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## ••• БІОТЕХНОЛОГІЯ ••• BIOTECHNOLOGY •••

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### **Creation of cornea biograft A.S.Kavelina**

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In recent years, the transplantation of cultured stem cells is considered the most promising method that allows influencing the process of human tissue reparation. Ophthalmologists are studying the possibility of introducing the new technology called ex vivo cultivation of epithelial stem cells to clinic. The technology enables to avoid possible complications connected with limbal autograft transplantation. Different researches by means of methods in vitro and in vivo demonstrated that amniotic membrane can be successfully used as a substrate for epithelial stem cells cultivation and their further application in the treatment of severe ocular surface disease. Peculiarities of the tissue structure and functions of amniotic membrane define the number of characters that can attract researchers and clinicians to use limbal and conjunctival epithelial covering in the treatment of corneal disease.

**Key words:** *limbal stem cells, amniotic membrane.*

### **Створення біотрансплантанта рогівки А.С.Кавеліна**

В останні роки в літературі розглядають трансплантацію культивованих стовбурових клітин як найбільш перспективний метод, що дозволяє впливати на хід репарації тканин організму людини. Офтальмологами вивчається можливість застосування в клініці нової технології, званої культивуванням ex vivo епітеліальних стовбурових клітин, яка дозволяє уникнути потенційних ускладнень, пов'язаних з пересадкою лімбального аутографта. Різними дослідниками in vitro та in vivo було продемонстровано, що амніотична мембрана може бути успішно використана в якості субстрату для культивування епітеліальних стовбурових клітин та їх подальшого застосування в лікуванні важкої патології поверхні ока. Особливості тканинного складу і функції амніотичної оболонки визначають ряд якостей, наявність яких приваблює дослідників і клініцистів до використання при лікуванні патології корнеальних, лімбальних і кон'юнктивальних епітеліальних покривів.

**Ключові слова:** *лімбальні стовбурові клітини, амніотична мембрана.*

### **Создание биотрансплантанта роговицы А.С.Кавелина**

В последние годы в литературе рассматривают трансплантацию культивированных стволовых клеток как наиболее перспективный метод, позволяющий влиять на ход репарации тканей организма человека. Офтальмологами изучается возможность применения в клинике новой технологии, называемой культивированием ex vivo эпителиальных стволовых клеток, которая позволяет избежать потенциальных осложнений, связанных с пересадкой лимбального аутографта. Разными исследователями in vitro и in vivo было продемонстрировано, что амниотическая мембрана может быть успешно использована в качестве субстрата для культивирования эпителиальных стволовых клеток и их последующего применения в лечении тяжелой патологии поверхности глаза. Особенности тканевого состава и функции амниотической оболочки определяют ряд качеств, наличие которых привлекает исследователей и клиницистов к использованию при лечении патологии корнеальных, лимбальных и конъюнктивальных эпителиальных покровов.

**Ключевые слова:** *лимбальные стволовые клетки, амниотическая мембрана.*

#### **Introduction**

Over the past decade in corneal pathology the importance of degenerative diseases has been increased which are becoming one of the leading causes of blindness, leading to permanent incapacity for

work and invalidization of working-age population (Gorgiladze, Ivanovskaya, 1992; Kasparov, 2009; Novitsky, 2003). A significant amount of work, both by domestic and foreign authors, are devoted to the study of etiology, pathogenesis and treatment of this disease (Gorgiladze, Ivanovskaya, 1992; Drozhzhina et al., 2011; Popandopulo et al., 2014; Pasechnikova et al., 2011; Bonini et al., 2003; Davis, Dohlman, 2001; Lambiase et al., 1999).

Functional failure of the corneal surface is associated with dysfunction of the epithelium of varying degrees of severity, which morphologically manifests as a type of cell metaplasia – limbal deficiency syndrome (LDS) (Mackie, 1995; Pushker et al., 2001). Two types of LDS are defined. The first involves a pathological condition caused by the direct destruction of limb cells. The second type of the syndrome occurs in the form of a slowly progressive disease (Lambiase et al., 1999). The most common causes of the occurrence of LDS are: viral infections, chronic injuries, inflammatory diseases of the eye surface, wearing of contact lenses, hereditary corneal dystrophy, consequences of chemical burns, injuries, paralysis of the 5th cranial nerve, surgery on the cornea and on other parts of the eyes, etc. Impairment of a corneal sensitivity is observed in some systemic diseases (diabetes, multiple sclerosis, leprosy, vitamin A deficiency and others), and also as a result of the toxic effects of certain medications – applying of topical anesthetic, timolol, non-steroidal anti-inflammatory drugs, dexamethasone and others (Drozhzhina et al., 2011; Kasparov, Trufanov 2003; Pasechnikova et al., 2011; Azuara-Blanco et al., 1998; Bonini et al., 2003; Tsai et al., 2000).

In the clinical course of neurotrophic corneal diseases three stages have been provided. The first stage is characterized by a point keratopathy, epithelium hyperplasia and its irregularity, superficial neovascularization and the formation of turbidity in the stroma. The second stage is characterized by the presence of persistent epithelial defects, stromal edema, descemet membrane folds, it's possible inflammatory reaction in the anterior chamber, sometimes with the presence of a sterile hypopyon. For the third stage, the formation of corneal ulcers is typical, which can be complicated by melting stroma, perforation and causes total loss of vision (Mackie, 1995).

Medical treatment of neurotrophic keratopathy (keratitis) is one of the most difficult in ophthalmology. The complexity of the treatment of the cornea disease under consideration is associated with a variety of etiologic factors, pathogenesis peculiarities and clinical manifestations of the disease, long-term therapy, as well as the development of serious complications (Kasparov, 2009; Lambiase et al., 1999; Pushker et al., 2001; Solomon et al., 2003; Tseng, Tsubota, 2001). Therefore, the improvement of existing and development of new treatments of this severe disease of the cornea is the actual problem of ophthalmology.

For stimulating of the regeneration of corneal tissue the conservative and surgical methods are used. With tectonic purpose as a temporary coverage of the corneal surface, different materials are applied and methods of their use: therapeutic soft contact lenses, transplantation of a donor cornea and conjunctiva, amniotic membrane (AM), cultured stem cells, limbal autograft and allograft transplantations (Demin et al., 2002; Kasparov, 2009; Kim, Tseng, 1995; Koizumi et al., 2001; Lee, Tseng, 1997; Mackie, 1995).

Today in ophthalmology ex vivo using of cultured corneal stem cells is considered as the most perspective method, which permits to avoid potential complications associated with limbal autograft transplantation and allowing stimulating repair processes in pathological conditions of the neurotrophic cornea genesis.

By experimental studies it have been proved that for a long-term cultivation and reproduction of various types of epithelial cells the presence of a nutrient medium or substrate, a sufficient amount of growth factors and cytokines is necessary (Bonini et al., 2003; Koizumi et al., 2000, 2001; Sato et al., 1998; Von Versen-Hoynck et al., 2004). Nowadays, there are different models of cultivation of limbal stem cells (LSC). A method of culturing corneal epithelial cells on basement membrane obtained from rabbit corneas has been proposed (Friend, 1982). Later on the possibility of using of other substrates, such as a hydrogel coated with fibronectin, collagen matrices, contact lenses, fibrin, etc., has been studied for ex vivo cultivation of the epithelium (Kasparov, 2009; Francisco, 2000; Lambiase et al., 1999; Tseng, Tsubota, 2001).

Recently various in vitro and in vivo studies have demonstrated that AM can be successfully used as a substrate for the cultivation of the LSC with a subsequent application in the treatment of severe ocular surface pathology, coursing with LDS (Demin et al., 2002; Kasparov, Trufanov, 2003; Novitsky, 2003; Dua et al., 2004; Meller et al., 2000; Solomon et al., 2003). Due to the content of significant amounts of cytokines, growth factors, metalloproteinases, antimicrobial factors AM contributes to the cultivation on the own surface of epithelial cells. And moreover, existing of biologically active substances in the structure of the amnion reduces apoptosis, contributes to the renewal and increase of regenerative activity of the remaining regional stem cells, encourages the movement and fixation on the surface of the cornea of mature epithelial cells,

reduces the processes of fibrosis, inhibits neovascularization (Koizumi et al., 2000; Von Versen-Hoyneck et al., 2004).

The purpose of the study is creation of biotransplantant on the AM foundation with layered culturing of limbal stem cells and squamous cells of the human cornea.

### **Materials and methods**

Isolation of corneal cells has been carried out in the laboratory of cell and tissue cultivation, V.K.Husak Institute of Urgent and Reconstructive Surgery, Ukrainian Academy of Medical Sciences, in appliance with GMP standards in compliance with aseptic conditions and in accordance with the laws of Ukraine. The resulting cell cultures have been tested for contamination by infectious agents.

The donor cornea has been washed in phosphate buffered saline. Areas of palisades of Vogt have been allocated. For obtaining limbal epithelial cells, containing in its composition stem cells, limbal graft has been cut out from the entire circumference of the limb. The obtained corneoscleral ring has been placed in a sterile Petri dish (d=60 mm), repeatedly washed with PBS, crushed to a size of 2×2 mm and gently placed on the bottom of the culture flask, treated with collagen.

Then, flasks have been filled with nutrient medium containing DMEM/F12, L-glutamine, ascorbic acid, fetal bovine serum, epidermal growth factor (EGF) and fibroblast growth factor (bFGF), penicillin, streptomycin, insulin isoproterenol, transferrin, hydrocortisone. Cultivation have been carried out in a CO<sub>2</sub> incubator, containing 95% atmospheric humidity at a temperature of 37°C. After reaching a confluent layer passaging has been performed. Visualization of cell lines has been carried out with the help of phase-contrast microscopy by our previously worked protocol (Grin et al., 2011). Medium has been changed every three days.

For the isolation of squamous epithelial cells (SECs) the central portion of the cornea has been used by scraping the upper epithelial layers of the cornea with a further enzymatic treatment by trypsin-EDTA solution 0.25% («Sigma», USA) for 7–10 minutes at 37°C. Further culturing has been carried out in a nutrient mixture DMEM/F12 media, 1:1 («Sigma», USA) supplemented with serum and growth factors for 7–10 days in CO<sub>2</sub> incubator at 37°C, with 5% CO<sub>2</sub> and 95% humidity. During this period, the change of the activity of cell proliferation and their morphology have been observed, and time intervals of changes in the characteristics of cell cultures also have been marked.

At formation of a monolayer, cell lines have been passaged with 0.5% solution of trypsin («Sigma», USA) in a ratio of 1:1 to a solution of Versene ("Biolot", Russia).

AM has been defrosted at ambient temperature, washed three times in a solution of PBS («Sigma», USA). Then it has been carefully smoothed out on sterile foil and cut into small pieces of 1×1cm<sup>2</sup>. Samples have been placed in the alveolar plateau (μ-Slide 8 well, IBIDI, GmbH). 10 thousand of LSC in 200 mcl of nutrient medium have been placed on the surface of the amnion. Medium has been changed every 48 hours (Popadopulo, 2014). After reaching confluent monolayer on the surface of the AM, suspension of SECs of the cornea has been placed and cultured for 3 days under the same conditions. Immunohistochemical staining has been performed. To evaluate the morphological structure of AM and cultured cells of the human cornea the staining with hematoxylin and eosin by Romanovsky, immunohistochemistry have been used.

For the proving of the stemness of limbal cells of the human cornea, a number of markers have been used, the most demonstrative among which are p63-stem cell marker, cytokeratin 19, a marker of limbal basal cells, cytokeratin 3.12 marker of stem cell differentiation, CD 117, ALDH3A1, CD 34, CD 45, α-SMA, keratan sulfate.

### **Results and discussion**

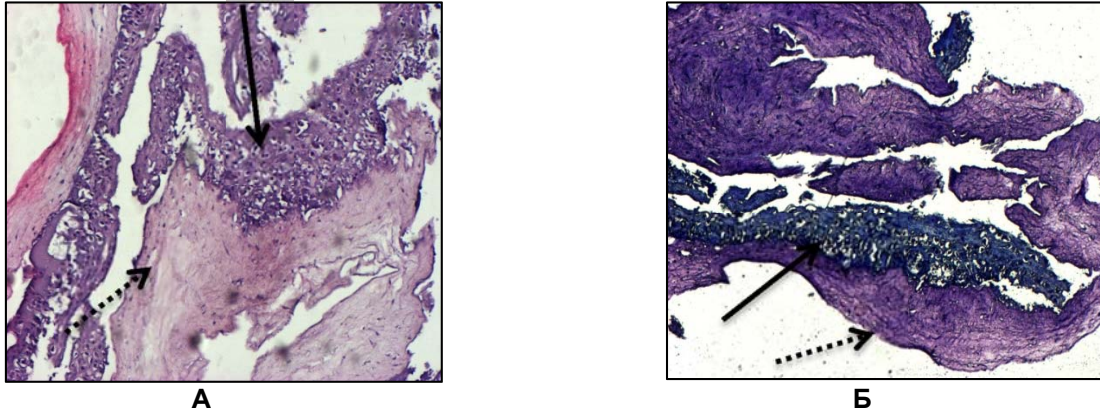
In the study the purpose of layered culturing of two cell subpopulations on the surface of AM has been achieved, taking into account the properly prepared membrane and selected nutrient media.

Throughout the entire cultivation of LSC the expression of nuclear p63 marker has been observed, required to maintain the compartment of population of stem limb cells. The presence of cytokeratin 3.12 indicates the differential potential of the LSC population. The presence of corneal crystalline ALDH3A1 determines transparency of the cornea. The marker CD 117 is important in cell survival, proliferation and differentiation. LSC have not expressed the following markers: CD 34, CD 45, α-SMA, keratan sulfate.

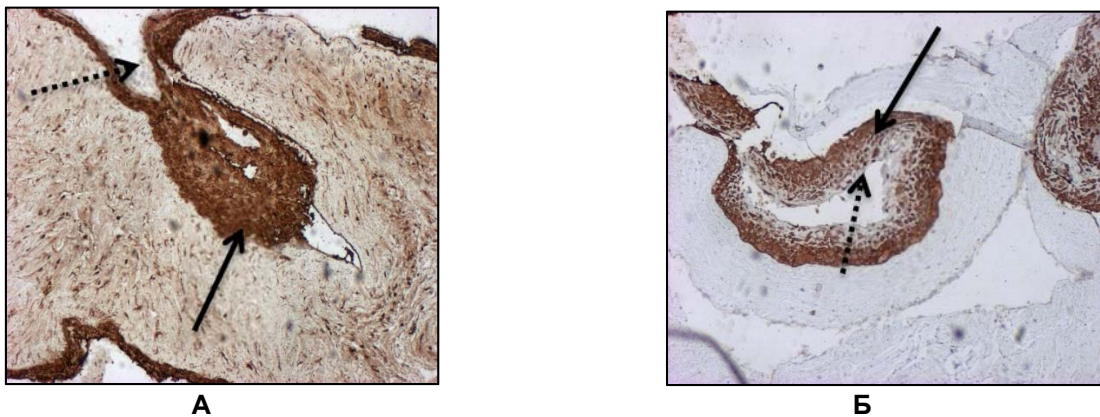
The specific antibodies which have been selected to identify the LSC do not demonstrate positive staining of SECs of the human cornea.

Cultivation of LSC and SECs of the human cornea on AM within 5–7 days makes it possible to form on its surface 2 monolayers of cells with strong intercellular interconnections.

The biogenic material which serves for providing favorable conditions of the regeneration of the corneal epithelium is biologically active and is able to restore temporarily or permanently the anatomical integrity and functional usefulness of the epithelium. Constant availability of reserve of sterile and easy to use surgeon material is also important.

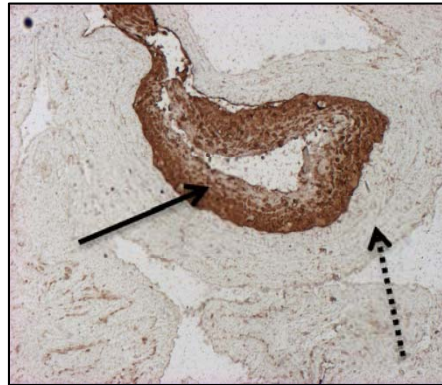


**Figure 1. Microphotos of cultured LSC and SECs (black arrow) on the surface of AM (dotted arrow): staining with hematoxylin and eosin, an increase of 45 (A); by Romanovsky (B), an increase of 35**



**Figure 2. Microphotos of cultured LSC (black arrow) and SECs (dotted arrow): immunohistochemical staining with cytokeratin 19 (A), an increase of 30; cytokeratin 03.12 (B), an increase of 30**

Thus, conducted studies have shown that the use of AM as a carrier of cultured cells simulates image of peculiar niche for corneal cells. The biological significance of stem cells (SC) is that they are playing a leading role in the organization of multicellular organisms and are central element of the structural and functional units of tissues and organs. Proliferative potential of the SC in renewing tissues is very high and the life span of the population of SC can greatly exceed the life span of the organism. For the development of new SC great importance has the microenvironment, where the contact with the surrounding cells and interaction with the matrix and growth factors occur.



**Figure 3. Immunohistochemical staining for specific marker P63: LSC and SECs (black arrow) of cultured on the surface of AM (dotted arrow), an increase of 30**

This concept can serve as the basis for an alternative method for the treatment of severe corneal pathology, accompanied by the LDS with the creation of optimal conditions for stimulation of regeneration of the corneal epithelium, which has all the prerequisites for the widespread introduction of this method in the practice of ophthalmology in Ukraine.

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