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Cultivation and characterization of limbal epithelial stem cells in rabbits

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Abstract

Purpose: In the last decades, strong evidence emerged regarding the presence of stem cells located at the corneal limbus. Our objective was to find a way to isolate and cultivate rabbit corneal stem cells *in vitro*, into an epithelial tissue. *Materials and Methods*: Two *in vitro* systems were developed to culture rabbit corneal stem cells: (1) limbal biopsies used as explants and cultivated on fresh denuded amniotic membrane and (2) a monolayer culture obtained by enzymatic treatment of the corneal biopsies. Genetic characterization (PCR) was performed. Specific triggers were used to induce differentiation of corneal stem cells. *Results*: At four weeks, 16 explant samples out of 18 cultures showed good expansion, ranging from 1 cm to 2 cm. Genetic characterization showed similar expression of genetic stem markers for corneal stem cells and placental stem cells, previously characterized (stem cell factor, Oct3/4, Vimentin, Nestin and Neurofilament). Corneal stem cells showed high Rhodamine efflux and were effective progenitors for neuronal, myocardial, osteogenic and endothelial lineage. *Conclusions*: In one month, it was possible to grow enough epithelial tissue with preserved proliferative state to allow transplantation on the cornea.

Keywords: stem cells, rabbit, cornea, cell culture.

Introduction

In the last decade, intensive research on corneal stem cells brought new hope for the treatment of ocular surface diseases that usually lead to blindness. Stem cell deficiency caused by corneal burns, Stevens Johnson syndrome, pemphigoid, or herpetic recurrent disease could be specifically addressed by therapies that use bioengineered tissues, cultivated in laboratory. We can now create organs, like trachea (first transplanted organ in 2008) [1], but bioengineered cornea is further away.

Stem cells are pluripotent, slow cycling cells, capable to maintain genomic stability through almost error free DNA transcriptions [2]. Under certain conditions, they give birth to progenitors that can proliferate rapidly into differentiated cells. Corneal stem cells are located in the peripheral cornea, at the limbus, in the basal epithelial layer, in a microenvironment forming stem cell niche [3]. Different concepts about the precise morphology and function of the niche exist and there are reports finding stem cells in central cornea or stroma [4]. Our understanding of the mechanisms implicated in corneal stem cell homeostasis, proliferation and differentiation path is incomplete. Stem cell markers are under investigation, comparing markers expressed by central and peripheral cornea, or identifying markers that usually are found in adult mesenchymal and hematopoietic stem cells or embryonic stem cells [2]. So far, there is no specific marker for corneal stem cells. The markers may be found among the complex of differentiation (CD, not covered in this study), the genes that maintain pluripotency (Nanog, Oct3/4, Sox2), the cell's cytoskeleton intermediate filament proteins (Vimentin, Neurofilament, Nestin) or Stem Cell Factor (SCF, a cytokine that plays an important role in hematopoiesis). Low retention of Rhodamine (Rho) can be a marker of corneal stem cells [5]. Finally, cell differentiation into multiple cell types is an important proof for pluripotency. Ethics restricts most of the research on embryonic stem cells, but there is no restriction on adult type cells, corneal stem cells included.

Purpose

Our objective was to find a way to cultivate rabbit corneal stem cells *in vitro*, into a tissue that could repair the ocular surface. Isolation and characterization of stem cells was an important part of the study.

Materials and Methods

Limbal biopsies

All animal experimental protocols were approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania.

Limbal biopsies were performed on rabbit eyes, from the species *Lepus californicus*, 3 kg male adults, under general anesthesia (Ketamine–Xylazine combination). A 2×2 mm superficial limbal biopsy at 12 o'clock was harvested and transported to the lab in basal culture medium.

Cultivation protocol

Explant cultures

The biopsies were further fragmented mechanically into smaller pieces and placed on denuded fresh amniotic membrane (AM), placed with stromal face down. Surface AM epithelium was removed through trypsinization (0.25% Trypsin). The pieces were incubated in culture medium at 37^0C into a 5% CO_2 atmosphere. Eighteen explant cultures were initiated. Cultures were observed with reverse phase microscope (Olympus CKX41 and Axiovert Zeiss) and photos were taken each three days.

Monolayer cultures

Some of the fragments were used for the characterization of the stem cells. An enzymatic digestion was applied – 0.25% Trypsin EDTA + 0.1% Collagenase type IV (Gibco) for five minutes in an orbital shaker at 37^0C, followed by vigorous pipetting, inactivation with medium with fetal serum (50 mL) and then centrifuged at 1200 rpm for seven minutes. Cell suspension was filtered through Filcon device (70 μm) to get a monocellular suspension and centrifuged again. Cell viability was determined with Trypan blue exclusion. Cells were seeded on Cole flasks, 25 cm^2, in culture medium and incubated at 37^0C into a 5% CO_2 atmosphere. Culture medium was first changed at day 7, after the initiation of cell adhesion, and then each 3–4 days until 70–80% confluence was reached. First passage was done at three weeks, the next passages at 3–7 days, changing the medium each 2–3 days.

Culture medium

Our previous research (unpublished) determined the use of the culture medium: Dulbecco-MEM/F12 (Sigma), 20% Fetal Calf Serum (FCS), Penicillin (100 IU/mL) – Streptomycin (100 μg/mL), 2 mM L-Glutamine, 1% non-essential amino acids (NEA), 1 mM Sodium Pyruvate, 55 μM β-Mercaptoethanol. Epithelial (EGF, 10 ng/mL) and fibroblastic (FGF, 20 ng/mL) growth factors were added. Fifteen percent dilution of FCS was used for secondary cultures (for characterization protocol).

Stem cell characterization

Rhodamine 123 efflux assay

Isolated cells in monolayer type culture were used. Cells were trypsinized and centrifuged after Trypsin inactivation. Cells were then numbered and 2×10^4 cells/well were resuspended in 90 μL PBS and placed onto 96-well cell culture plates. HeLa cellular line with the same cell concentration was used as negative control (differentiated cervix carcinoma line). After the cells adhered, after 16 hours, the wells were washed three times with PBS and 10 μL Rhodamine solution (Sigma Aldrich) was added in 90 μL of PBS/well (final concentration of 1 μg/mL). The culture microplates were incubated at 37^0C for 30 minutes in darkness, under 7% CO_2 atmosphere. Wells were then washed with cold PBS with $Ca^{2+}Mg^{2+}$ (4^0C).

Fluorescence intensity was measured with Synergy 2 (BioTek) plate reader, at 488 nm. Probes were worked in triplicate.

Statistical analysis was performed with GraphPad Prism 5 software, one-way ANOVA and Dunnett's multiple comparison test ($p<0.05$).

Genotypic characterization

Genotypic characterization was performed with reverse transcription – polymerase chain reaction (RT-PCR). RT-PCR allows a semi-quantitatively analysis of mRNA, a highly sensitive technique in which a very low copy number of RNA molecules can be detected (even from one cell).

Total RNA was isolated from rabbit cornea cells (C) and from human placenta mesenchymal stem cells (P). Both were cultivated as an adherent monolayer culture until 80% confluence. The placental cells have already been extensively investigated and they were used as a comparative control for messenger RNA expression. mRNA extraction was performed on cultures with 80% confluence, placed in six wells culture trays. Guanidinium thiocyanate-phenol-chloroform extraction (TRIzol Reagent, Invitrogen) was employed as described in literature [6], according to the manufacturer instruction.

One μg of total RNA was used for reverse transcription with the ImProm Reverse Transcription System (Promega). Only mRNA was transformed into complementary-DNA (cDNA) by using oligo-dT primers in the reaction mixture, together with: AMV reverse-transcriptase 15 u/μg; buffer solution (10 mM Tris-HCl, pH 9; 50 mM KCl; 0.1% Triton X-100); dNTP solution, 1 mM each; $MgCl_2$ 5 mM; recombinant ribonuclease inhibitor 1 u/μL; ultrapure nuclease-free water. The described mixture was incubated at 45^0C for 45 minutes, followed by heating at 95^0C for five minutes and cooling at $0–5^0$C for another five minutes. Subsequently, the cDNA obtained was stored at -20^0C until used.

Amplification was performed under standard conditions, using the components of the GoTaq PCR Core System II kit (Promega) on a Techne TC3000 thermal cycler (Bibby Scientific Ltd). The cDNA amount was generally 5–10 ng/μL and negative controls were always prepared (ultrapure water instead of standard DNA). The reaction also included: Taq polymerase 0.025 u/μL; buffer solution; dNTP solution 0.2 mM each; $MgCl_2$ 1.5 mM or 3 mM; primers and nuclease-free ultrapure water. Human primers were used in both types of cells because both the primers and the amplified portion of the gene have very similar nucleotide sequences in humans and rabbits. The program used was a standard amplification scheme, in which the melting temperatures of specific primers varied: denaturation 95^0C, two minutes; 35–45 cycles at 95^0C for 30 seconds; one minute at 50^0C or 56^0C, depending on primers; 72^0C, two minutes; 72^0C, five minutes; storage at 4^0C.

The primer sequences and the size of the amplification product are indicated in the table below (Table 1).

The amplification scheme used different temperatures (50^0C and 56^0C) and different Mg^{2+} concentrations (normal and double). The PCR products were then separated by electrophoresis on 2% agarose gel and photographed with a UV transilluminator.

Table 1 – *The genes, primer sequences and the size of the amplification product*

Genes	Primer sequences (5'→3') forward and reverse	Size of the product [bp]	Melting temperature [^{0}C]
Oct3/4	aggagtcccaggacatcaaag; tcgtttggctgaataccttc	146	56
Vimentin	ttcagagagaggaagccgaaaac; tttaagggcatccacttcacag	422	56
Nestin	aaacagggcctacagagccagatc; aaacagggcctacagagccagatc	320	50
Neurofilament	tgaagatggctttggatattgagat; tcttcttttgcttcttcagactct	391	56
Stem Cell Factor (SCF)	tatttaatcctctcgtcaaaac; agaattcttcaggagtaaagag	369	56

Stem cell differentiation

Neuronal differentiation

Stem cells were cultivated in differentiation medium, in two steps: N1 medium for 48 hours (DMEM high glucose/F12, 10% FCS, EGF 10 ng/mL, FGF 10 ng/mL, N2 supplement ×100, B27 supplement ×50) and N2 medium for the others 14–21 days (similar with N1 medium, supplemented with 0.5 mM IBMX – 3-Isobutyl-1-methyl-xanthine – and 1 μM *all-trans* Retinoic Acid).

Osteogenic differentiation

Simple differentiation medium (DMEM, 15% FCS, 10 nM Dexamethasone, 1 μg/mL Insulin, 50 μg/mL Ascorbic Acid) and complex differentiation medium (enriched with BMP2 3 ng/mL and TGFβ 2 ng/mL).

Cardiac differentiation

Cells at passages 9–12 were used. Stem cells were seeded on chamber slides on collagen substrate at 2×10^5 concentration/well in DMEM/F12 medium with 15% FCS, until confluence. Cells were exposed to 5-Azacytidine (Sigma), with different concentrations (5, 10 and 15 μM) for 24 hours, with one exposure/week. Cultivation period was one month and 19 days.

Endothelial differentiation in fibrin gel

Fifty thousands (5×10^4) cells/dish were seeded on a Fibrin substrate in endothelial differentiation medium: 199 medium and serum-free medium (ratio 3:1) with VEGF 10 ng/mL and PDGF 10 ng/mL. Serum free medium consist of CS-C medium with 1% ECGF ×100 (Endothelial Cell growth Factor-Sigma). Capillary tubes formation was visualized under a reverse phase microscope after 24 hours.

Limitations of the study

The authors acknowledge some limitations of this study: the biopsy was most likely to contain some stromal cells (although probably a negligible amount), the possibilities to analyze cell proliferation on AM were limited, microscopic examination was difficult on AM; cell differentiation was observed only morphologically, without differentiation markers.

Results

The cultures progressed slowly in the first week, with only a few cells advancing at the edge of the explants. After one week, the proliferation state increased, with cells extending from the explants, showing epithelioid appearance (Figure 1). From there, the cell proliferation increased exponentially, cells developing into a multi-layered tissue with up to 2 cm diameter after one month. Sixteen explant samples from 18 cultures were successful, resulting into tissue formation. The two failures were caused by explant detachment from the AM, due to culture processing.

Rhodamine levels were found low in corneal lines (c2 and c2') compared to control (HeLa cellular line) (Figure 2).

RT-PCR showed that genetic expression of corneal stem cells was quite similar to that of placenta stem cells, especially for Oct3/4, Nestin and Vimentin. A difference was noted for the Neurofilament genes, with more isomorphs for rabbit corneal stem cells, at the 56^0C protocol. SCF was also expressed in corneal stem cells, but to lesser extent than in placenta stem cells (Figures 3 and 4).

Corneal stem cells were effective progenitors for neuronal, myocardial, osteogenic and endothelial lineages. Exposed to specific differentiation media, rabbit corneal stem cells developed morphological aspects characteristic to different tissues. Retinoic Acid or IBMX supplemented media induced morphological changes characteristic to neural cells (cells with smaller body and dendritic extensions) (Figure 5). Proliferation of these neural cells continued for up to 30 days, with apoptosis levels increasing parallel to decreasing levels of proliferation, following differentiation.

Osteogenic differentiation (in both osteoinductive media, simple and complex) showed cells with elongated cellular bodies and dendritic aspects (Figure 6). Characteristic for osteogenic differentiation were osteogenic nodules, observed at three weeks. At two months, calcium deposits (crystals) were observed.

For myocardial differentiation, the influence of different 5-Azacytidine concentration, treatment numbers and substrate type were followed. Concentration of 10 μM of demethylating agent 5-Azacytidine sustained both cellular proliferation and characteristic morphological changes (elongated cells at first, then round or polygonal cells) (Figure 7, up).

Endothelial differentiation showed capillary tubes formation, a functional characteristic of endothelial cells, observed from the first 24 hours, longer and better align at 48 hours, with network formation at 72 hours (Figure 7, down).

Discussion

Stem cells are undifferentiated cells with renewing capacity while maintaining a stable genome. They are capable of differentiation into specific lineage cells. Embryonic stem cells are the reference point in stem cells research, but there is increasing interest in adult stem cells, found in many tissues including blood, liver, brain, skeleton, muscle, intestine, skin and cornea.

Mesenchymal stem cells are the focus in adult stem group.

The most well known corneal stem cells are located at the limbus, in the basal layer of the epithelium and they are of course, of epithelial origin. Recently, stromal mesenchymal cells were described, just underneath the epithelial stem niche [7]. In our case, the biopsies were taken at a superficial level but they were most likely to contain superficial stroma, with the possibility of harboring mesenchymal stromal stem cells. Most of the material was of an epithelial origin, so the study truly investigated the genetic material of the epithelial cells, with little contamination from the stroma.

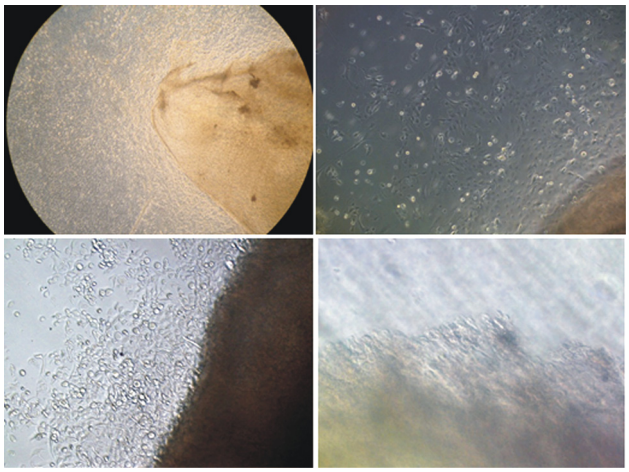

Figure 1 – ***Phase contrast images in white light microscopy, cell proliferation near the explant (×10, ×20, ×20, ×40).***

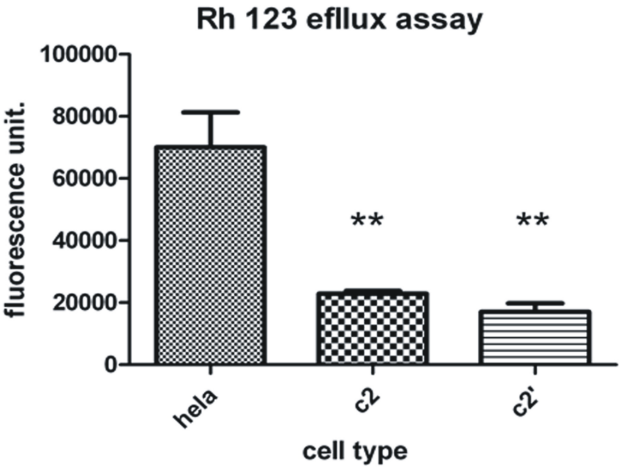

Figure 2 – ***Rhodamine 123 efflux assay show significant statistical difference between isolated stem cells from cornea and HeLa cells – Dunnett's multiple comparison test. Corneal lines c2 and c2' against control, HeLa line.***

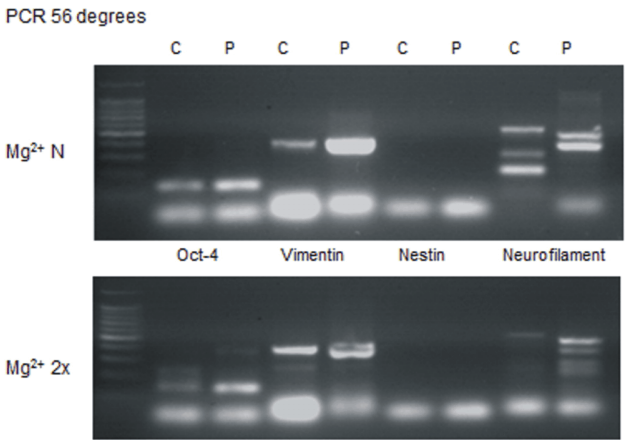

Figure 3 – ***RT-PCR results at 56^{0}C, under normal and double concentration of Mg^{2+}: electrophoresis for Oct-4, Vimentin, Nestin, Neurofilament.***

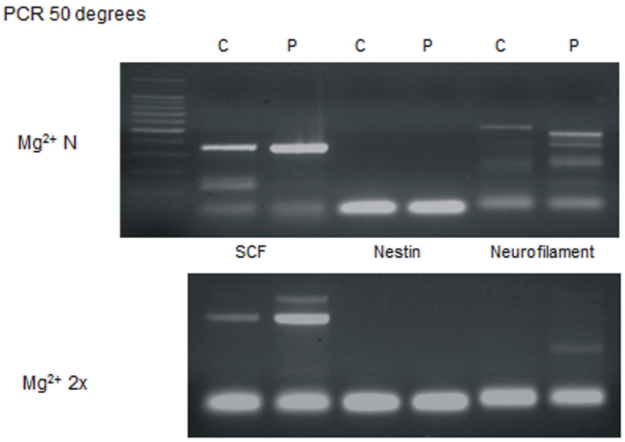

Figure 4 – ***RT-PCR results at 50^{0}C, under normal and double concentration of Mg^{2+}: electrophoresis for SCF, Nestin, Neurofilament.***

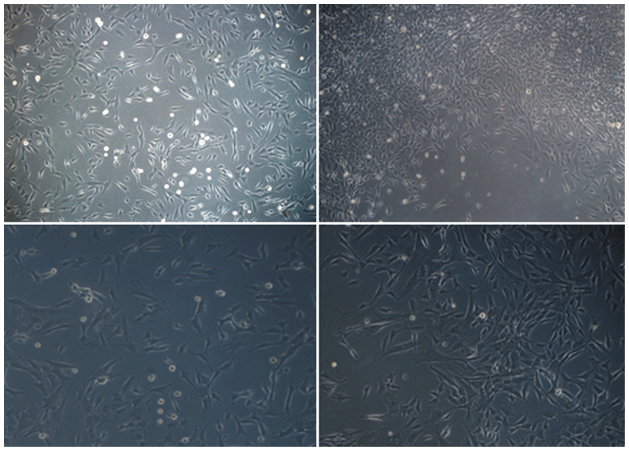

Figure 5 – ***Neural differentiation at 24 hours and 48 hours, with media containing Retinoic Acid (up) and IBMX (down). Phase contrast image, ×10.***

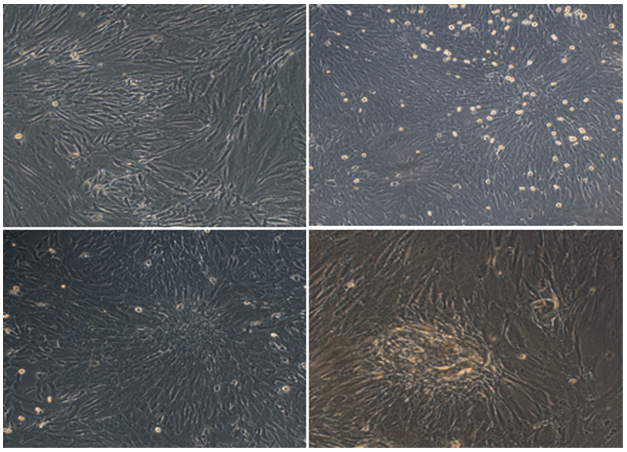

Figure 6 – ***Osteogenic differentiation at 24 hours, 48 hours and 60 days. Osteogenesis nodules at three weeks (lower right). Phase contrast microscopy, ×10.***

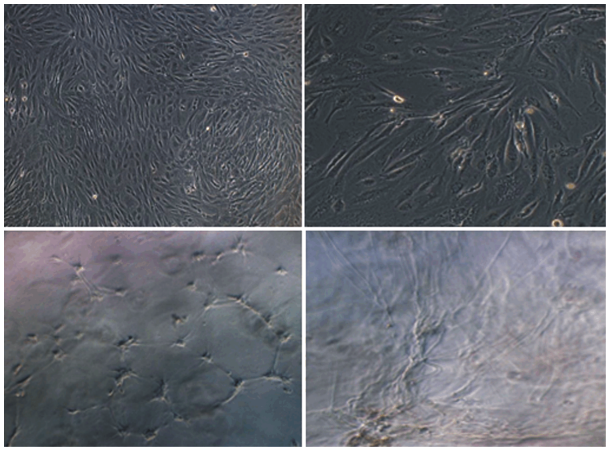

Figure 7 – ***Cardiac differentiation at 48 hours and 24 days (up). Endothelial differentiation at 48 hours and 72 hours (down). Phase contrast microscopy, ×10, ×20, ×40, ×40.***

To date, no direct methods have been established to identify the corneal stem cells because of the lack of specific molecular markers, although a variety of stem cells associated markers have been proposed [8]. Some of these markers are only expressed in the peripheral cornea (at the limbus), others only at the basal layer of the peripheral cornea, marking small cells with little cytoplasm and big nuclei, thought to be limbal stem cells [9].

The main characteristics of the stem cells are pluri-potency and the auto-regeneration capacity, properties conferred by the presence of cellular transcription factors, one of the most studied being Oct3/4 (encoded by the POU 5F1 gene). mRNA Oct3/4 is expressed in the embryonic stem cells, the germ cells and the adult stem cells. Oct3/4 (or Oct4) is one of the earliest transcription factors found during embryonary development, capable to generate at least three transcripts (Oct4A, Oct4B, and Oct4B1) and four protein isoforms (Oct4A, Oct4B-190, Oct4B-265, and Oct4B-164) [10]. It is essential to the formation of the internal cellular mass and to the auto-regenerative function. It has a double function, in pluripotency and cell differentiation. Oct3/4 is not the only factor to keep the cell in a pluripotency state. It is associated with Sox2, NANOG and TCF3. Oct3/4 was reported to also instruct stem cell fate through a gene dosage effect. If Oct3/4 level drops 50% from the endogen baseline, the undifferentiated embryonic stem cell will lose pluripotency and will start differentiating into trophectoderm. If the level increases more than 50% from the baseline, the embryonic stem cell will engage towards endoderm and mesoderm [11]. Either Sox2 or Oct3/4 may trigger the differentiation, each functioning as a molecular rheostat to control the self-renewal and pluripotency of embryonic stem cells [12].

Nestin, vimentin and neurofilament are intermediate filament proteins present in non-differentiated cells. Intermediate filament protein members are expressed in specific cell types, for example, keratin in epithelial cells, vimentin in mesenchymal cells, neurofilament and nestin in neuronal cells, desmin in muscular cells and glial fibrillar acidic protein in glial cells. Nestin is a class VI intermediate filament protein that was originally described as a neuronal stem cell marker during central nervous system development. Nestin is also expressed in non-neuronal immature or progenitor cells in normal tissues and also in several tumor cells. Nestin may be a marker for newly synthesized tumor vessels and a therapeutic target for tumor angiogenesis [13]. A study from 2012 found that cardiac resident nestin-positive cells may present proliferative advantages. A subpopulation of nestin-positive cells identified in the normal heart expressed cardiac progenitor transcriptional factors and may directly contribute to myocardial regeneration following ischemic damage [14]. A study from 2010 found increased levels of nestin and also vimentin in limbal basal epithelial cells, compared to central basal epithelial cells in rabbit corneas [15]. Nestin presence in the limbal corneal cells is an indicator for the immature, undifferentiated state of these cells and may indicate multi-potentiality and regenerative potential [16].

Stem cell factor (SCF) is a cytokine that binds to the c-Kit receptor. SCF can exist both as a transmembrane protein and a soluble protein [17]. SCF plays an important role in the hematopoiesis during embryonic development. SCF is also expressed in adult corneal epithelial cells and plays a role in corneal wound healing. It is unclear whether this is an appropriate marker for limbal stem cells [18].

The isolated corneal limbal cells from monolayer cultures used in our study, showed many of the genetic characteristics of the stem cells. Rabbit corneal stem cells expressed similar Oct3/4 isoforms with placenta stem cells. The genetic expression was also similar regarding nestin, vimentin and to a lesser extent the SCF. A slight difference appeared at the neurofilament genes, with more isoforms for the rabbit cells, confirmed also at RT-PCR 56^0C protocol.

Rhodamine 123 is a fluorescent dye that binds to active mitochondria in cells, thus reflecting the metabolic state of the cell [19]. Rho efflux is mediated by P-glycoprotein (a transmembrane protein carrier, ATP-Binding Cassette – ABCG2), encoded by the multidrug resistance gene-1 [20]. Stem cells in normal tissue and their malignant counterparts share the multidrug resistance gene transporter activity as a major mechanism of self-protection, implicated in the excretion of toxins from cells [21]. Stem cells have the capacity to eject Rho. Cell sorting based on Rho dye efflux provides an additional parameter for purification of human stem cells, for instance hematopoietic stem cells [22]. Rho dye efflux was also used to isolate the cancer stem-like cells, replacing Hoechst 33342 dye, which is more expensive [23].

Corneal limbal cells showed high levels of plasticity, a characteristic of the pluri- and multi-potent stem cells, embryonary or adult. This property allowed LSC's to transdifferentiate into mesodermal and endodermal tissue type. The characteristic morphological changes in myocardial expansion, with elongated cells at first, then round or polygonal cells was consistent with myotube-forming cells as reported by Martin-Rendon *et al.* [24]. Induced cell morphology could be explained by remodeling and assembly of the myofibrils on actin stress fiber-like cables that serve as a template, coupled by the focal adhesions to the extracellular environment to establish a polygonal morphology [25].

Cultures using limbal explants are preferred by most investigators because the procedure is easier and more time efficient than other methods, for example cell suspension, which in turn may be superior in stem cell content [26].

Cultivating cells on AM allowed us to obtain a multilayered epithelium with a basement membrane-like structure. The use of AM in ocular surface reconstruction (as a biologic cover over the bare stroma, after the removal of abnormal conjunctival tissue that invades cornea in various diseases) is based on the rabbit model reported by Kim and Tseng [27], in which the amniotic membrane is thought to inhibit conjunctival overgrowth and provides a good substrate for normal epithelial migration. AM is known to have immune tolerance with incomplete expression of HLA, promotes healing, down regulating proinflammatory factors and containing growth factors. The basement membrane in AM contains glycoproteins and collagens common to basement membranes found in cornea and conjunctiva. Koizumi *et al*. showed that amniotic membrane used as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits is feasible [28]. When compared, stem cell cultures on denuded AM appear to advance 10 times faster than on AM with intact epithelium [29].

Koizumi *et al.* also advocates that the amniotic membrane may decrease the antigenicity of the corneal epithelial cells cultivated on it [29]. Another study from 2008 showed that there is possible to cultivate limbal epithelial stem cells into tissue to be used as allograft [30]. The success of this technique was 33%, on seven cases. Shimazaki *et al.* did not find high success rate after allografting of the cultivated limbal stem cells [31]. In a study from 2012, we have demonstrated the use of cultivated epithelium as allografts, but the result was the loss of graft transparency and vascularization [32]. There is need for more research, regarding the size of the graft, the importance of the remaining quantity of the host epithelial cells and the mechanisms of the immunological response in the allograft.

Conclusions

In one month, it was possible to cultivate epithelial tissue with preserved proliferative state that could be used for transplantation. Isolated cells from limbal cornea expressed stem cell markers: Oct3/4, SCF, Nestin, Vimentin and Neurofilament and showed high Rhodamine efflux. Limbal cells differentiated into different tissues: neuronal, myocardial, osteogenic and endothelial, a proof of pluripotency.

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