Transplant of Limbal Epithelial Stem Cells on Bioresorbable Scaffold

DOI: 10.17691/stm2017.9.4.05
Received August 23, 2017

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The aim of the study was to develop a method to culture limbal epithelial stem cells (LESC) including their subsequent transplantation onto a scaffold based on bioresorbable fibrin glue for further autotransplantation into the eye of a recipient with limbal insufficiency.

Materials and Methods. Two methods of limbal tissue cultivation were used: 1) with preliminary enzymatic and mechanical tissue dissociation; 2) with planting the entire limbal specimen on a plastic base. Bioresorbable glue from Evicel (Johnson & Johnson, Russia) was used as a scaffold. Immunocytochemical phenotyping was performed using the markers of differentiated cells of the cornea, limbus and conjunctiva and the markers of stem cells.

Results. Immunocytochemical studies have confirmed the presence of cells whose marker profile was characteristic for LESC. The developed method resulted in a transplant based on LESC cultured on a bioresorbable scaffold.

Conclusion. Culturing a limbus biopsy specimen without preliminary dissociation allows one to preserve cell composition and proliferative potential; the method significantly increases the growth of limbal stem cells.

Key words: limbal epithelial stem cells; limbal stem cells deficiency; transplant; scaffold.

The diseases caused by deficiency of limbal epithelial stem cells (LESC) are characterized by corneal opacification, chronic eyeball inflammation, neovascularization, conjunctiva pannus formation, and recurrent erosions; all those changes ultimately lead to a significant decrease in visual acuity [1]. Limbic insufficiency can be caused by burns, Stevens–Johnson syndrome, cicatricial pemphigoid, trachoma, ionizing radiation, ultraviolet radiation, congenital diseases (e.g., aniridia), keratitis and similar keratopathies [2, 3].

Stem cells have been proposed for the pathogenetic treatment of limbic insufficiency; the techniques depend on the type of this deficiency (full or partial, congenital or acquired) [4–6]:

1) limbus autografts from a healthy eye or from close relatives. A disadvantage of the method is the risk of developing limbal deficiency in a previously intact eyeball, obviously this approach cannot be used for bilateral lesions or in hereditary diseases;

2) allogeneic limbus transplants from dead

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bodies. The main disadvantage is that a long-term immunosuppressive therapy is needed;

3) autologous limbal cells cultured on various scaffold (amniotic membrane, collagen scaffold, etc.). The use of this method is limited by technical complexity and cost.

Thus, the existing methods of cultivation and transplantation of limbal cells do not provide stable regeneration of the corneal epithelium and do not maintain the regenerative potential of the corneal epithelium long enough to prepare for a successful corneal transplantation.

Located in the folds of the Vogt palisades, LESC make up a special pool of stem cells in the area of the basal epithelium of the limbus [7, 8]; the LESC participate in cornea restoration after injuries. LESC divide asymmetrically, i.e., one daughter cell allows maintaining the stem cell properties and stem cell pool; the other daughter cell becomes a transient amplified cell and can then differentiate into pterygoid cells, which in turn transform into the terminally differentiated corneal epithelial cells [9]. As compared with other somatic stem cells, LESC have their own characteristics: a small size, a high nuclear-cytoplasmic ratio, the absence of differentiation markers. In addition, these cells are characterized by the presence of α-enolase, the EGF receptors, the pigment, as well as vimentin and cytokeratin 14, 15, 19 [10, 11]. The major positive markers of LESC are factor p63 and the G-2 subunit of ABCG2 protein [12, 13]. Among the commonly used negative markers of stem cells are cytokeratin 3 (CK3) and cytokeratin 19 (CK19), as well as the protein connexin 43, which are typical for differentiated cells. CK3 is a marker of terminal differentiation; it is present in all differentiated cells of the corneal and limbal epithelium, but not in cells of the conjunctival epithelium. CK19 is present in all conjunctival and limbal epithelial cells, as well as in the peripheral basal cells of the cornea. Unfortunately, until now no specific LESC marker has been identified, so a combination of positive and negative markers is the most reliable way of phenotyping the putative LESC of the limbal epithelium.

The aim of the study was to develop a method for culturing limbal epithelial stem cells for further planting onto a scaffold from fibrin glue and subsequent transplantation to recipients with limbal stem cells deficiency.

Materials and Methods

Cell culture. Sampling of limbal cells was performed on an adult rabbit under the operating room conditions. A sample of 2–3 mm long and about 200 μm thick was excised from the limbus together with ~1.5 mm of the cornea and the conjunctiva. All experimentations on animals were carried out in accordance with the guidelines specified in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and with the National standard of the Russian Federation 33044-2014 “Principles of Good Laboratory Practice”, and with the ethical principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (adopted in Strasbourg on 18.03.1986 and confirmed in Strasbourg on 15.06.2006). The study protocol was approved by the Ethics Committee of the Nizhny Novgorod State Medical Academy.

All tissue processing and cell cultivation were performed under aseptic conditions in a laminar flow cabinet. The limb tissue was washed several times in Hanks’ solution (PanEko, Russia) containing the antibiotic gentamicin at 100 μg/ml (Krka, Slovenia).

For planting the culture, two methods were used: 1) mechanical and enzymatic dissociation of the limbus tissue followed by cell sedimentation onto the plastic surface; 2) planting the limbus tissue directly on the plastic base without preliminary dissociation.

Method 1. The isolated tissue of the limbus was mechanically and enzymatically disaggregated: crushed with two scalpels to a size of ~1 mm^3 and then incubated in 0.25% trypsin solution (Invitrogen, USA) for 15 min at 35.5°C. To inactivate trypsin, an excess of Dulbecco’s modified Eagle’s medium and Ham’s F12 — DMEM-F12 (Invitrogen, USA) + 10% fetal bovine serum (PanEco, Russia) was added to the suspension. The suspension was then centrifuged for 5 min at 1,000 rpm. The pellet was resuspended in the growth medium: DMEM-F12 + 10% fetal bovine serum. The suspended cells obtained from one biopsy specimen were poured into 3 ml of the medium in a plastic Petri dish of 60 mm in diameter. There the cells got attached to the bottom and then actively proliferated.

Method 2. The excised limbus sample was mechanically separated into 5–8 mm fragments using two scalpels, and then placed in 60 mm plastic Petri dishes filled with the growth medium (DMEM-F12 and 10% fetal bovine serum).

The organotypic tissue samples and dissociated cells were cultured at 35.5°C and 5% CO_2 in a MCO-18AIC incubator (Sanyo, Japan) until a cell monolayer of 90–100% confluency was reached. One ml of the medium was replaced every 3 days.

The development of the cultures was assessed using an inverted microscope DM1000 (Leica Microsystems, Germany). The rate of culture growth was quantified by the time needed to form a cell monolayer of 90–100% confluence.

After the formation of a cell monolayer, limbal stem cells were removed from the plastic surface by trypsinization and transplanted onto a bioresorbable carrier — the scaffold consisted of a translucent fibrin glue film located at the bottom of a culture plate well of 15.4 mm in diameter. The scaffold with attached cells was cultured at a constant temperature of 35.5°C, under 5% CO_2 in a humidified incubator (MCO-18AIC).

Immunocytochemical phenotyping of cell cultures. The protocol for cell staining in the culture included fixation with 70% ethanol, incubation with primary antibodies — monoclonal mouse antibodies

to CK19, clone Ks19.1 (Lab Vision Corporation, USA) and to CKpan, clone AE1/AE3 (Lab Vision Corporation, USA). In the case of immunofluorescent staining, primary rabbit polyclonal antibodies to the SOX2 protein (Abcam, USA) or to the vimentin protein [EPR3776] — Cytoskeleton Marker (Abcam, USA) were also added to the mixture. The reaction products were visualized using the UltraVision Quanto Detection System (Lab Vision Corporation, USA) according to the manufacturer's protocol but without blocking the peroxidase activity and without protein blocking; in this case, the cell nuclei were further stained with Mayer's hematoxylin. Alternatively, the visualization step was carried out using secondary goat anti-mouse antibodies attached to fluorescent probes — Goat Anti-Mouse IgG H & L — Alexa Fluor® 488 (Thermo Fisher, USA) and anti-rabbit antibodies Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody — Alexa Fluor® 555 (Thermo Fisher, USA); in this case, the nuclei were stained with Hoechst 33342 (BD Pharmingen, USA). The cells stained with fluorescent markers were examined using a confocal fluorescence laser scanning microscope LSM 710 (Carl Zeiss, Germany).

Results

Cultivation of dissociated limbal cells on a plastic base before their transplantation onto a scaffold. In the cultured dissociated limbal cells, the growth of a cell monolayer was different from that in the organotypic limbus tissue cultures.

In Method 1, the primary culture (day 1) was actually a cell suspension (Figure 1 (a)). Few cells began attaching to the plastic bottom after 1–2 days. On day 5, the culture of dissociated limb cells consisted largely of phenotypically heterogeneous cells; most of the cell mass formed a suspension, and only few cells were attached to the bottom of the Petri dish (Figure 1 (b)). Subsequently, the attached cells proliferated, forming multicellular clumps on the bottom. The cell cultures reached 90–100% confluency after about 3 weeks (22.0±2.6 days) upon planting of the dissociated cells (Figure 1 (c)).

In Method 2 (non-dissociated tissue samples), the primary culture (day 1) contained only a limbus tissue specimen measuring 5–8 mm (Figure 2 (a)). On day 2, the limbus tissue was seen attached to the plastic bottom; in the lower tissue layers, cells (heterogeneous by phenotypic composition) were found actively proliferating (Figure 2 (b)). Upon growing, the culture covered most of the bottom with a homogeneous monolayer that formed a thin film visible to the naked eye. Here, the rate of cell proliferation and monolayer formation was significantly higher than that observed in the cultures of dissociated cells (Figure 2 (c)). The 90–100% confluence stage was reached after 7.5±0.5 days.

Thus, both methods of cultivation resulted in a growth and proliferation of limbal stem cells, sufficient for transplantation onto the fibrin glue scaffold. As mentioned, the cell growth rate was higher in the cultures without preliminary dissociation of limb tissue.

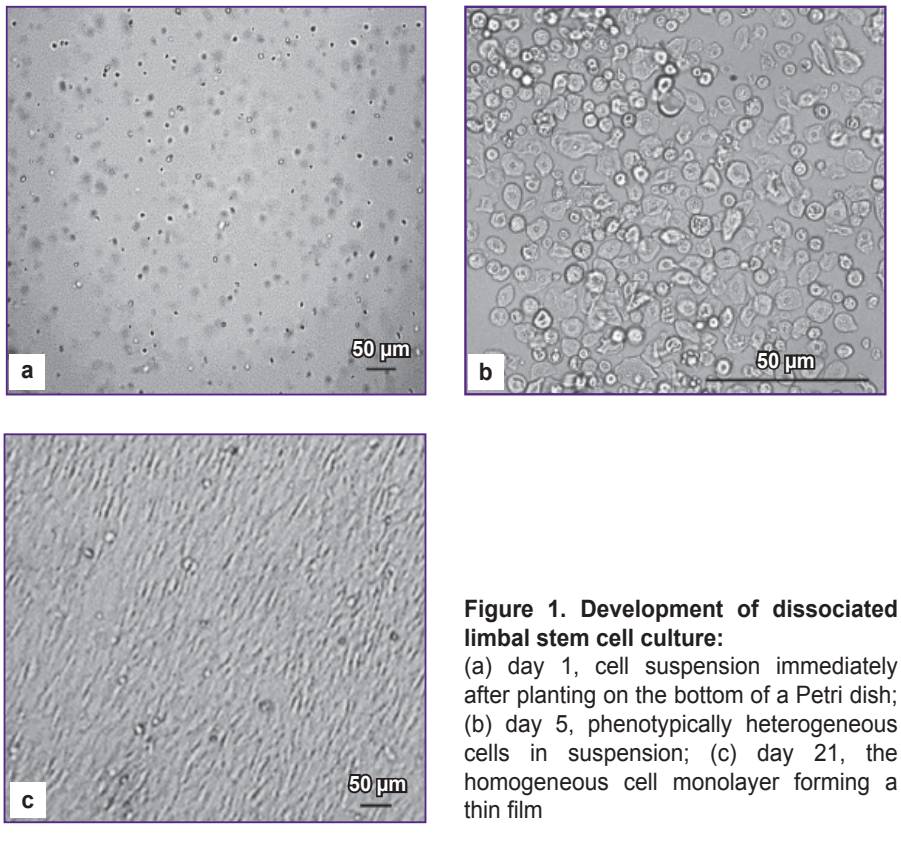

Figure 1. Development of dissociated limbal stem cell culture:
(a) day 1, cell suspension immediately after planting on the bottom of a Petri dish; (b) day 5, phenotypically heterogeneous cells in suspension; (c) day 21, the homogeneous cell monolayer forming a thin film

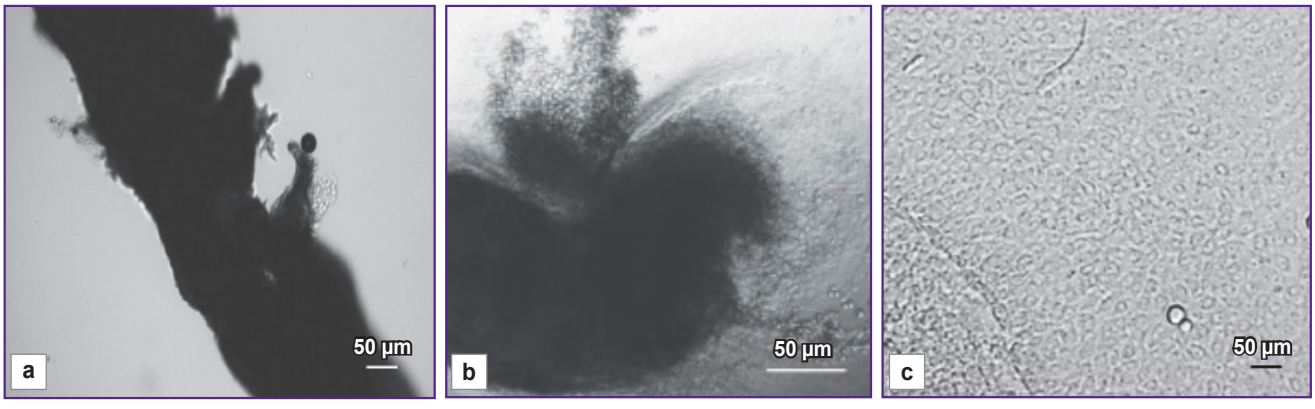

Figure 2. Development of limbus tissue specimen without prior dissociation:
(a) day 1, a specimen of the limbus immediately after placement on the substrate; (b) day 3, the limbus tissue attached to the plastic surface, in the lower part of the sample, phenotype-diverse cells actively proliferate; (c) day 5, the homogeneous cell monolayer forming a thin film

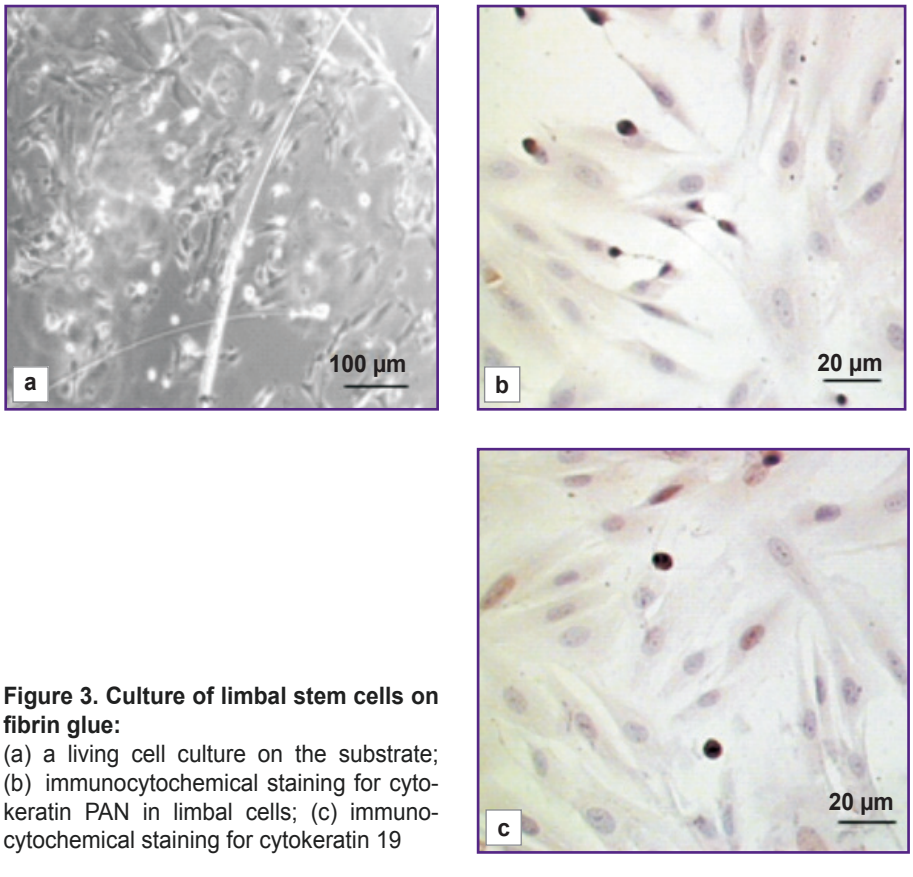

Figure 3. Culture of limbal stem cells on fibrin glue:
(a) a living cell culture on the substrate; (b) immunocytochemical staining for cytokeratin PAN in limbal cells; (c) immunocytochemical staining for cytokeratin 19

Transplantation of limbal epithelial stem cells onto the scaffold. Following the enzymatic removal from the Petri dish and upon planting on the scaffold, the cells gradually attached to the fibrin glue layer and proliferated on it (Figure 3 (a)). A cell monolayer was formed within 15–17 days with the cells obtained by Method 1 and within 5–6 days with the cells obtained by Method 2.

Phenotyping of limbal stem cells in culture. Immunocytochemical and immunofluorescent staining revealed the presence of isolated differentiated cells with some amounts of cytokeratins (Figure 3 (b), (c)). Those were small, rounded cells with the cytoplasm well-defined after staining with fluorescent-labeled antibodies to proteins CKpan and CK19 (Figure 4). A small number of SOX2-positive nuclei were also present (Figure 4, *top row*). The culture consisted largely (>95%) of vimentin-positive cells (Figure 4, *bottom row*).

The results of the immunocytochemical and

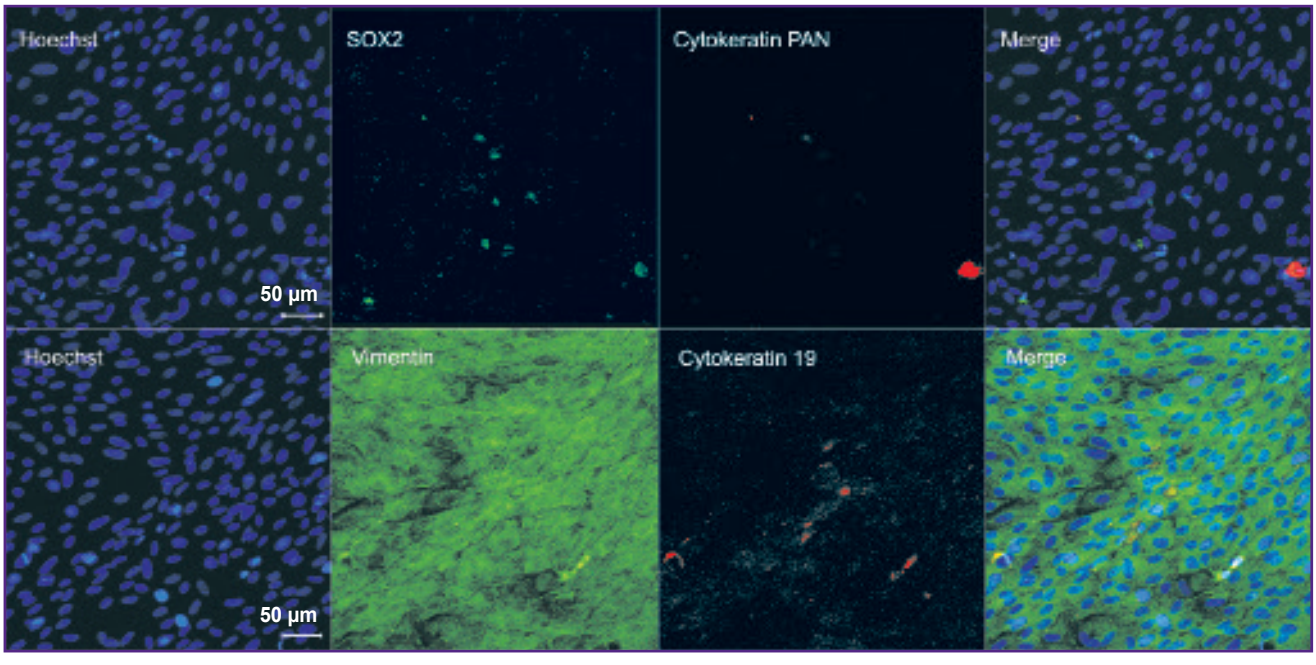

Figure 4. Expression of markers on limbal stem cells in culture:
top row — a small number of cells with SOX2-positive nuclei (*green color*); also present is a rounded cell positive for cytokeratin PAN (*red color*); the cell nuclei are stained with Hoechst 33342 (*blue color*); *bottom row* — positive staining for vimentin (*green color*) in most of the cultured cells; several cells show co-expression of vimentin with cytokeratin 19 (*red color*). The cell nuclei are stained with Hoechst 33342 (*blue color*)

immunofluorescence analysis indicated that most of the cultured cells were vimentin-positive with small numbers of SOX2-, CKpan- and CK19-positive cells. The absence of the differentiation markers (CKpan and CK19) and the presence of the "stem" cell marker (vimentin) proved that the culture consisted mostly of limbal stem cells.

Thus, the proposed method for cultivation of a limbus tissue specimen without preliminary dissociation allows preserving its cell composition and proliferative potential; the method significantly increases the growth rate of limbal stem cell. The level of 90–100% confluence is reached within a week in cultures initiated with a whole tissue specimen, but it takes 3 weeks to cultures with preliminary tissue dissociation.

Conclusion. The present approach to culturing a monolayer transplant of limbal epithelial stem cells on bioresorbable fibrin glue Evicel can be used as a prototype for producing cells needed to treat limbal deficiency in the eye.

Financial Support. The study was not funded by any sources.

Conflicts of Interest. The authors claim no conflicts of interest.

References

1. Pozdeeva N.A., Tonaeva H.D., Borzenok S.A. Allolimbal transplantation in the treatment of patients with insufficiency of limbal stem cells in case of congenital aniridia. *Meditsinskiy al'manakh* 2014; 1(31): 74–77.

2. Kuzmenko V.V., Stupnikova T.V., Kheyfets Yu.B., Vavilova L.M. Application of stem cells in ophthalmology. *Razrabotka i registratsiya lekarstvennykh sredstv* 2015; 4(13): 128–133.

3. Freeman J.M. Associated with aniridia keratopathy. *Prakticheskaya meditsina* 2015; 1–2(87): 62–69.

4. Pasechnikova N.V., Grin V.K., Drozhzhina G.I., Popandopulo A.G., Ivanova O.N., Kavelina A.S. Three-dimensional graft of limbal cornea cells. *Vestnik neotlozhnoy i vosstanovitel'noy meditsiny* 2012; 13(1): 99–102.

5. Popandopulo A.S., Kavelina O.N., Ivanova G.I., Drozhzhina A.G. The role of limbal cells in regeneration of cornea. *Tavricheskiy mediko-biologicheskiy vestnik* 2013; 1–2(61): 158–160.

6. Gundorova R.A., Makarov P.V., Terskikh V.V., Vasil'ev A.V., Khodzhabekyan G.V. Prospects of application of new biotechnology methods in regulation of corneal regeneration. *Vestnik oftal'mologii* 2004; 120(6): 49–52.

7. Yoon J.J., Ismail S., Sherwin T. Limbal stem cells: central concepts of corneal epithelial homeostasis. *World J Stem Cells* 2014; 6(4): 391–403, https://doi.org/10.4252/wjsc.v6.i4.391.

8. Joseph A., Powell-Richards A.O., Shanmuganathan V.A., Dua H.S. Epithelial cell characteristics of cultured human limbal explants. *Br J Ophthalmol* 2004; 88(3): 393–398, https://doi.org/10.1136/bjo.2003.018481.

9. Nieto-Miguel T., Calonge M., de la Mata A., López-Paniagua M., Galindo S., de la Paz M.F., Corrales R.M. A comparison of stem cell-related gene expression in the progenitor-rich limbal epithelium and the differentiating central corneal epithelium. *Mol Vis* 2011; 17: 2102–2117.

10. Kurpakus M.A., Maniaci M.T., Esco M. Expression

of keratins K12, K4 and K14 during development of ocular surface epithelium. *Curr Eye Res* 1994; 13(11): 805–814, https://doi.org/10.3109/02713689409025135.

11. Yoshida S., Shimmura S., Kawakita T., Miyashita H., Den S., Shimazaki J., Tsubota K. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci* 2006; 47(11): 4780–4786, https://doi.org/10.1167/iovs.06-0574.

12. Ksander B.R., Kolovou P.E., Wilson B.J., Saab K.R., Guo Q., Ma J., McGuire S.P., Gregory M.S., Vincent W.J., Perez V.L., Cruz-Guilloty F., Kao W.W., Call M.K., Tucker B.A., Zhan Q., Murphy G.F., Lathrop K.L., Alt C., Mortensen L.J., Lin C.P., Zieske J.D., Frank M.H., Frank N.Y. ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* 2014; 511(7509): 353–357, https://doi.org/10.1038/nature13426.

13. De Paiva C.S., Chen Z., Corrales R.M., Pflugfelder S.C., Li D.Q. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 2005; 23(1): 63–73, https://doi.org/10.1634/stemcells.2004-0093.