

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

**TISSUE ENGINEERING OF THE HUMAN CORNEA
USING *EX VIVO* METHODS**

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Introduction

The cornea is the clear, curved, anterior part of the eye that has a lamellar structure consisting of 5 layers (from anterior to posterior): corneal epithelium, Bowman's layer, stroma, Descemet's membrane and corneal endothelium. The renewal of the epithelium is continuous: the outermost cells lost by desquamation are replaced by cells moving centripetally and towards the surface originated from the limbal basal epithelium. The limbus is approximately 1.5-2 mm wide rim located at the corneo-scleral junction. It contains the limbal epithelial crypts, where limbal epithelial stem cells (LESCs) reside in a specific and highly regulated microenvironment, the niche. LESCs are unipotent stem cells that maintain a relatively undifferentiated state in the niche, but have a high proliferative potential and can undergo asymmetric division and migrate towards the surface and centripetally, progressively becoming more differentiated until they lose their proliferation ability and become mature central epithelial cells (CECs). Decreased number of stem cells or disturbances in the stem cell microenvironment due to injury can cause painful epithelial defects, stromal scarring, corneal neovascularization, conjunctivalization that results in impaired vision and ultimately vision loss. LESC deficiency (LESCD) treatment depends on the severity of the disease: in more severe cases invasive methods may be needed. One treatment option is the replacement of the stem cell population by grafting limbal biopsies from the patient's healthy eye or using allografts, but this is restricted by the lack of donor tissues and the need for immunosuppression. The use of autogenic grafts have good results without the requirement of systemic immunosuppressive treatment, but taking large grafts from the patient's fellow/healthy eye carries the risk of inducing LESCD in that eye and it is not even possible in bilateral cases. To avoid this, an *ex vivo* expansion of small biopsies from the patient, a patient's relative or cadavers has been developed. In case of cultured limbal epithelial cell therapy (CLET), the cultivation of LESCs is usually supported by different synthetic or biological scaffolds that assist the formation of cell sheets, which are then transferred to the affected eyes. The most widely used carrier is human amniotic membrane (HAM), as it is an avascular tissue that possesses anti-inflammatory and anti-angiogenic properties. Co-cultivation of LESCs on feeder layers is also a common culturing method, using inactivated murine 3T3 fibroblasts. Despite intense research in this field, in the last couple of years, the real characteristics of long-term *ex vivo* expanded LESCs have not been investigated.

The cornea stroma's main component is water and collagen rich extracellular matrix, mostly type I and type V collagen organized into lamellae. The uniformly spaced lamellar structure maintains the transparency of the cornea. The corneal stroma mesenchymal-like stem cells (CSMSCs) are multipotent stem cells located in the central corneal stroma that have a potential to differentiate *in vitro* into fat, bone and cartilage, but also keratocytes and neural cells. They possess some immunosuppressive features, suggesting that they can play an important role in immune regulation of the surrounding microenvironment and regeneration of corneal stroma and epithelium. It has been shown that CSMSCs express important markers of MSCs, but are negative for markers of hematopoietic lineage and activated cells. Schnyder corneal dystrophy (SCD) (previously known as Schnyder crystalline corneal dystrophy) is a rare type of corneal disease that appears bilaterally as a subepithelial opacification under the Bowman's layer. The corneal opacity is caused by abnormal deposition of intracellular and extracellular esterified and unesterified phospholipids and cholesterol in the stroma, later affecting the Bowman's layer as well. The word crystalline in the earlier name of the condition refers to the yellow-white needle-shaped deposits that were considered necessary for the diagnosis, but it has been proved that only about 50% of the patients develop crystals, hence the change in the terminology. Although SCD is a progressive disease, the clinical severity varies among different individuals. The condition is hereditary, and it is linked to the UbiA Prenyltransferase Domain Containing 1 (UBIAD1) gene located on chromosome 1 (1p36.3). Currently, there is no treatment available to stop or slow down the progression. Electron microscopic examination shows multilamellar bodies (MLBs) in the stroma of the affected corneas. MLB formation is associated with a presence of excess lipid. The latter are round shaped vesicles with 100-2400 μm diameter, with an ultrastructure consisting of multiple, concentric electron dense lamellae rich in lipoprotein. While the lipids can appear in physiological conditions, their appearance is also notable in some pathological changes (e.g. lysosomal storage diseases). The process of MLB formation is proved to be an autophagy dependent mechanism. Autophagy is the controlled degradation of sequestered cytoplasmic components by lysosomal hydrolytic enzymes. It is essential in maintaining the cellular homeostasis. Under stress conditions autophagy is upregulated to protect the cell from potential damage. The lysosomal nature of autophagic vacuoles (AVs) and MLBs indicate a similar pathway in their formation. In general, autophagy can be stimulated by starvation and rapamycin and inhibited by 3-methyladenine (3-MA).

Aims of the study

1. To isolate and long-term cultivate cornea LESC's in media containing serum as the only growth supplement without the use of scaffolds or special surface treatment.
2. To characterize the long-term *ex vivo* expanded LESC's by morphological (light-microscopy, TEM) and molecular biology techniques (immunofluorescent staining, flow-cytometry, cell viability test).
3. To compare the expression of the long-term *ex vivo* cultured LESC's surface markers to the results previously obtained from short-term *ex vivo* cultured LESC's.
4. To isolate and long-term cultivate human corneal stroma-derived stem-like cells (CSMSC's) from the central cornea to induce MLB formation.
5. To investigate the effect autophagy has upon MLB formation in long-term expanded CSMSC cultures.
6. To examine the long-term CSMSC cultures as a possible model for studying Schynder corneal dystrophy (SCD).

Materials and methods

Tissue collection and isolation

All tissue collection complied with the Guidelines of the Helsinki Declaration and was approved by the Regional Ethical Committee at the University of Debrecen, Hungary (DEOEC RKEB/IKEB 3094/2010 and 14415/2013/EKU-183/2013). Limbal and corneal tissue collection was done within 24 hours of biologic death from cadavers. In case of LESC isolation, rectangular-shaped tissues were dissected from the superficial layer using a lamellar knife placed tangential to the surface being cut. In case of CSMSC isolation, a corneal button was cut off using a lamellar knife. The epithelium and endothelium were scraped. The central part of the cornea stroma (approximately a 6-7 diameter cube) was then cut into multiple small pieces.

Cell culturing

LESC explants were plated into 24-well cell culture plates and viscoelastic material (ProVisc, Alcon, Fort Worth, TX, USA) was added on top of the tissues to support adherence. The cultures were maintained in 1 mL Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% Fetal Calf Serum (FCS, Sigma-Aldrich, St. Louis, MO, USA), 200mM/mL L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 1% Antibiotic/Antimycotic Solution (PAA, Pasching, Austria) at 37°C, 5% CO₂. The corneal stroma explants were seeded into 24-well cell culture plates, cultivated in 1 mL low glucose DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich) and 1% Antibiotic/Antimycotic Solution (PAA, Pasching, Austria), at 37°C, 5% CO₂. The medium was changed on alternating days on both cultures, and the cells were maintained at passage 0. The growth of the cultures was monitored under phase contrast microscope on a regular basis.

After long-term (> 3 months) expansion, the CSMSC cultures were treated to test for presence of autophagy. Autophagy induction was carried out using 50 nM rapamycin (RAP) (Sigma-Aldrich) treatment for 24 hours, and by starvation using serum free media, also for 24 hours. For the inhibition of autophagy, the cultures were treated with 10mM 3-MA (Sigma-Aldrich) for 24

hours. The cell cultures forming a membrane were then lifted and collected from the cell culture plates for further analysis. At this time the average age of the cultures was 239 ± 130 days ($n = 3$).

Cell viability assay and sterility test

To assess cell viability of LESC cultures, CellTiter-Glo® luminescent cell viability assay and traditional trypan blue dye exclusion test was used. The long-term LESC cultures were regularly tested for absence of *Mycoplasma* contamination (Mycoalert PLUS Mycoplasma Detection Kit, Lonza, Cat. No.: LT07-710) by an accredited microbiology laboratory (University of Debrecen).

Immunofluorescent staining

The tissue samples were embedded in paraffin, sectioned and prepared for immunofluorescent labeling. A 4',6-diamidino-2-phenylindole (DAPI) staining was used to visualize the nuclei. The LESC samples were characterized for markers of stemness (ABCG2, CK15, CK19, Vim), proliferation (p63 α , Ki-67), limbal epithelial cells- (CK8/18) and differentiated corneal epithelial cell markers (CK3 and CK12). For assessing the composition of extracellular matrix, collagen I, IV and V staining was performed. The CSMSC tissues were investigated for autophagy and the effects of its induction or inhibition by microtubule-associated protein 1 light chain 3 (LC3) and p62 protein, also known as sequestosome 1 (SQSTM1) staining. The visualisation and imaging of immunofluorescent staining was performed by a ZEISS Axio Observer. Z1 microscope (ZEISS, Oberkochen, Germany) and an EVOS FL microscope (Advanced Microscopy Group, Bothell, WA). For analyzing the images Image J software version 1.50 (National Institutes of Health, Bethesda, MD) was used.

Immunophenotyping of LESC by flow cytometry

The phenotype of the long-term cultured LESC was determined by multicolor flow cytometry using FITC-, PE- and APC- conjugated antibodies against selected surface CD47, CD90/Thy-1, CD117/c-kit, CD146/MCAM, CD166/ALCAM, CXCR4 (all from R&D Systems, Minneapolis, MN, USA). The samples were measured on a FACS Calibur flow cytometer (BD Biosciences Immunocytometry Systems) and the data were analyzed using Flowing Software 2.5 (PerttuTerho, Turku Centre for Biotechnology, University of Turku, Finland).

Transmission electron microscopy (TEM)

The LESC tissues were fixed overnight at 4°C in a freshly prepared fixative containing 2% glutaraldehyde in cacodylate buffer (pH 7.4). The CSMSC tissues were fixed for 24 hours at room temperature in a freshly prepared fixative containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer. After fixation, the samples were washed in PBS (3x 5 mins), and were kept in PBS at 4°C until further processing. For both LESC and CSMSC cultures, 1% osmium tetroxide was used for post-fixation, followed by dehydration in ascending ethanol series and immersion in propyleneoxide for 20 minutes. Finally the samples were embedded in Epon (Electron Microscopy Sciences, Hatfield, PA). For ultra thin sectioning (60-70 nm thick section), a Leica Ultracut Ultramicrotome UCT (Leica, Wetzlar, Germany) was used, followed by staining of the samples with uranyl acetate and lead citrate, then examined using a Tecnai 12 transmission electron microscope (Phillips, Amsterdam, the Netherlands).

Statistical analysis

The percentage of cells positive for a given marker was determined by counting the positive cells in a visual field with the help of nuclear staining by same three independent individuals. The results are expressed as mean \pm SD or SEM. Each experiment was performed at least three times, and each sample was tested in triplicates. Statistically significant difference was determined with Student's t-test and a value of $p \leq 0.05$ was considered significant.

Results

Tissue engineering of the human cornea limbal epithelial stem cells

LESCs cultured *ex vivo* start growing out from the explants after 5-7 days as revealed by phase contrast microscopy, and form a monolayer within 2 weeks, to then produce a 3-dimensional (3D) multilayer structure. After long-term expansion, the stratified outgrowth forms a macroscopically visible, thick membrane that fills out the well of the cell culture plate that could be air-lifted by forceps. Immunofluorescent staining of these sheets revealed a different distributional pattern of molecules and markers in the 3D cell outgrowth proximal to the explants versus distal to the explants. From the markers expressed by differentiated corneal epithelium, CK3 and CK12, the former was negative in both proximal and distal to the explants, but the latter showed a mild positivity proximal to the explants (18.1±1.6%), and an even weaker expression in the distal outgrowing cell sheets (7.4±2.4%). ABCG2 - a putative marker of stemness, was positive in 6.0±1.7% of the outgrowths proximal to the explants and 9.8±2.0% distal to the explants. The expression of pluripotency marker CK15 close to the limbal explants was 15.6±2.5%, while in the cells far from the explants was 54.3±1.2%. CK19 staining was observed in 12.0±1.3% of the cells near to the explants, and in 20.3±2.7% of the cells farther from the explants. Co-staining of the nuclear localized p63 and cytoplasmic Vim, also part of the putative stem cell markers, were found to be positive in 8.6±0.1% and 67.3±3.7% of the cells, respectively. The proliferation marker Ki-67 showed positivity in 5.0±0.1% of the cells. Staining was positive for collagen I, IV and V throughout the outgrowing cell sheets, creating a self-assembled, natural scaffold for the expanding 3D cell culture. DAPI nuclear staining showed that cells were embedded in the outgrowing multilayered ECM outside the explant tissue, but could not be found in the donor tissues any longer, after long-term cultivation.

The results of cell viability test showed a higher than 90% cell viability that was similar compared to the short term cultures. The TEM analysis showed the stratified ultrastructure of the long-term *ex vivo* cultures and revealed desmosomes (cell-to-cell junctions) between adjacent cells.

Immunophenotyping of the long-term cultivated LESCs was compared to the surface marker expression profile of short-term LESCs. Comparing the expression of CD117/c-kit (0.6±0.1%), a

marker of pluripotent hematopoietic progenitor cells in the short-term cultures versus long-term cultures, the disappearance of this marker could be observed in the latter. CD34 was not expressed on either the long-term ($0.8 \pm 0.3\%$) or the short-term LSCs. Chemokine receptor CXCR4 ($22.4 \pm 10.3\%$) which plays an important role in the regulation of stem cell migration was abolished in long-term compared to short-term LSC cultures. High expression of CD47 ($95.0 \pm 2.6\%$) indicates the viability and immunocompetence of both culture types. No expression of endothelial-related marker CD31/Platelet endothelial cell adhesion molecule (PECAM) could be detected on any of the cell types, excluding endothelial-related contamination. Alterations in the cell surface adhesion molecules that are involved in the interaction with the surrounding ECM and other cells were further investigated, which revealed that CD146/MCAM ($45.0 \pm 63.0\%$) and CD166/ALCAM ($64.16 \pm 12.7\%$) are significantly decreased, while CD44/homing-associated cell adhesion molecule (H-CAM) insignificantly increased ($73.4 \pm 3.1\%$) ($p < 0.001$) in the long-term LSC cultures compared to the short-term ones.

Tissue engineering of the human corneal stroma - 3D model for crystalline dystrophy

Phase contrast images of *ex vivo* cultivated corneal stroma grafts showed cell outgrowth from the explant assuming fibroblastic morphology within 2 weeks of cultivation, and continuous monolayer formation during the weeks 2-3. Upon reaching confluence, the cultures underwent stratification and formed a 3D structure after 4-5 weeks. Immunofluorescent staining of long-term 3D stroma showed *de novo* ECM deposition abundant in multilamellar collagen type I, similar to a tissue of the native corneal stroma. TEM analysis of the long-term 3D stroma revealed heavy MLB formation, with AVs being seen in the untreated long-term cultivated corneal stroma. The effect of RAP treatment and serum deprivation/starvation enhanced the expression of MLBs and AVs, while the 3-MA treatment attenuated it. The mitochondria appeared to be intact under 3-MA treatment also. Similarly, immunohistochemical analysis of the long-term 3D stroma for the typical autophagy markers LC3 and p62, showed that before treatment, the 3D corneal stroma tissue contained positive AVs, while further induction of autophagy by RAP treatment and serum starvation enhanced the amount of AVs; treatment by 3-MA resulted in attenuated LC3 and p62 expression, and therefore presence of decreased number of AVs.

Discussion

Tissue engineering has been a promising and a rapidly developing area of science in the last decades, while the use of stem cells in regenerative medicine to repair tissue damage and restore normal tissue function has been widely investigated. Adult stem cells from the cornea have great potential for applications in regenerative medicine and stem cell-based disease modeling. In our study, a successful cultivation of long-term LESC without use of any scaffolds was performed, and characterization of such cultures morphologically and immunophenotypically was achieved. After more than three months of cultivation without passaging, the cells developed a transparent, intact 3D sheet structure that could be harvested without the need of proteolytic enzymes, only using a forceps for lifting or air-lifting. The microscopic morphology of these *ex vivo* structures revealed a well-organized, stratified tissue, similar to that found *in vivo*. The disappearance of the cells from the donor tissue explants suggests that, during the long-term cultivation process, the cells migrated out from the explants and moved towards the cell culture surface of the culture plate, while depositing their own ECM. As collagen I is the most abundant protein in the corneal stroma *in vivo*, this could serve as a suitable self-assembled scaffold for corneal tissue engineering. Furthermore, our cultures could synthesize collagen IV, commonly known as a major component of the basement membrane, and collagen V, as well as demonstrate stratification without any direct induction. This structure could replace the need for using a different biologic or synthetic scaffold or a feeder-layer to attain a stratified, 3D structure and by removing any foreign components that could have even a minimal antigenicity, we could eliminate the risk of immunogenic reactions. TEM examination showed desmosomes in the ultrastructure of the cultures that indicated an adequate cell-to-cell adhesion, and can provide a high mechanical strength.

Several markers were suggested for the identification of LESC, but to date, a specific marker has not been found, therefore, a combination of putative stem cell markers and lack of differentiation related markers is in use. The absence, or presence of a relatively low expression of corneal differentiation markers (CK3 and CK12, respectively) in our cells implies that the cells are in a relatively undifferentiated state after long-term cultivation. Positive expression of ABCG2, CK15 and CK19 indicates that even after long-term cultivation, a small amount of cells maintain stem cell characteristics in a 3D environment. The high expression of Vim (characteristic for

cells located in the limbal crypts) around the limbal explants can probably be explained by the presence of tissue explants or other matrix associations existing in the cultures, and further proves the presence of undifferentiated progenitor cells. The presence of Ki-67, a marker associated with cell proliferation and growth, that showed a low expression distal to the explants, proves that after the long-term cultivation, the cells were capable of maintaining a low proliferative capacity. It has been shown that the expression of p63 α a nuclear progenitor marker and a putative LESC marker indicates a success of limbal transplantation, since cultures with more than 3% p63 α positive cells have better prognosis post-transplantation. Our samples revealed high expression of p63 around the explants (8.6 \pm 0.1%), which is a promising result for the outcome in future clinical use. The expression CD47, a cell viability and immunoregulatory marker was still high after more than 3 months of cultivation, indicating high viability and inhibition of phagocytosis. We found that the expression of CD117/c-kit an early progenitor/pluripotent stem cell marker, and CXCR4, a chemokine receptor for proliferating cells, diminished in long-term cultures. A possible explanation for the decline in the expression of these markers could be that after confluence and stratification was achieved, the space for further expansion became restricted. The expression of the mesenchymal/ epithelial marker CD90/Thy-1 increased over time and up to 7-fold in the long-standing cultures versus the short-term LESC cultures. Although CD90/Thy-1 has been located on cornea epithelial cells as well, its expression on long-term cultures of LESCs might refer to a commitment or a trans-differentiation potential towards epithelial lineage when cultured over longer periods of time. The changes in the examined adhesion molecule expression (CD44/HCAM, CD144/VE-Cadherin, CD146/MCAM and CD166/ALCAM) in the long-term culture versus the short-term cultures could be associated with the development of ECM deposition.

In the second part of our study, *ex vivo* long-term cultures of CSMSCs was established, that could possibly model Schnyder corneal dystrophy, and a mechanism for its potential resolution is being proposed. The microscopic morphology of these long-term cultures showed a well-structured, multilayered 3D tissue resembling the cornea stroma structure *in situ*. Immunofluorescent staining revealed that a *de novo* synthesized ECM in the 3D structure is composed mainly of collagen I, the main structural protein in the corneal stroma. Examination of the ultrastructure of our 3D cultures revealed that after long-term cultivation, spontaneous intracellular structural changes develop, as abundant numbers of MLBs appeared. It has been

shown that the biogenesis of MLBs in these cells is an autophagy-dependent mechanism. In our samples, the presence of autophagy was proved by immunofluorescent staining with markers for autophagy (LC3 and p62) after autophagy induction with RAP and serum starvation treatment, as well as inhibition with 3-MA treatment, which showed an inversely related increase and decrease in the expression of these markers, respectively. TEM results showed that autophagy inhibition reduces the number of MLBs and leads to formation of dense vacuoles that are processed or degraded through the MLB pathway. When cells were treated with RAP or exposed to serum starvation, the number and size of MLBs increased. The intracellular changes becoming apparent in the morphology of CSMSC after long-term cultivation is similar to that seen in SCD, as large amounts of MLBs accumulate in the cells' cytoplasm. Likely, the long-term cultures undergo spontaneous intracellular changes resembling MLBs and that of SCD. MLB formation in long-standing CSMSC cultures could serve as a potential *ex vivo* model for studying corneal stroma diseases, including SCD. Inhibition of autophagy can decrease the formation of MLBs, which may lead to a novel treatment of the disease in the future.

Summary

We successfully isolated, expanded and long-term cultivated LESC. The outgrowing cells were able to migrate from the explants, create a stratified structure and deposit a 3D ECM, generating a transparent tissue that could be easily manipulated. The cells expressed stemness-related markers, but also a commitment toward epithelial differentiation. After long-term cultivation, the migration and proliferation potential diminished. The cultures expressed a sufficient amount of biomarkers or fingerprint that can indicate a possible clinical success in case of future transplantation.

Long-term cultivated CSMSCs over 3 months and without any passaging, are hereby considered as nearly as close model for studying corneal dystrophy as the *in vivo* condition. The process of producing and self-assembling the corneal stroma *ex vivo*, with strong expression of collagen type I, and the evidence for presence and inhibition of autophagy in these 3D structure, thus decreasing the formation of MBLs, can serve the role of slowing down the progressive loss of stromal transparency in SCD or any other condition involving MLBs and/or autophagy degradation pathway involvement. This can certainly have basic and transplantational

implications, serving as a model for *ex vivo* studies on SCD, as well as therapeutic developments for treatment of this rare, but sight-threatening corneal dystrophy.

The thesis is built upon the following publications:

1. **Szabó DJ**, Noer A, Nagymihály R, Josifovska N, Andjelic S, Veréb Z, Facskó A, Moe MC, Petrovski G. **Long-Term Cultures of Human Cornea Limbal Explants Form 3D Structures Ex Vivo - Implications for Tissue Engineering and Clinical Applications.** *PLoS One*. 2015 Nov 18;10(11):e0143053. PMID: 26580800

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2. **Szabó DJ**, Nagymihály R, Veréb Z, Josifovska N, Noer A, Liskova P, Facskó A, Moe MC, Petrovski G. **Ex vivo 3D human corneal stroma model for Schnyder corneal dystrophy - role of autophagy in its pathogenesis and resolution.** *Histol Histopathol*. 2018 May;33(5):455-462. doi: 10.14670/HH-11-928. Epub 2017 Sep 5. PMID: 28872183.

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