SOUTH-EAST EUROPEAN JOURNAL of OPHTHALMOLOGY

# Ex vivo cultivation of limbal epithelial stem/progenitor cells: comparison of two culturing techniques

Zala Lužnik[1], Marko Hawlina[1], Marina Bertolin[2], Elvira Maličev[3], Tina Cirman[3], Andreja Nataša Kopitar[4], Alojz Ihan[4], Stefano Ferrari[2] and Petra Schollmayer[1]

[1]*Eye Hospital, University Medical Centre, Ljubljana, Slovenia.*
[2]*Fondazione Banca degli Occhi del Veneto, Venice, Italy.*
[3]*Blood Transfusion Centre of Slovenia.*
[4]*Medical Faculty Ljubljana, Institute of Microbiology and Immunology*

**Keywords**
*limbal stem cell deficiency disease, limbal epithelial stem cells, tissue engineering*

**Abstract**

Ex vivo culturing of limbal stem cells necessitates establishment of appropriate culturing conditions. Different approaches of limbal stem cell transplantation have their advantages and disadvantages. Focus of this review is comparison between the two most commonly used techniques for ex vivo limbal stem cell culturing and their use in ocular surface disease therapy. Additionally, we present our first results of ex vivo limbal stem cells culturing in Slovenia.

## Introduction

Corneal epithelium is renewed by stem cells (SC) located in the basal epithelial layers of the limbal epithelium in a special supporting microenvironment known as the stem cell niche (1). When the SC containing limbal epithelium is partially or totally damaged, a blinding and painful disease of limbal stem cell deficiency (LSCD) ensues (1, 2). Total and severe LSCD is difficult to manage. Transplantation of limbal epithelial SCs (LESC) is necessary to restore vision (2, 3).

Conventional surgical treatment methods depend mainly upon whether one or both eyes are affected (2). In case of total bilateral LSCD, large pieces of healthy donor limbal tissue can be transplanted from an allogeneic source (allografting from cadaveric or living related donor) (4). However, although high dose systemic immune suppression is required, the immunological rejection of transplanted allogeneic limbal tissue is reported to occur in over 50% of patients (5). If one eye is affected by total LSCD, limbal tissue from the healthy other eye can be used (autografting), potentially putting the donor eye at risk (4).

To overcome these issues, cultivated limbal epithelial transplantation (CLET) that transplants ex vivo expanded LESC from a small amount of limbal tissue biopsy (1×2 mm) was first described by Pellegrini et al. in 1997 (6, 7). Since then, CLET has evolved to be the most successful alternative to whole limbal transplantation in patients with unilateral disease and offers a therapeutic chance to patients with severe bilateral disease (8), with a significantly lower immune rejection rate occurring after allogeneic CLET (from 3,3% [5] to 20% [9]). Although direct comparison of reported clinical trials between centers is difficult due to the wide

Corresponding author's information:
Zala Lužnik, MD, Eye Hospital, University Medical Centre Ljubljana, Grabovičeva 46, 1000 Ljubljana - SLOVENIA
Tel: (+386) 51316960, Fax: (+386) (0)15221960, E-mail: zala_luznik@yahoo.com, zala.luznik@kclj.si

diversity of culture protocols used and the etiologies of LSCD treated, with the overall success rate of 76% (7), CLET appears to be a promising treatment modality for LSCD (7).

A variety of culturing techniques have been developed (10), with two culturing techniques being most commonly reported and clinically used: the limbal cell suspension technique and the limbal explant technique (8), which are the focus of the current review. Additionally, our first results of ex vivo limbal stem cell culturing will be presented.

## Limbal stem cells

Stem cells are cells with unlimited or prolonged self-renewal capacity that can generate at least one type of highly differentiated progeny (1). Corneal SCs are concentrated and maintained in special supporting microenvironment known as the SC niches (3, 7). The niche plays an important role in the maintenance of SC properties and is tightly regulated by factors from the surrounding tissue (e.g. from vasculature; the special basement membrane composition – specific isoforms of collagen IV, laminin and fibronectin; paracrine hormones from limbal fibroblasts in the underlying stroma) (11).

Certain stimuli from the microenvironment (e.g. corneal epithelial damage) can on demand activate LESCs for tissue regeneration (3). They can increase their own population or differentiate into transient amplifying cells (TAC) (12). The TACs have a limited proliferating capacity with a short life span (12). They generate post-mitotic and terminally differentiated cells that are located in the supra-basal and superficial corneal layers (12) *(Figure 1)*. During the differentiation process the cells change in their morphology and express different molecular markers (12). The undifferentiated limbal basal epithelial cells express the keratin pair K5/K14, K19, and are devoid of the cornea-specific keratin pair K3/K12 (1, 12).

Thus, for the ex vivo cultivation of limbal stem cells appropriate culturing conditions (a laboratory surrogate limbal niche) needs to be established for the survival and proliferation of limbal cells (3).

## Identification of LESC in the cultured epithelium

Results of clinical studies have shown that optimal clinical outcomes of CLET primarily depend on the quality of the limbal cultures, where the accurate determination of SC content in the corneo-limbal epithelial grafts represents the best quality indicator (13). Thus, much effort has been put into developing safe, standardized and optimal in vitro culturing conditions that would retain limbal SC function and prevent their terminal differentiation in culture (7).

The SC potential of cultured cells can be determined with the help of functional or molecular test (14). Functional evaluation with multiple serial passages (e.g. colony forming efficacy [CFE] test) or clonal analysis testing still represent the gold standard tests for SC identification in culture (14).

Although during the last years a progress has been made towards the development of molecular markers that may distinguish limbal stem cells from other corneal cells, a specific limbal stem cell marker is still not identified (12). Thus, a combination of differentiation-associated markers (e.g. K3/K12) and putative stem cell-associated markers (e.g. ABCG2, K19, p63) may provide a suitable tool for the identification (12). Transcriptional factor $\Delta Np63\alpha$ is currently thought to be the best positive marker to determine the percentage of limbal SCs in corneo-limbal grafts, as a positive clinical correlation was already presented (13, 14).

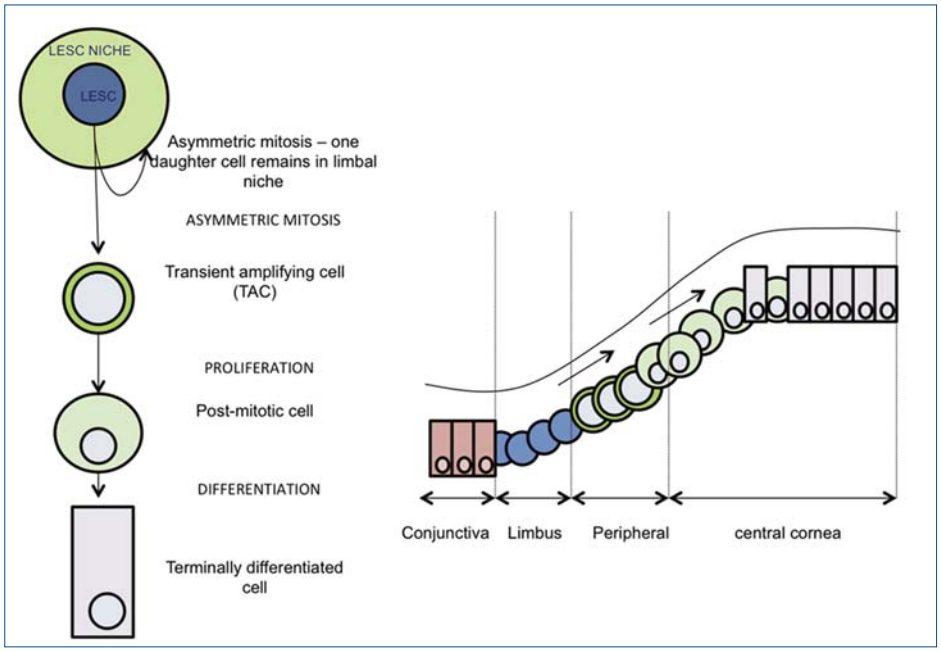


*Figure 1. Limbal stem cell proliferation and differentiation of daughter cells*

## Culture techniques

Despite many variations in the culturing protocols (e.g. using xenogeneic or xenogeneic-free media, different scaffolds, murine feeder layers, air-lifting), the procedure involves three basic steps: (i) harvesting epithelial SCs from a donor source, (ii) culturing the cells ex vivo, and (iii) transplanting the generated epithelial sheets or grafts into a diseased corneal surface on a carrier system.

To date, two main protocols for the expansion of limbal epithelial cells are being used in clinical trials, either the cell suspension technique or the limbal explant technique (7).

In the cell suspension technique the small limbal biopsy is enzymatically processed (e.g. with trypsin) (6). The obtained suspension of single cells is then co-cultivated on a mitotically inactivated 3T3-J2 murine fibroblast feeder layer (6). The feeder layer cells are inactivated with mitomycin or by irradiation. The primary limbal cultures are cultivated in standard laboratory conditions for 7-10 days (13). The sub-confluent cultures are trypsinezed and re-seeded into a fibrin glue embedded culture plate in the presence of a feeder layer (13). The secondary limbal cultures are cultured for another 1-2 weeks before they are clinically used (13).

A small amount of cells from the primary limbal culture (approximately 30,000 cells) are used for quality control tests: 1) to assess the percentage of LESC, 2) their clonogenic potential (through a CFE test), and 3) the presence of cell culture contamination (e.g. conjunctival cells, fibroblasts) (13). The quality control tests are repeated on the final product from cells isolated from the edges of the secondary culture, before the central part of the epithelial graft is transplanted onto the patient's eye (13). These cells are also used to evaluate the levels of endotoxin and potential bacterial/viral contaminants within the graft (13).

Pellegrini and colleagues reported the first successful reconstruction of the ocular surface in LSCD with this technique, using confluent secondary autograft cultures (6, 13, 14). However, up to now, this technique still requires the concomitant use of xenogeneic material (e.g. murine 3T3 fibroblast feeder layer and fetal bovine serum (FBS) in the growth medium), which may poses the potential risk of interspecies pathogen transfer and of initiating immunological rejections (15).

The limbal explant culturing technique uses unprocessed limbal biopsy tissue, which is most commonly cultured on a cryopreserved human amniotic membrane (AM) (2, 12). The AM is composed of three layers: the epithelial layer, a thick basement membrane layer, and a stromal matrix layer (16). It is well documented that the amniotic epithelium produces basic fibroblast, hepatocyte and transforming growth factor, which may modulate proliferation and differentiation of limbal cells (17). On the other hand, the amniotic membrane stromal matrix also suppresses the expression of certain inflammatory cytokines that originate from the ocular surface epithelia, including interleukins (IL-1, IL-2, IL-8), interferon, tumor necrosis factor, fibroblast growth factor and platelet-derived growth factor (18), suggesting that AM may be ideal for restoring an ex vivo limbal niche for LEMC expansion. In addition, as limbal explants are not enzymatically processed, the epithelial stem cells are usually co-cultured with some of the underlying limbal stromal cells (11).

The limbal explants are seeded directly onto the fastened AM, usually on the epithelial side of the AM (19). In some protocols the AM epithelium is enzymatically removed (denuded AM) (19). In the explant technique, the progenitor limbal epithelial cells start to proliferate and migrate out of the explant covering the whole scaffold in approximately 2–3 weeks (15, 19). The composite graft is then transplanted to the diseased eye surface after fibrovascular pannus removal (19).

Compared to the suspension technique, the technique is less demanding. The xenogeneic murine feeder layer is not obligatory needed for cell expansion and in some culturing protocols FBS was already successfully replaced with human serum (15, 19). As no enzymatic processing is needed, the initial risk of harming SCs might be overcome (20) and the SCs can be cultured with their own supporting surrounding niche cells. However, on the other hand, the migration of SCs out of the limbal explant is still controversial, with some studies suggesting that only TACs can migrate and proliferate (14, 20). Additionally, functional tests (e.g. CFE) for SC identification are usually not routinely underdone (14).

## Clinical outcomes of autologous and allogeneic CLET

In the past 20-years, various studies reported the clinical outcome results from CLET (10). As different culturing techniques, inclusion criteria and follow up protocols were used; direct comparison of the clinical outcome results between different centers is not possible (7). However, in general, patients treated for chemical or thermal corneal burns had a higher success rate than patients with immunological (autoimmune) corneal diseases (e.g. ocular pemphigoid and Stevens-Johnson syndrome) (10). Probably, the pathological processes that influence the immunological conditions create an ongoing hostile environment for the transplanted stem cell survival (7).

Allogeneic CLET represented 1/5 of all reported cases, with an average clinical success rate of establishing a stable ocular surface of 73% (7). In comparison, in autologous CLET the outcome is approximately 77% (7).

## First results of ex vivo limbal culturing in Slovenia

Before clinical trials can be performed, a safe and validated protocol for limbal stem cell culturing needs to be established. Therefore, an experimental study was started in March 2014 in Ljubljana Eye Hospital, after the National Medical Ethics Committee approved (123/02/14, date: 25. 3. 2014) all the procedures and protocols. The research followed the tenets of the Declaration of Helsinki.

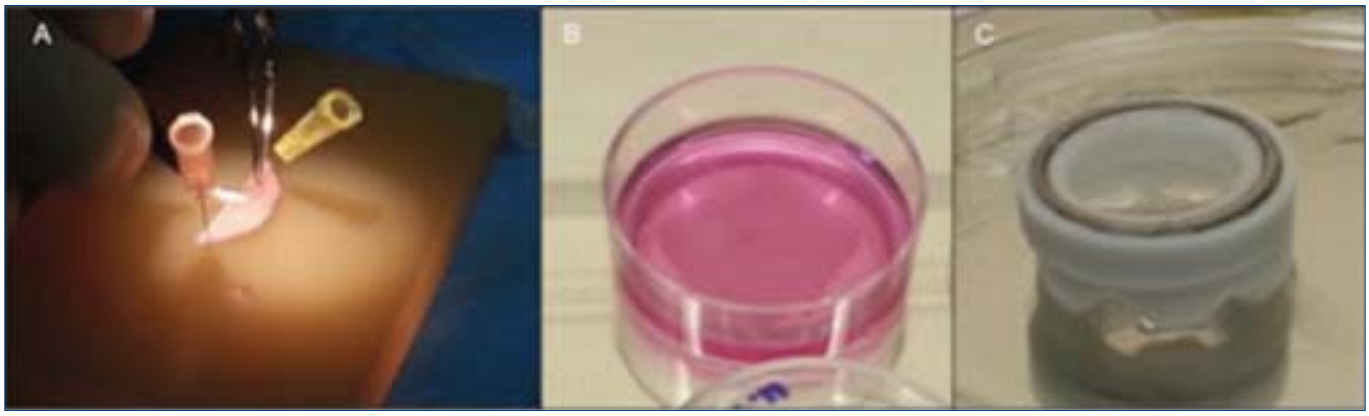


*Figure 2. Limbal explant culture preparation. Corneo-scleral rim excision of limbal explant biopsy (1 × 2 × 0.25 mm in size) under sterile conditions (A). Limbal explant cultures on intact AM fastened onto a 35-mm culture plate with fibrin glue (Beriplast CSL Behring) with the epithelial side facing up (B). The AM fastened with the inter-lockable plastic rings (C), epithelial side up, which was a generous gift from the Veneto Eye Bank Foundation (Venice, Italy)*

The purpose of the research was to validate our culturing protocol using a medium supplemented with only human serum and AM as a substrate carrier for the cultivation of limbal epithelial cells, using the limbal explant technique (21). Cryopreserved human AM were provided by the Blood Transfusion Centre of Ljubljana, Slovenia, and were preserved according to the method described by Koizumi et al (22). After the central corneal buttons were used for corneal transplantation purposes, we used the preserved cadaveric human corneo-scleral rims for limbal explant preparation. As shown in *Figure 2A*, each limbal rim was cut into 2 $mm^2$ sized samples (2×1 mm wide and 0.25 mm deep into the stroma) under sterile conditions. Each limbal explant with the epithelium side down was directly placed into a well of twelve-well plastic culture plates (control) or on cryopreserved intact human AM (on the epithelial or stromal side), in a feeder cell-free culture system (21) to study the effect of AM orientation. To establish successful limbal explant cultures, the AM has to be properly fixated, in order not to let it floating in the medium. Therefore, at the beginning of our research study, the intact AM was fastened onto a 35-mm culture plate with fibrin glue (Beriplast CSL Behring) with either the epithelial or stromal side facing up *(Figure 2B)*. This enabled us to successfully observe the outgrowing cells with an inverted microscope until 3-4 weeks, when the fibrin glue was degraded and the AM started to float again. Recently, we have fastened the AMs with inter-lockable plastic rings (*Figure 2C* – a generous gift from the Veneto Eye Bank Foundation, Venice, Italy), epithelial side up or down. The explants were cultured in a xenogeneic-free culturing medium, which was a 1:1 mixture of Dulbecco modified Eagle medium (DMEM) and Ham's F12 medium (GibcoTM / Thermo Fisher Scientific) containing 10% or 20% human serum (Sigma Aldrich, Italy) and antibiotics 50 $\mu g\ ml^{-1}$ gentamicin and penicillin (GibcoTM/Thermo Fisher Scientific), at 37 °C under 5% $CO_2$ and 95% humidity. The medium was renewed every 2–3 days. Growth was observed under an inverted microscope. To further determine the cultured cell phenotype we

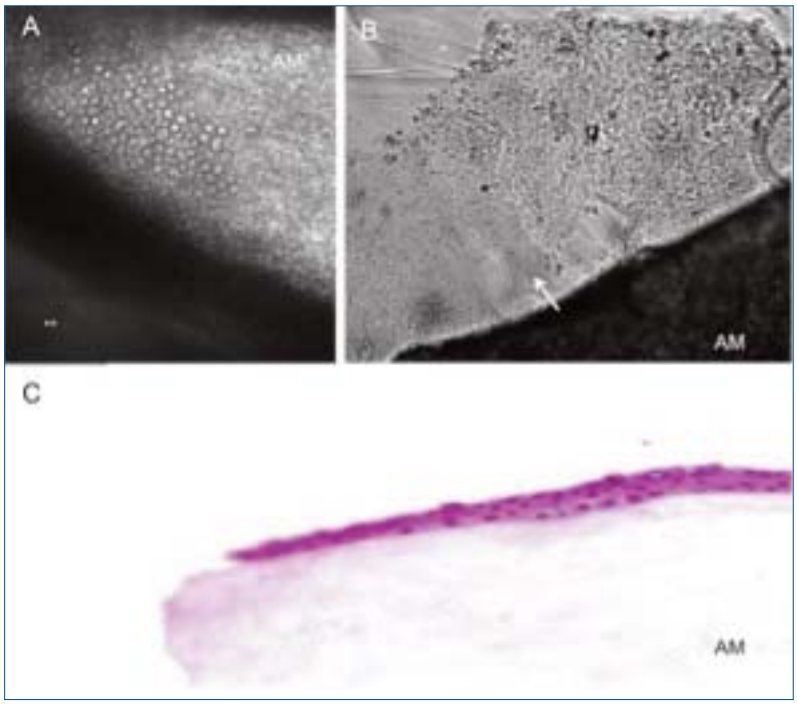


*Figure 3. Limbal explant outgrowth of cells cultured on AM after 3 days of culture and after 3 weeks of culture. The cells adjacent to the limbal explants were more uniform, smaller and had larger nuclei (A), whereas after 3 weeks of culture an epithelial outgrowth was observed at the AM border (white arrow) (B) (A,B: Magnification:10x). Light microscopy showed limbal epithelial cells cultivated on intact AM (stromal side) produced a well-stratified cell layer; (HE: 10x). (**; explants; *; plastic plate)*

performed immunocytochemistry studies. The expression of mesenchymal stem cell markers (CD73, CD90, CD105), proliferation and putative progenitor markers (CD184, CD117), and epithelial markers (MUC, CK7, CK12) was determined by flow cytometry. Immunohistology staining of limbal cultures on AM was tested for pancytokeratin, p63, and Ki67.

On the 3rd day of cell culturing, the first cell outgrowths from the limbal explant samples were noticed *(Figure 3)*. Morphologically the cells were round-shaped with scarce cytoplasm. After one week the cell proliferation accelerated and a change in cell phenotype was observed. Some cells cultivated on culture plates without AM became more elongated and spindle shaped. The cells cultivated on AM had retained an epithelial cell structure, which was further confirmed by histology examination. Interestingly, morphological and immunostaining analyses revealed two distinct stem cell population types, which could be identified over prolonged culturing time periods (21). Expression of mesenchymal stem cell markers and CD184 was significantly higher ($p<0.05$) in cultures cultivated without AM (21). However, no statistically significant difference was observed in CD117 expression (21). The cells cultivated on AM retained an epithelial cell structure, which was further confirmed by histology examination. Histology revealed limbal epithelial growth and p63, Ki67 positive cells on both sides of AM (21).

To sum up, with our first experiments we observed that limbal cells cultivated on AM exhibited a lower expression profile of the tested mesenchymal stem cell markers and CD184 marker as limbal cells cultivated on plastic culture plates (21). However, CD117 expression was similar. Histology confirmed limbal epithelial cell growth on both sides of AM, with no morphological differences, or positivity of cells for p63 and Ki67 (21).

## Future challenges

Although almost two decades have passed since the first CLET, most clinically used culture techniques are still based on the use of xenobiotic or allogeneic products, such as murine feeder layers, human amniotic membranes, media containing serum of bovine or human origin and supplements such as cholera toxin (7). The presence of animal derived products carries not only the theoretical health risk of pathogen transmission (e.g. prions, viruses, bacterial endotoxins) and host immune reaction and graft failure initiation, but are also being biologically variable (23).

Thus, the major aim of current research studies on cultured LESC is to avoid the use of xenobiotic material, to identify novel bio-functional scaffolds that replicate the biological stem cell niche and alternative autologous stem cell sources (such as nasal or oral mucosal cells, mesenchymal stem cells, hair follicle cells, dental pulp stem cells, induced pluripotent stem cells, umbilicard cord and embryonic stem cells) (7).

## Conclusion

Transplantation of cultured limbal stem cells is already successfully used in specialized centers for the treatment of advanced limbal stem cell deficiency. Different approaches of limbal stem cell transplantation have their advantages and disadvantages. Ex vivo culturing of limbal stem cells necessitates the establishment of appropriate culturing conditions. With a multidisciplinary approach, we would like to establish a safe and validated protocol for limbal epithelium culturing in Slovenia, which could be also clinically used.

## References


1. Bakhtiari P, Djalilian A. Update on limbal stem cell transplantation. Middle East Afr J Ophthalmol. 2010; 17: 9–14.

2. Meller D, Pires RT, Tseng SC. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. Br J Ophthalmol. 2002; 86: 463–71.

3. Li W, Hayashida Y, He H, Kuo CL, Tseng SC. The fate of limbal epithelial progenitor cells during explant culture on intact amniotic membrane. Invest Ophthalmol Vis Sci. 2007; 48: 605–13.

4. Ahmad S, Osei-Bempong C, Dana R, Jurkunas U. The culture and transplantation of human limbal stem cells. J Cell Physiol. 2010; 225: 15–9.

5. Oh JY, Ko JH, Lee HJ, Kim MK, Lee JH, Wee WR. The antigenicity of ex vivo cultivated human corneal limbal epithelial and stromal cells: temporal changes in vitro. Cornea. 2010; 29: 1302–7.

6. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R in De LM: Long-term restoration of damaged corneal surface with autologous cultivated corneal epithelium. Lancet. 1997; 349: 990–3.

7. Baylis O, Figueiredo F, Henein C, Lako M, Ahmad S. 13 years of cultured limbal epithelial cell therapy: a review of the outcomes. J Cell Biochem. 2011; 112: 993–1002.

8. Burman S, Sangwan V. Cultivated limbal stem cell transplantation for ocular surface reconstruction. Clin Ophthalmol. 2008; 2: 489–502.

9. Qi X, Xie L, Cheng J, Zhai H, Zhou q. Characteristics of immune rejection after allogenic cultivated limbal epithelial transplantation. Ophtalmology. 2013;120: 931–6.

10. Haagdorens M, Van Acker SI, Van Gerwen V, Ní Dhubhghaill S, Koppen C, Tassignon MJ, Zakaria N. Limbal Stem Cell Deficiency: Current Treatment Options and Emerging Therapies. Stem Cells Int. 2016;2016:9798374.

11. Polisetti N, Agarwal P, Khan I, Kondaiah P, Sangwan VS, Vemuganti GK. Gene expression profile of epithelial cells and mesenchymal cells derived from limbal explant culture. Mol Vis. 2010; 16: 1227–40.

12. Schlötzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. Exp Eye Res. 2005; 81: 247–64.

13. Di Iorio E, Ferrari S, Fasolo A, Böhm E, Ponzin D, Barbaro V. Techniques for culture and assessment of limbal stem cell grafts. Ocul Surf. 2010; 8: 146–53.

14. Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, et al. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J Cell Biol. 1999; 145: 769–82.

15. Kolli S, Lako M, Figueiredo F, Mudhar H, Ahmad S. Loss of corneal epithelial stem cell properties in outgrowths from human limbal explants cultured on intact amniotic membrane. Regen Med. 2008; 3: 329–42.

16. Higa K, Shimmura S, Shimazaki J, Tsubota K. Ocular surface epithelial cells up-regulate HLA-G when expanded in vitro on amniotic membrane substrates. Cornea. 2006; 25: 715–21.

17. Sato H, Shimazaki J, Shinozaki N. Role of growth factors for ocular surface reconstruction after amniotic membrane transplantation. Invest Ophthalmol Vis Sci. 1998; 39:S428.

18. Solomon A, Rosenblatt M, Monroy D, Ji Z, Pflugfelder SC, Tseng SC. Suppression of interleukin 1alpha and interleukin 1beta in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. Br J Ophthalmol. 2001; 85: 444–9.

19. Zakaria N, Possemiers T, Dhubhghaill SN, Leysen I, Rozema J, Koppen C, et al. Results of a phase I/II clinical trial: standardized, non-xenogenic, cultivated limbal stem cell transplantation. J Transl Med 2014; 12: 58.

20. Grueterich M, Espana EM, Tseng SC. Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol. 2003; 48: 631–46.

21. Lužnik Z, Hawlina M, Maličev E, Bertolin M, Kopitar AN, Ihan A, Ferrari S, Schollmayer P. Effect of Cryopreserved Amniotic Membrane Orientation on the Expression of Limbal Mesenchymal and Epithelial Stem Cell Markers in Prolonged Limbal Explant Cultures. PLoS One. 2016 Oct 10;11(10):e0164408.

22. Koizumi N, Fullwood NJ, Bairaktaris G, Inatomi T, Kinoshita S, Quantock AJ. Cultivation of corneal epi- thelial cells on intact and denuded human amniotic membrane. Invest Ophthalmol Vis Sci. 2000 Aug; 41(9):2506–13.

23. de Araujo AL, Gomes JÁ1. Corneal stem cells and tissue engineering: Current advances and future perspectives. World J Stem Cells. 2015; 7:806–14.

## Acknowledgments

This research work is a result of doctoral research, in part financed by the European Union, European Social Fund and the Republic of Slovenia, Ministry for Education, Science and Sport in the framework of the Operational programme for human resources development for the period 2007 – 2013.

We like to thank the reaserch team at FBOV and the director dr. Diego Ponzin for enabling the collaborative research project.

Z.L. was the recipient of a Boehringer-Ingelheim travel grant and SEE-ARVO travel grant in 2016.