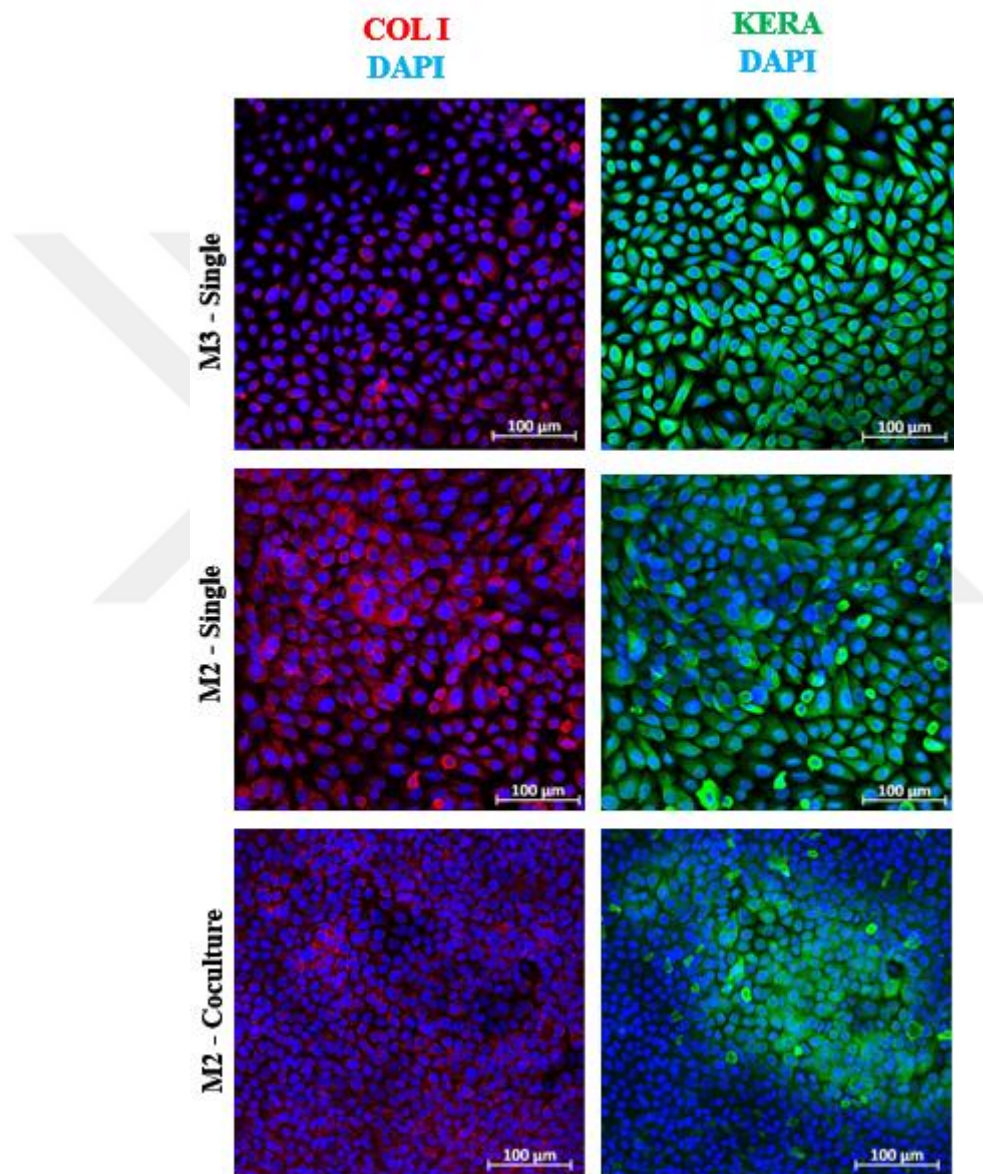


Figure 41. The results of ICC performed using corneal keratocyte-specific markers COL I and KERA for hCKCs are presented. The hCKCs were cultured under single-culture conditions in M3 and M2 media, as well as under co-culture conditions in M2 medium, after a two-week culture period.

Single-culturing hKCs in the M2 medium enhances the expression of COL1 and KERA. In the co-culture condition, although the expression of basic markers decreased, cell density increased. Based on these results, it can be concluded that the co-culture condition promotes keratocyte proliferation while slowing down extracellular matrix formation.

3.5. Tissue Engineering

This thesis study involved conducting CTE research using corneal epithelium and endothelium on PGS membranes in 2D culture, while corneal stroma was cultured in peptide hydrogels in a 3D setting. Detailed descriptions of these studies are provided below.

3.5.1. Corneal Epithelium Tissue Engineering

PGS is a biocompatible, biodegradable, and elastic polymer. Due to these properties, it has been utilized in tissue engineering studies of myocardial, vascular, cartilage, and retinal tissues ^[157] In this study, project member Kamal Asadipakdel produced three different transparent PGS pre-polymers with polymerization reaction times of 50, 75, and 105 minutes (Figure 41). Under 130°C heat application for 48h, pre-polymer films were crosslinked. After testing the transparency and mechanical properties, HCEC viability and phenotype analyses were performed on PGS membranes subjected to cell culture.

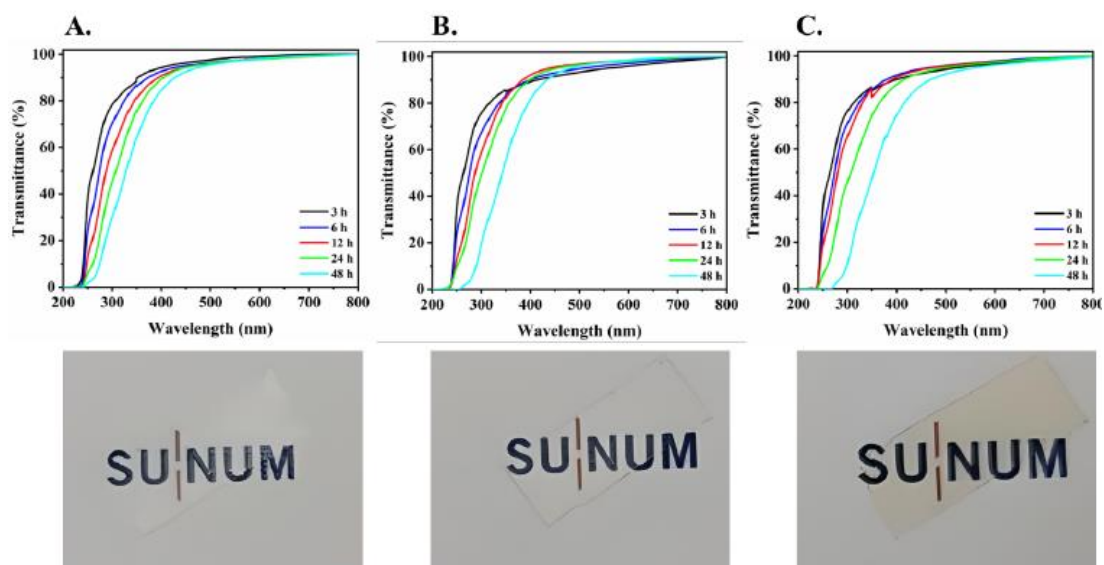


Figure 42. Transparency properties of PGS membranes. (A) UV-Vis spectroscopy results of PGS membranes polymerized for 50 minutes, (B) for 75 minutes, and (C) for 105 minutes, along with photographs taken after 48 hours of crosslinking.

The PGS membranes obtained by 50 minutes of pre-polymerization provided 84% transparency, while 75 minutes of pre-polymerization achieved 81%, and 105 minutes of pre-polymerization had 74% transparency. Natural corneal tissue exhibits 87% transparency^[76] Due to their transparency properties, which closely resemble those of natural corneal tissue, PGS membranes are suitable for use in CTE studies.

After seeding cells onto PGS membranes, a 2-week cell culture process was conducted. At the end of the culture period, biocompatibility assessment was performed first (**Figure 42**). Cell viability was observed in all three PGS membranes. Maximum viability was achieved in membranes polymerized for 50 and 105 minutes.

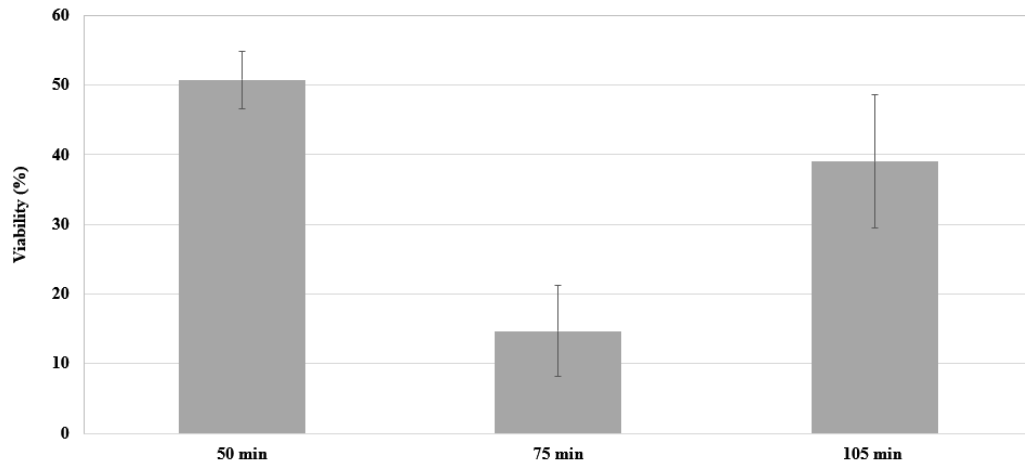


Figure 43. Viability percentage values of hCECs cultured on 50, 75, and 105 min polymerized PGS membranes (n=4).

After a 2-week culture period, ICC was conducted using the fundamental CEC markers CK3 and ZO1 (**Figure 43**). hCECs cultured on PGS membranes produced with 105 minutes of polymerization exhibited higher CK3 and ZO1 expression than the control group.

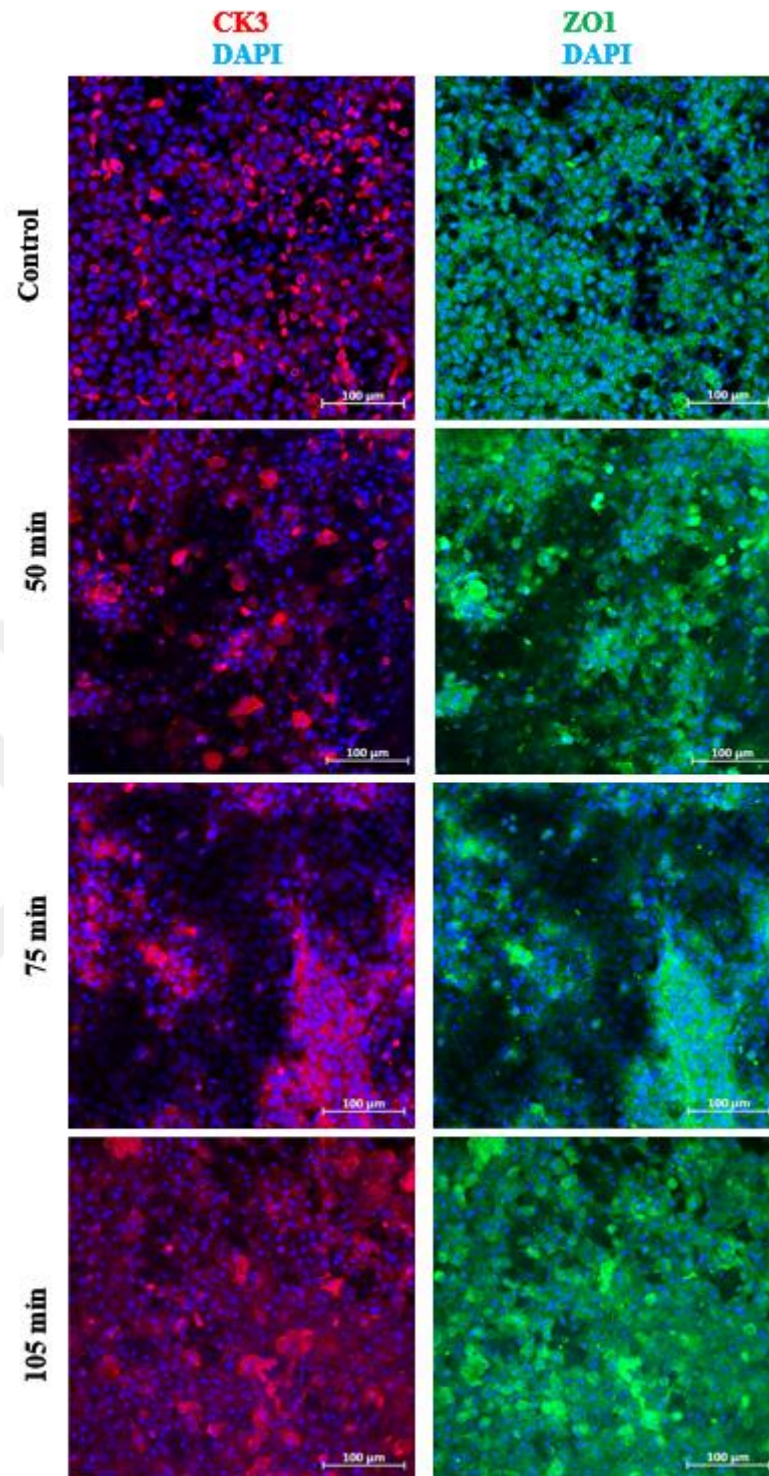


Figure 44. ICC results of hCECs after 15 days cultured on PGS membranes pre-polymerized at different periods.

The results indicate that the transparent PGS membranes are suitable for CTE studies. Furthermore, PGS membranes obtained with a 105-minute polymerization duration support CEC cell proliferation and the production of fundamental CEC markers

3.5.2. Corneal Keratocyte Tissue Engineering

RADA (Ac–RADARADARADARADA–CO–NH₂) is a synthetic peptide that, in aqueous environments, self-assembles into β -sheet nanofibers, forming a peptide hydrogel. The RADA hydrogel exhibits high similarity to the native 3D tissue ECM, making it suitable for use in various tissue engineering studies ^[158].

In this project, project member Ebru Demir aimed to mimic the corneal stroma by producing a 3D hydrogel scaffold. Hydrogels were created using RADA alone, uncrosslinked RADA - PGS nanoparticles (PGS-NP), and crosslinked RADA - PGS-NP hydrogels. The cross-linking of RADA peptide with PGS nanoparticles was achieved using EDC/NHS.

After seeding hKC cells onto a sterile tissue scaffold, a 15-day cell culture process was initiated, and cell characterization was conducted using biocompatibility and ICC assays. CCK-8 biocompatibility assay results indicate that the RADA peptide hydrogel does not exhibit toxic effects on hKC cells (**Figure 44**). However, this peptide hydrogel is not durable for long-term cultures. Factors such as pH, temperature, and ionic strength in the culture medium affect the stability of the RADA peptide hydrogel ^[159]. Therefore, a hydrogel combining RADA with PGS-NP was prepared to enhance mechanical strength. Crosslinking RADA with PGS-NP increased both mechanical strength and cell viability (98%), which was higher than the positive cell control's (92%) viability. Negligible viability (0.2%) was obtained in the uncrosslinked RADA – PGS-NP hydrogel. Based on this result, it can be said that crosslinking RADA and PGS-NP supports hydrogel stability and enhances cell viability.

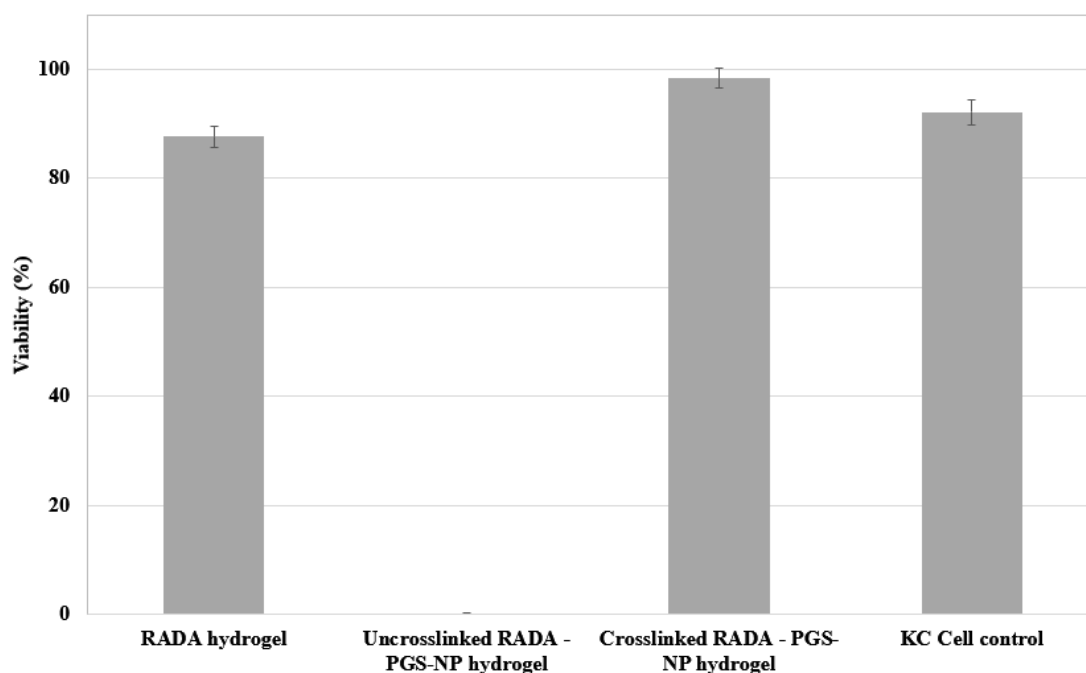


Figure 45. Viability percentages of hKCs after 15 days culture period on RADA peptide hydrogel, crosslinked and uncrosslinked RADA – PGS-NP hydrogels (n=2).

After a 15-day culture in the hydrogel, cell characterization was performed using COL I and KERA staining, which are fundamental keratocyte markers. The results were compared with the control group, which consisted of 2D cultured hKCs (**Figure 45**). Based on the obtained results, it can be stated that 2D cultured hKCs exhibit higher COL I and KERA expression. This outcome might be attributed to differences in the microenvironment. In 3D cultures, cells are exposed to a more complex microenvironment in terms of environmental factors and cell-cell interactions, which can lead to different gene expression profiles ^[160].

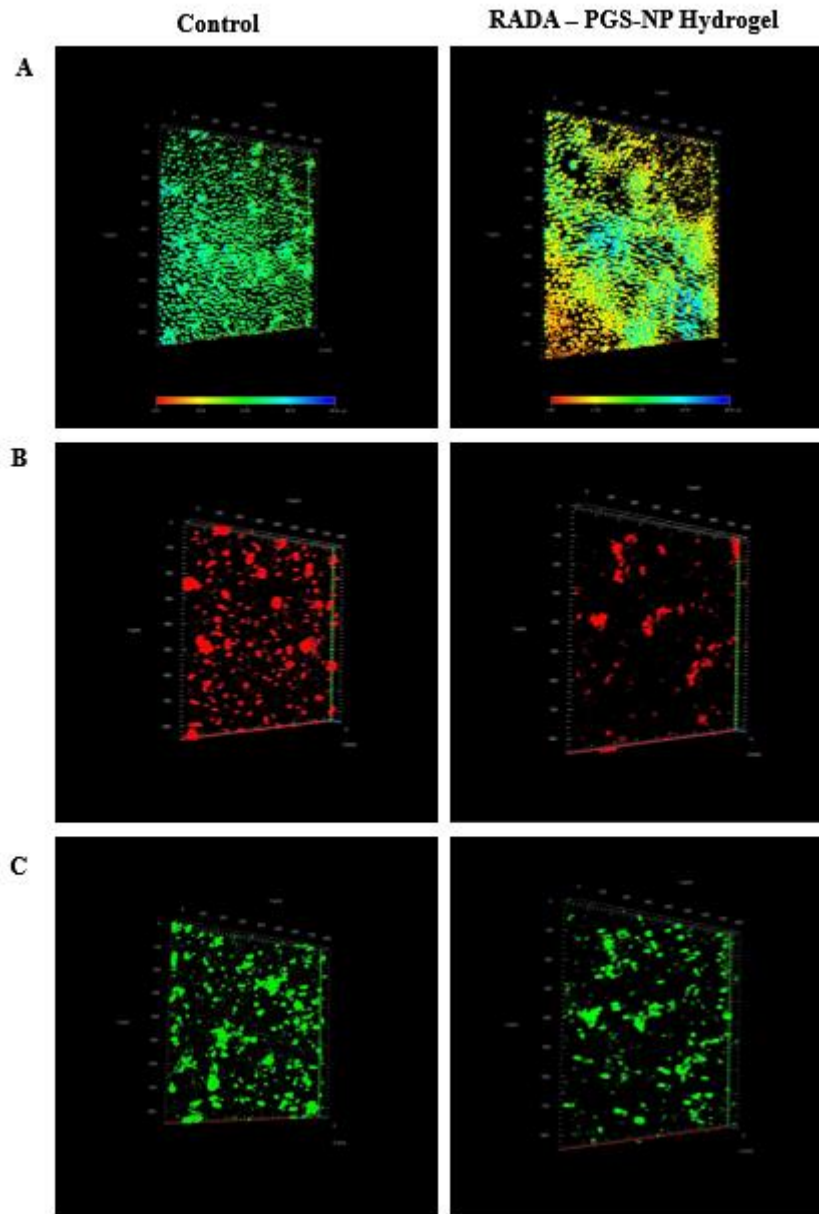


Figure 46. ICC results of 2D cultured hKCs as control group and 3D cultured hKCs on hydrogel. (A) 3D distribution of cells, (B) COL I staining, (C) KERA staining.

4. Conclusion

The cornea is a transparent, avascular tissue located at the outermost part of the eye. The most common method for treating corneal diseases is keratoplasty, which is corneal transplantation. The success of the keratoplasty procedure is influenced by many parameters, such as the donor's age, the presence of health conditions like diabetes, and whether the donor cornea has been damaged. CTE studies are of great importance to reduce dependence on donor corneas in the treatment of corneal diseases.

This thesis aims to isolate primary cells from donor human corneas obtained from an eye bank and mimic corneal layers. Accordingly, CEC, KC, CEnC, and limbal stem cell enzymatic and explant cultures were first conducted from the donor cornea. The phenotype and genotype characterizations of the isolated primary and immortalized cells were performed. The results indicate that immortalizing the isolated primary cells at passage 1 supports cell viability and subculture. Additionally, it was proven through ICC and gene expression studies that using immortalized passage 12 KCs in CTE studies is appropriate. It has been shown that limbal stem cells are a source for CEC and KC and that these stem cells have the potential to differentiate into corneal cells with the adjustment of the media content.

The phenotype and genotype analyses of the CEC, KC, and CEnC lines obtained for use in CTE studies have proven their suitability for use in CTE studies. Additionally, corneal cells in co-culture were characterized and compared with single cultures. The results show that corneal cells in co-culture exhibit high barrier integrity and proliferation and the expression of main cell markers.

The cell culture studies conducted on PGS membranes used to mimic Bowman's and Descemet's membranes within this thesis have shown that CECs maintain their viability on this membrane and express specific CEC markers. RADA peptide hydrogel was chosen to mimic the corneal stroma layer. The high water retention capacity of the hydrogel ensures the high water content of the stroma layer. Additionally, it was aimed to mimic stromal collagen fibrils with the β -sheet conformation provided by RADA peptides. Due to the loss of stability of RADA hydrogel during the culture process caused by temperature, pH, and ions in the medium, a hydrogel scaffold was compensated via the incorporation of PGS-NP. The cell culture results on the tissue scaffold obtained by crosslinking RADA, and PGS-NP shows that the hydrogel maintains its structural integrity throughout the culture period and that KCs exhibit high viability and express

key KC markers. This study presents a work that can serve as a source for CTE, corneal cell isolation and culture, and corneal and limbal cell characterization.

5. References

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