

***In vitro* expansion and characterization of corneal stem cells isolated from an eye with malignant melanoma**

O. SAMOILĂ^{1,2)}, OLGA SORIȚĂU³⁾, M. CĂLUGĂRU^{1,2)},
 LĂCRĂMIOARA TOTU¹⁾, S. ȘUȘMAN⁴⁾, CRISTINA CRISTIAN²⁾,
 CARMEN MIHAELA MIHU⁴⁾

¹⁾Ophthalmology Clinic, Cluj-Napoca

²⁾"Iuliu Hațieganu" University of Medicine and Pharmacy, Cluj-Napoca

³⁾"Prof. Dr. Ion Chiricuță" Oncology Institute, Cluj-Napoca

⁴⁾Department of Histology,

"Iuliu Hațieganu" University of Medicine and Pharmacy, Cluj-Napoca

Abstract

Purpose: The objective of this study was the identification, characterization and *in vitro* replication of the human corneal stem cells, taking into consideration the difficulties in obtaining sufficient corneal material from living donors. The study explored a variety of stem cell markers, usually found in embryonic or adult mesenchymal stem cells. Culture medium and replication substrates had to be identified, with no data available on this subject in our country (there are no other reports on corneal stem cells in Romania, to our knowledge). **Materials and Methods:** Corneal epithelial limbus was harvested from an enucleated eye, containing also a choroid malignant melanoma. Stem cells from the limbus were isolated and cultivated *in vitro*. Expression of specific stem cell markers was evaluated with immunocytochemistry. **Results:** Corneal stem cell expansion in primary culture was slow, achieving 70–80% confluence after 28 days. Stem cells were easily isolated in standard medium, showed fibroblastoid morphology and were positive for certain stem cell specific markers in immunocytochemical staining: Oct3/4, SOX2, Nanog, SSEA4, CD44, CD90, CD133, and CD34. They also expressed pan-cytokeratin. Donor age (72 years) and the presence of a malignant tumor close to limbal stem niche could have had an impact on the proliferation rate and the characteristics of the corneal stem cells. **Conclusions:** Isolated limbal cells were adult type stem cells with an epithelial orientation. The characterization of these cells with immunocytochemistry allowed us to observe surface markers that other stem cells also express.

Keywords: limbal, cornea, stem cell, cell culture.

Introduction

The cornea represents a transparent tissue localized at the anterior pole of the ocular globe, closing the scleral foramen, functioning as a convex lens. Histologically, it presents three layers: a pluristratified non-keratinized epithelium at the surface, stroma and endothelium. The homeostasis of the ocular surface is dependent on the health of the corneal epithelium and surrounding conjunctival epithelium. The healing of the corneal epithelium is realized through the proliferation of the basal epithelial layer to the surface and centripetally. In general, the lateral proliferation is limited; thereby, extensive epithelial destructions involving the periphery will be followed by conjunctival invasion and corneal vascularization, with the loss of the corneal transparency, and in the end the loss of vision.

In the last decade, increasing number of studies pointed out the presence of stem cells (LSC's) in the peripheral cornea (at the sclerocorneal limbus). These stem cells are essential in the process of corneal healing, functioning as a deposit of epithelial progenitors. Adult stem cells, maintained in a multipotent state at the level of corneal stem niches are activated by unknown factors (as a response to corneal injury) into transient activated

cells (TAC), which move centripetally and to the surface, becoming differentiated epithelial cells.

Many ocular disorders can destroy the LSC's. Without these cells, the corneal epithelium disappears, being replaced by cells with conjunctival origin, leading to vascular invasion. The most common corneal pathologies concerning stem cell deficits are the chemical burns (alkali), but also Stevens Johnson syndrome, recurrent herpetic disease, trachoma, ocular pemphigoid, contact lens use, aniridia. So far, there is no efficient treatment and the corneal transplant is not efficient in these cases.

The extensive research on LSC's brought a new hope for this category of patients. The LSC niche was described, with potential structures harboring LSC's, like limbal crypts and focal stromal projections. A map was made with stem cell density at the limbus [1]. In the present, it is under investigation the possibility of the *in vitro* corneal stem cells replication, starting from a little amount of corneal tissue to obtain enough tissue needed for transplantation on the injured eye. When there is a stem cell deficit in a corneal sector, there is the possibility to expand *in vitro* the few cells left, using tissue culture techniques. With all the remarkable progress, there are many unknowns concerning corneal stem cells. From controversies related to the exact

localization to those related to the functioning of stem microclimate, the factors that activate stem cells to TAC and even the specific corneal stem markers, the research area it is vast.

There are several approaches for the cultivation of the corneal LSC's. Adult limbal progenitors normally are mitotically quiescent *in vivo* but can be activated to proliferate *in vitro*. Without fibroblast feeder layers, they quickly differentiate and lose stemness. The current practice employed for the expansion of the corneal limbal stem cells is the use of amniotic membrane with or without the feeder layer [2].

The objective of this study was the replication of the corneal stem cells *in vitro*, the identification and characterization of the human corneal stem cells, taking in consideration the difficulties in obtaining sufficient corneal material from living donors. The study explored a variety of stem cell markers, usually found in embryonic or adult mesenchymal stem cells (ranging from the surface markers to genes involved in pluripotency – Nanog, Oct3/4, SOX2). Culture medium and replication substrates had to be identified, with no data available on this subject in our country (there are no other reports on corneal stem cells in Romania, to our knowledge).

Materials and Methods

Patient' data

Human corneal epithelial stem cells were harvested with a limbal biopsy from a living donor, under patient consent. The study was approved by the Ethics Committee of "Iuliu Hațieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. A 72-year-old patient presenting a malignant ciliary body melanoma (with ultrasound and angiographic documentation) in the left eye was scheduled for the extraction of the eye globe (enucleation), as a curative treatment. The surgical intervention respected the standard enucleation protocols. At the end of intervention, after the extraction of the eye, a 3–4 mm wide ring was harvested from the peripheral cornea, at the limbus with superficial dissection, without perforation of the eye (which was sent for the histological examination).

Histopathology on the enucleated eye

The enucleated eye was fixed with 10% formalin, sectioned along the longitudinal diameter, passing through the tumor and the optic nerve. The tumor fragments were then embedded in paraffin wax and cut in 5 µm thin sections and stained with Hematoxylin–Eosin (HE).

Isolation protocol for the corneal stem cells

The corneal limbus was placed in a culture medium and transported as soon as possible at the laboratory for processing. The piece was transferred on a Petri dish where it was washed two times with PBS and a mechanical processing was done with sterile instruments. An enzymatic digestion was applied with 0.25% Trypsin EDTA, 0.1% Collagenase type IV (Gibco), for 5 minutes in an orbital shaker at 37°C, followed by vigorous pipetting, inactivation with 50 mL medium containing fetal serum and centrifugation at 1100 rpm for 7 minutes.

The cell pellet was resuspended in complete medium and filtrated through Filcons device (with 70 µm network) to obtain a monocellular suspension and centrifuged one more time. The viability of the cells was determined with the exclusion of the Trypan Blue stain. The cells were seeded on 25 cm² Cole flasks, resuspended in 5–7 mL of culture medium – DMEM high glucose/F12-HAM (1:1 ratio), 15% Fetal calf serum, 1% Antibiotics, 2 mM Glutamine, 1% Non-essential amino acids, 55 µM beta-Mercaptoethanol, 1 mM Natrium Pyruvate (all reagents purchased from Sigma) and incubated at 37°C in an atmosphere with 7% CO₂. In day 7 after isolation, after proofing the adhesion of the first cells, the medium was changed. When 70–80% cellular confluence was reached after four weeks of cultivation, the first passage was realized by trypsinisation. Half of the obtained cells were frozen and stored in liquid nitrogen. After 6th passage, we supplemented the culture medium with growth factors: 20 ng/mL basic Fibroblast Growth Factor (bFGF) and 10 ng/mL Epithelial Growth Factor (EGF).

Immunocytochemistry

Cells at 6th passage cultivated in two wells chamber slides were fixed with 4% Paraformaldehyde after washing three times with PBS. Membrane permeabilization was done with 0.1% Triton X-100 in PBS for 20 minutes at room temperature. 10% BSA (Bovine serum albumin) blocking solution in PBS was added for 20 minutes at room temperature, to avoid unspecific antibody bound. For the expression of stem cells specific markers, primary antibodies diluted at 1:50 ratio were used. The secondary goat anti-mouse antibodies IgG1 marked with FITC (Fluorescein Isothiocyanate) and Texas Red – PE (Phycoerythrin) (Santa Cruz Biotechnologies) were used at the same dilution with primary antibodies for the identification of SOX2, Nanog (R&D), Oct3/4, SSEA4, CD34, CD133, CD73, CD44, CD90, pan-cytokeratin (PCK, Santa Cruz Biotechnologies) (all tagged with FITC), CD105, CD29 and CD49e (BD Biosciences) (all tagged with PE). Incubation with the primary antibody was performed at 4°C during the night and in the case of the secondary antibody for 45–60 minutes at 37°C. The samples were counterstained with an antifade medium containing DAPI (4,6-diamidino-2-phenylindole) in order to evidence the nuclei and were examined using an inverted phase Zeiss Axiovert equipped with soft image analysis AxioVision Rel 4.6, using filters at 488, 546 and 340/360 nm. Image acquisition was done with an AxioCam MRC camera.

Results

Limbal stem cells obtained from a 72-year-old patient presenting a malignant ciliary body melanoma were isolated, characterized and cultivated *in vitro*.

The female patient presented good vision, partial amputation of the temporal vision field, brownish discoloration of anterior sclera, close to cornea at nine o'clock, the site of scleral tumor invasion (Figure 1A), with a dome shaped tumor localized nasally to optic disk (Figure 1B). Fluorescein angiography revealed a dome shaped mass with double vascularization, characteristic to malignant melanoma (Figure 1C).

Echography confirmed the diagnosis, finding a tumor localized nasally at ciliary body with posterior extension and secondary retinal detachment.

The histopathology exam confirmed the clinical findings, identifying a malignant melanoma of the ciliary body, epithelioid type (Figure 1D), with extension to the choroid (causing the detachment of the retina – Figure 1E) and sclera (Figure 1F).

After isolation of cells from human cornea, adhesion of first cells occurred after 2–3 days after initiation of

primary culture. Cells showed a round and elongated morphology as the adhesion process was complete, towards a fibroblastoid typical morphology. Their expansion in primary culture was slow, achieving 70–80% confluence after 28 days, when the first passage was done (Figure 2A). Cells were frozen at the second passage. From this moment, the proliferation rate increased and further passages were performed at each 4–5 days (Figure 2B).

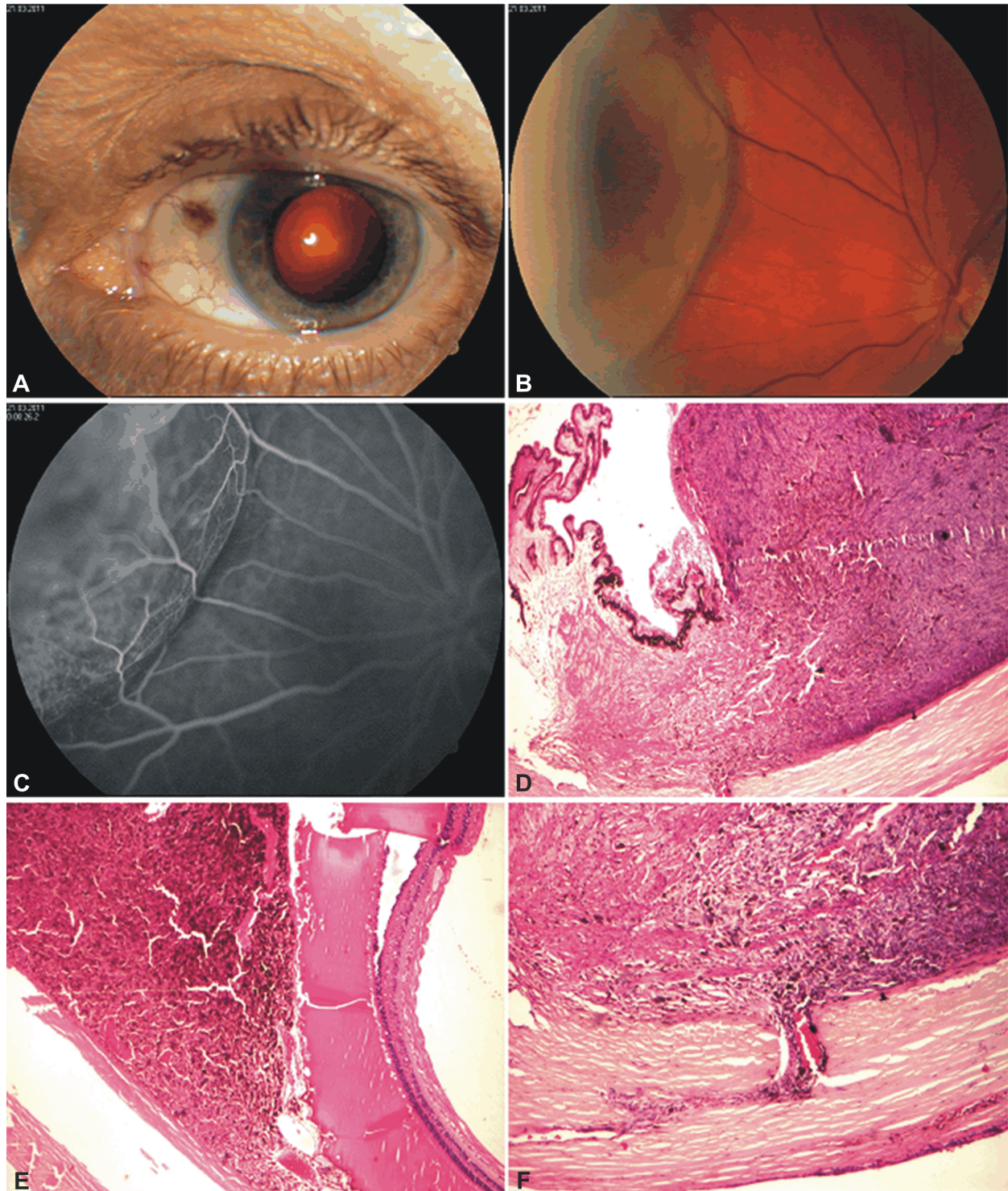


Figure 1 – (A) Biomicroscopic eye examination (scleral tumor extension). (B) Eye fundus photo (grey tumor nasally to optic disk). (C) Fluorescein angiography, late phase (double vascularization of the tumor). (D) Melanic cells at ciliary body, epithelioid type, HE stain, ob. $\times 2$. (E) Choroid invasion with melanic cells, retinal detachment, HE stain, ob. $\times 2$. (F) Ciliary muscle and scleral infiltration, HE stain, ob. $\times 4$.

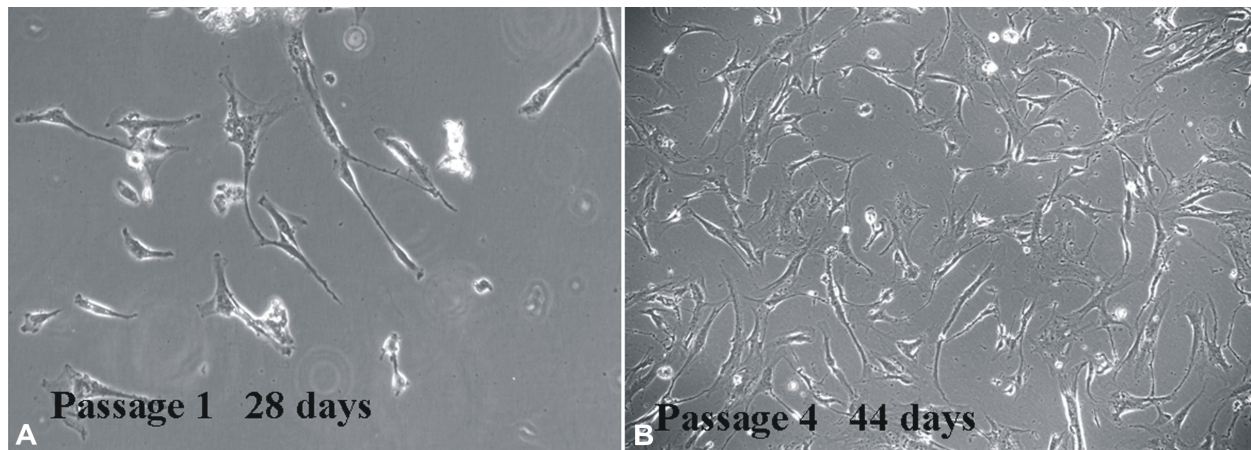


Figure 2 – (A) Phase contrast of adherent cells isolated from limbal cornea, after 28 days of cultivation in standard media, at first passage ($\times 100$). (B) Spindle-like shape of isolated cells at 4th passage ($\times 100$).

After the 6th passage, cells slowed the rate of growth and for this reason we added in the standard stem cell media growth factors: 10 ng/mL EGF and 20 ng/mL bFGF. For a short time, this supplementation of culture media proved to accelerate cell proliferation. Finally, cells entered in senescence and they stopped the proliferation. The cells became enlarged, flattened with multiple dendritic extensions (Figure 3, A and B).

In our study, isolated cells from limbal cornea showed morphologic and phenotypic characteristics of adult mesenchymal stem cells, with a strong capacity of adhesion to culture flasks. Stem cells were easily isolated

in standard medium, showed fibroblastoid morphology and were positive for certain stem cell specific markers in immunocytochemical staining: Oct3/4, SOX2, Nanog, and CD133 (Figure 4).

Staining with monoclonal antibodies against alkaline phosphatase, CD34 and CD73 revealed positivity for these markers (weaker for CD73) (Figure 5).

Positivity for CD44, CD90 and SSEA4 was observed. Cells were negative for CD29 and CD105. Typically, stem cells exhibited some orientation for epithelial progenitors as shown by weak positivity for pan-cytokeratin (Pan-CK) antibody staining (Figure 6).

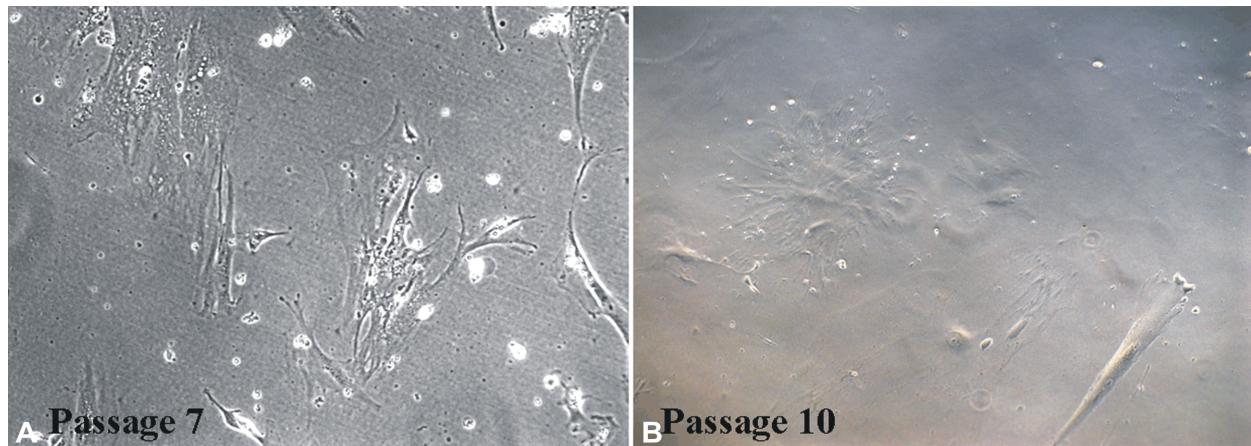


Figure 3 – (A) Phase contrast microscopic aspect after thawing stem cells at 7th passage, with signs of cellular senescence ($\times 100$). (B) Persistence of senescence characteristic morphology, at 10th passage, after cultivation in presence of EGF and bFGF ($\times 100$).

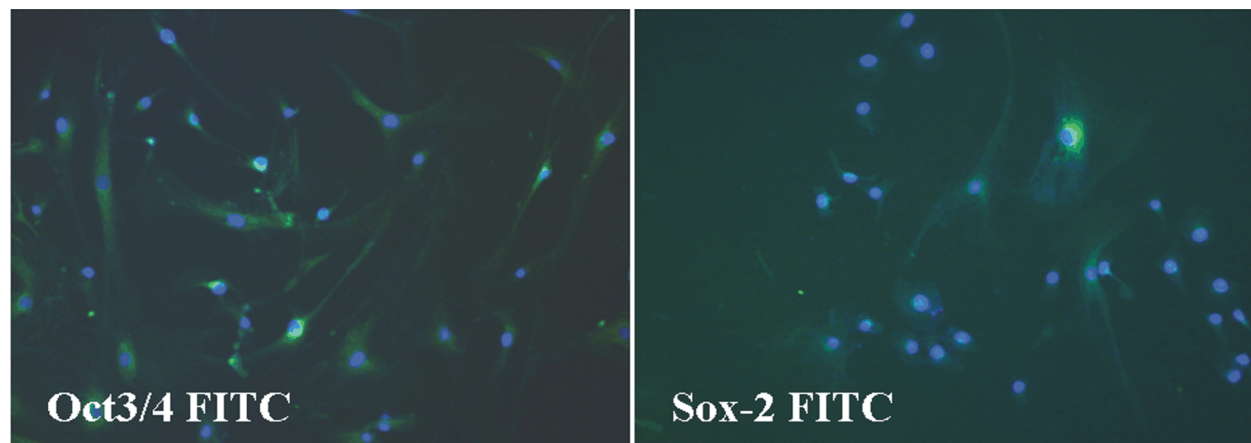


Figure 4 – Immunofluorescent staining for Oct3/4 and SOX2 (FITC, DAPI counterstaining, $\times 200$).

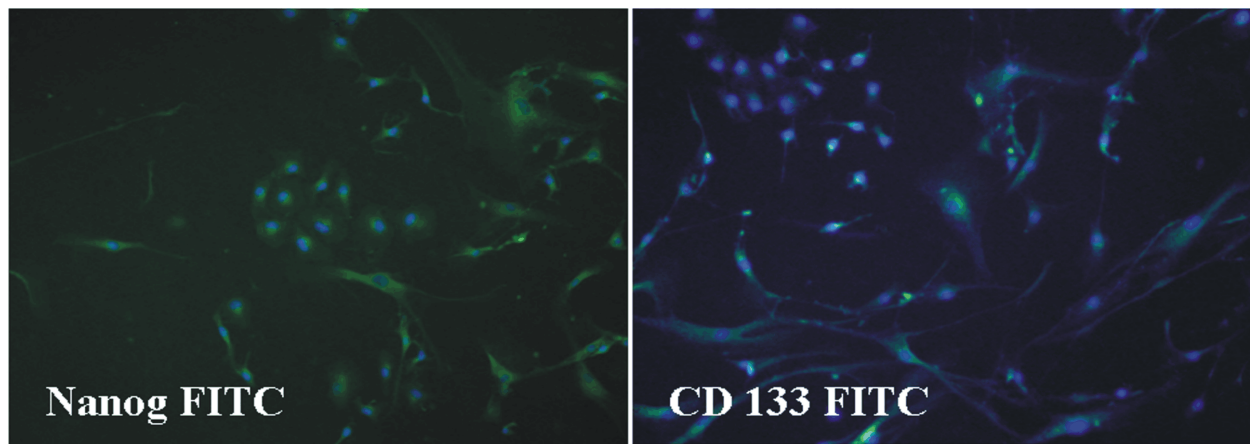


Figure 4 (continued) – Immunofluorescent staining for Nanog and CD133 (FITC, DAPI counterstaining, $\times 200$).

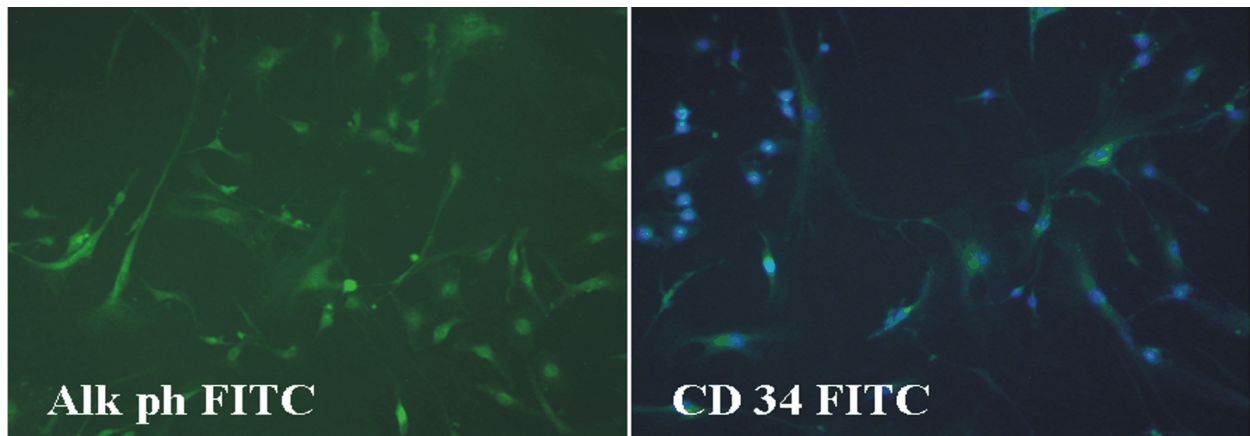


Figure 5 – Immunofluorescence staining for alkaline phosphatase and CD34, at 6th passage (FITC, DAPI counterstaining, $\times 200$).

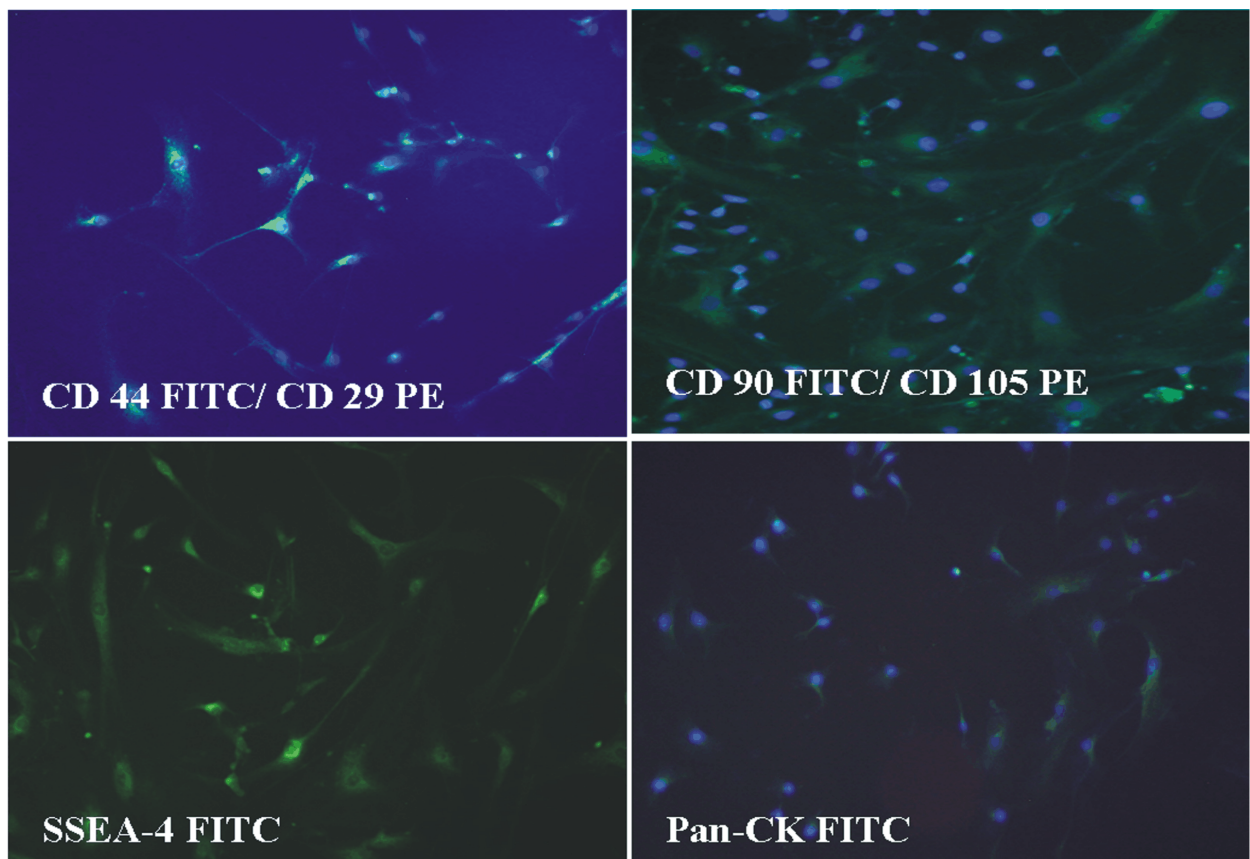


Figure 6 – Immunofluorescent staining for CD44, CD90, CD105, SSEA4, Pan-CK (FITC and PE, DAPI counterstaining, $\times 200$).

Discussion

Stem cells have already entered clinical trials to restore damaged cornea in cases of failure or deficiency of LSC's using autologous methods or allografts, with or without substrate, as reported in first studies in the late 80's [3, 4]. Isolation and cultivation of these cells raise many technical problems even in present days [5, 6]. In our study, proliferation was slow, cells reaching confluence after 28 days, and after the 6th passage cells entered in a senescence process. There are a couple of possible reasons. First, we did not use any substrate for this experiment. Current studies suggest the use of amniotic membrane substrate without surface epithelium to fasten the expansion [2] or a fibroblastic feeder-layer. We used collagenase digestion in cell isolation protocol, a method described by Chen S *et al.* to be more appropriate for obtaining limbal epithelial progenitor. This method maintains associated mesenchymal cells and preserves some basement membrane matrix [7]. Secondly, LSC's came from a 72-year-old patient, with a malignant melanoma growing for months inside the eye. Old age is a major risk factor for many diseases and disorders. Decline in the function of stem cells with age, like other cells of the body, results in an imbalance between cell loss and cell renewal [8]. Replicative senescence was first described in Hayflick's model, which suggested that normal diploid cells show a finite limit of cultivation *in vitro*, while malignant cells are capable of unlimited mitotic potential [9]. Today, we know that limited lifespan of normal cells is due to entry into a permanent non-proliferative stage of senescence, cells characterized by large, flattened body, associated with a high frequency of nuclear anomalies, often with the appearance of multinucleated cells or giant polyploid cells, positive for β -galactosidase at pH 6. The explanation at the molecular level is the shortening of telomeres, which beyond a certain limit is associated with apoptosis response induced by DNA damage [10]. Pellegrini G *et al.* demonstrated that telomere-dependent replicative senescence is a property for keratinocytes also, but telomerase activity is sufficient to extend the lifespan only for keratinocytes endowed with high proliferative potentials (which include stem cells), but not for transitory activated keratinocytes [11]. There are reports showing decrease in number of LSC's in mice, parallel with age [12]. Another study showed that the proliferative and migratory potential and the differentiation capacity of stem cells isolated from human periodontal ligaments decreased as age increased [13]. Donor age has to be considered when evaluating stem cells properties.

The uveal malignant melanoma represents the most frequent primary malignant tumor of the human eye. There are no etiopathogenetic and evolutionary connections with the cutaneous melanomas. The average age of appearance is 55 years, unilateral. Among the uveal components (iris, ciliary body and choroid), approximately 10% of the melanomas have their origins in the ciliary body. These may extend to the posterior in order to infiltrate the choroid, causing in the end secondary detachment of the retina. In the natural evolution, the uveal malignant melanoma presents

extraocular direct extension and hepatic metastases. If approximately half of the enucleated eyes present the infiltration of the emissary canals (intrasceral canals approached by ocular vessels and nerves), only 8–17% of the enucleated eyes present visible extraocular extension, on the scleral surface. The Callender classification is a good indicator of the evolution based on the result of the histological examination [14]. Thus, the uveal melanomas with fusiform cells A or B, the most frequent, have a good prognosis. The mixed types of uveal melanomas and especially the epithelioid ones have a bad prognosis (only 5% of the uveal melanomas are of the epithelioid type).

This case presents a rare and aggressive histological aspect (the epithelioid one) with extraocular dissemination (observed at the moment of the harvesting). We could only speculate about the influence of the melanoma tumor on the corneal stem cells. The association of this pathology might have had an influence (positive or negative) on the proliferation of the corneal stem cells, both *in vivo* and *in vitro*. Even if melanocytes were not found at the sclerocornean limbus, interference with the microclimate at the site of stem cells niches is possible. One more argument on the possible changes that the local stem microclimate had suffered is the assumption that all tumors arise from pluripotent stem cells, which express their own signal [15]. This particular tumor invaded the sclera close to the cornea, were corneal stem cells reside, placing the signals in the proximity of these cells.

Changes in the culture medium influenced the rate of proliferation. The culture medium supplemented with EGF and bFGF seemed to boost LSC's expansion *in vitro*, for a limited time.

The main characteristic of the stem cells is pluripotency and the auto-regeneration capacity, properties conferred by the presence of cellular transcription factors, the most studied being Oct3/4 (encoded by the *POU 5F1 gene*) and Nanog. mRNA Oct3/4 and Nanog are expressed in the embryo stem cells, the germ cells and the adult stem cells. They are essential to the formation of the internal cellular mass and to the auto-regenerative function. SOX2, another transcription factor, can bind DNA together with Oct3/4, controlling Oct3/4 expression [16]. Mutation in this regulatory master gene has been associated with bilateral anophthalmia (eyes not formed) [17]. SSEA4 (stage specific embryonic antigen) is expressed in the early embryonic stage, maintaining the non-differentiated state of the stem cells. Human corneal stem cells in our study expressed surface markers found on undifferentiated embryonic stem cells – Oct3/4, Nanog, SOX2 and SSEA4. A 2011 study suggested that SSEA4 was highly expressed by differentiated corneal epithelium at limbus, making SSEA4 a negative marker for stem cells [18]. In our case, cells with stem cell phenotype, proliferating *in vitro*, positively expressed this marker. These differences point out once more the difficulties encountered in LSC's research, sometimes leading to contradictory results. It is possible that at the limbus there are subsets of LSC's with slightly different phenotypes.

Alkaline phosphatase activity identifies pluripotent stem cell populations and it is down regulated in differentiated cells [19].

Further characterization was done with other surface markers, found on many adult mesenchymal stem cells, the CD's: CD34, CD133, CD29, CD73, CD90 and CD105 [7, 19, 20]. CD34 is a surface glycoprotein considered the most critical marker for hematopoietic stem cell. CD34+ population in bone marrow seems responsible for most of the hematopoietic activity [21].

The cells group we have isolated in peripheral cornea also expressed CD34+. Some reports suggested that LSC's with epithelial origin are CD34- [22]. Others like Perrella G *et al.*, found that human keratinocytes express CD133 and CD34, but this expression decreases with time in culture, with most but not all cells losing expression. Expression or down regulation of these molecules could represent different stages of activation of these cells [20].

Chen S *et al.* found that cells expressing pan-cytokeratin and those expressing CD34 are mutually exclusive [7]. Our findings imply the existence of a PCK+ and CD34+ group, cells with epithelial orientation expressing CD34. Recent studies of Pinnamaneni N and Funderburgh JL reported that as corneal limbal stromal stem cells differentiate to keratinocytes, CD34 is up regulated [23]. It is possible that isolated cells in our experiment were in a transition stage to a process of differentiation.

CD133 is another glycoprotein defining hematopoietic stem cells but also neural stem cells or cancer stem cells [24]. Cells we have cultivated *in vitro* showed the markers found also on stem cells of different origin: CD34, CD133 and CD90.

CD29 and CD105, implicated in cell adhesion, are also found on mesenchymal stem cell surface [25] and were not expressed on corneal stem cells.

✉ Conclusions

Isolated limbal cells were adult type stem cells with an epithelial orientation. The characterization of these cells with immunocytochemistry allowed us to observe surface markers that other stem cells also express. Age, associated pathology and previous therapies may affect the rate of proliferation and maintenance of these cells in culture. These factors may be involved in earlier triggering of senescence, with a practical and clinical importance of these factors in selecting a donor.

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Corresponding author

Ovidiu Samoilă, MD, Ophthalmology Clinic, “Iuliu Hațieganu” University of Medicine and Pharmacy, 3–5 Clinicilor Street, 400006 Cluj-Napoca, Romania; Phone +40726–386 832, e-mail: iovidius@yahoo.com

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