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CULTURE OF HUMAN LABIAL MUCOSAL EPITHELIAL CELL FOR USE IN PATIENTS WITH BILATERAL LIMBAL STEM CELL DEFICIENCY

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Aim: to obtain a stable population of the human labial mucosal epithelium without feeder cells through explant culture technique and simplified formulation of the culture media. **Materials and methods.** Labial mucosa samples were obtained from 6 patients in the operating room after the patients had signed an informed consent. Samples were trimmed of the substantia propria and cut into uniformed explants. Cell culture was done using DMEM/F12 (1:1) (1.05 mM calcium) and EpiLife (0.06 mM calcium) media, supplemented with 5% fetal bovine serum, antibiotic-antimycotic, insulin (5 μg/mL), hydrocortisone (5 μg/mL) and epidermal growth factor (10 ng/mL). Primary cells were stained for stemness and proliferative markers (anti-p63), intermediate filaments (anti-vimentin), and tight junction protein-1 (anti-ZO-1). Image analysis was performed in Fiji (ImageJ). **Results.** Primary cell culture was obtained from all the samples in both media. Cellular morphology was characterized as a classic "coble-stone" phenotype. 34.7% p63-expressing cells (median, n = 3) was detected in the 1.05 mM Ca medium, while ZO-1 expression was estimated at 17.05 μm per cell (median, n = 3). In cells cultured in 0.06 mM Ca medium, positive p63 expression was 39.2% (median, n = 3), while the length of the ZO-1 expression was 5.18 μm per cell (median, n = 3). **Conclusion.** This study presents a detailed protocol on how to obtain cell culture of human labial mucosal epithelium from a small biopsy with high proliferative activity without feeder cells condition. The 1.05 mM Ca medium promoted generation of the tight junction and may be used in *in vitro* epithelium differentiation models. In contrast, the 0.06 mM Ca medium maintained reduced level of maturation in the cell culture. Thus, the media formulations, cell culture source and method described in this study, may be used for transplantation of autologous labial mucosal epithelium in patients with bilateral limbal stem cell deficiency.

Keywords: labial mucosal epithelium, primary cell culture, cell growth factors, cornea, limbal stem cell deficiency.

INTRODUCTION

Normally, the human cornea is covered with a non-keratinized stratified squamous epithelium [1], which is renewed due to the limbal epithelial stem cells (LESCs) [2] located in the limb zone of Vogt crypts and focal stromal projections [3, 4]. If LESCs are extensively damaged, limbal stem cell deficiency (LSCD) develops [5], featured by the presence of non-transparent fibrovascular pannus at the site of the anterior epithelium, which prevents the passage of light through the cornea, thus causing blindness and poor vision [6].

The bilateral LSCD can develop in such diseases as aniridia, Stevens–Johnson syndrome, and corneal burns in both eyes [7]. To rehabilitate this group of patients, surgical methods of allogenic limb transplantation in combination with prolonged immunosuppression are offered [8]. Cell therapy with cultured oral cavity cells is a promising trend of treatment for the bilateral LSCD [9]. At the beginning of the method development, the transplantation of autologous cultured buccal epithelium was performed [10, 11], as it is morphologically similar to the anterior corneal epithelium, i. e. it is non-keratinized stratified squamous epithelium and stays in contact with the environment [12].

At the same time, there are epithelial zones with identical morphology in the mouth cavity as well as in the vestibule. These are the mucosal epithelium and the lower surface of the tongue, soft palate, and mouth floor [12]. According to literature data, cheek (buccal) epithelium has long remained the major source of cells for creating a therapeutic construct [9, 13]. At this, mucosal epithelium as a source of cells for the cell epithelium construct has not been earlier studied in detail.

According to literature reports, the inscription of cultural media for selective cell growth of the oral cavity

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epithelium assumes the presence of a common mitogen, L-glutamine, decontamination components, and factors stimulating the growth of the epithelium [14]. Most of investigators used the DMEM/F12 basal medium (1:1–1:3) containing 1.05–1.425 mM of Ca to obtain the buccal epithelium culture [14]. However, according to R.Y. Freshny, the low Ca medium (0.06–0.07 mM) is preferable as it supports the proliferative activity of cultivated epithelial cells, does not cause their differentiation and contributes to the elimination of fibroblast-like cells [15]. The earlier cultivation methods also included feeder cell layers and other xenogenic components, e. g. bovine pituitary extract. It has been shown in modern studies that feeder layers can be excluded [16], and among many specific factors, only three can be used to stimulate epithelium growth; namely, insulin, hydrocortisone, and epidermal growth factor (EGF) [17], each is produced by a number of companies complying with GMP rules.

The present study is aimed at obtaining a stable population of the human labial mucosal epithelium without feeder cells using the explant method and simplified formulation of the culture media.

MATERIALS AND METHODS

Formulation of the culture media

In the present study, the following culture media with 2 mM L-glutamine were used as basal: DMEM/F12 (1:1) (D6421, Sigma Aldrich) containing 1.05 mM of Ca and phenolic red, and EpiLife (MEPICFPRF500, Gibco) with 0.06 mM of Ca without phenolic red. EpiLife medium is similar in its formulation to the medium for keratinocytes MCDB 153 [18] and, unlike such basal media as DMEM/F12, contains nickel chloride, ammonium paramolybdate and metavanadate, sodium selenite, sodium metasilicate, tin chloride, and lipoic acid [19]. As a general mitogen, fetal bovine serum (FBS) (SH30109.03, HyClone Laboratories) was used at 5% concentration. To prevent pathogenic microflora growth, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B (A5955, Sigma Aldrich) were added to the culture medium. Among the specific factors used for stimulation of oral cavity epithelium growth were soluble human biosynthetic short-acting insulin, 5 μg/ml (Humulin Regular, Injectable solution, Elie Lilly & Company), 5 μg/ml hydrocortisone (injection suspension, 25 mg/ml, Pharmak), human recombinant epidermal growth factor (EGF), 10 ng/ml (FR-08000, PanEco). Prepared complete culture media were stored at +4 °C with no exposure to light.

Isolation of labial mucosal tissue

All studies were carried out in structural divisions of the S.N. Fedorov NMRC "MNTK Eye Microsurgery".

To obtain the primary cell culture of the epithelium, the remnants of free labial mucosa graft tissue were used, isolated during its transplantation at the planned surgery in the Reconstructive and Plastic Surgery Department. The study was approved by the Ethical Committee (protocol No. 88.5 of October 11, 2018). The tissue extraction was performed after the patient signed a voluntarily informed consent to the use of the tissue remnants for research purposes. The free graft was isolated according to the standard rules of aseptic and antiseptics. For this, the labial mucosa was treated with a sterile gauze sponge moistened in the Iodoftal solution (LLC "NEP MG") for one minute. Then the infiltration anesthesia of the zone of interest was performed with 2% lidocaine solution (GROTEX LLC). The labia were fixed with a holder, providing access to the mucous membrane. Then the mucous membrane was cut with the blade laterally from the frenulum to get a full-layer graft in the form of a petal. In the course of the operation, the graft required for transplanting was removed, and the remains of tissue were placed in the Borzenk–Moroz medium (LLC "NEP MG"). The edges of the formed mucous defect were covered with 5 to 7 separate nodal sutures. In the postoperative period, the patients observe hygiene of the oral cavity in accordance with the standard postoperative protocol.

Primary culture of labial mucosal epithelium

Cellular and tissue materials were processed in the Laboratory of Transplantology and Cell Biology of the Center for Fundamental and Applied Medical and Biological Problems in accordance with the established sanitary and epidemiological regime. To comply with aseptic conditions, the work with the materials was carried out in the working space of laminar boxes of II class of safety. All devices and tools were calibrated according to the standards and prepared in accordance with the aseptic requirements. The standard culturing conditions were taken to be the temperature mode of 37 °C, 5% CO_2 concentration and 100% humidity maintained in the incubator (NU5510, NuAir).

In total, 6 samples of the upper or lower labial mucosal membranes in the form of a narrow strip about 1.5–2.0 mm wide and less than 1 cm long were obtained from 6 patients. The tissue was transported and stored in the Borzenk–Moroz medium at +4 °C. The slices for the subsequent seeding were taken as follows: the tissue was transferred to the Petri dishes with the epithelial side down and, with microsurgical forceps and scissors, the submucosal part was separated until a whitish layer appeared before the epithelium layer. The resulting strip was cut into pieces with 1.0–1.5 mm transverse dimensions. In the course of the described manipulations, to prevent drying out, the Borzenk–Moroz medium was applied over the tissue and pieces. The stages of treat-

ment of the labial mucosal strip for seeding and primary cultivation of the epithelium are shown in Fig. 1.

The primary culture of the labial mucosal epithelium cells was obtained by the explant cultivation method. For this, the pieces obtained after separation of the subjacent tissue were placed with the epithelial side facing up (whitish layer downwards) on the cultural surface of Petri dishes (430165, Corning) or 4-well slide flasks (30104, SPL). Then the samples were left with an open lid for their primary "drying" for 1–2 minutes in laminar boxing conditions. Then, 40 µl of the complete culture medium was applied to the pieces, the lid of the culture dishes was closed, and the pieces were moved to standard incubator conditions for 3–4 hours. After that, 500 µl of the complete culture medium was added very slowly to the pieces cultivated under standard conditions. The cell growth was visually controlled with a phase-contrast inverted light microscope IX-81 (Olympus). The growth and morphology of cell culture were recorded. The culture medium was completely altered from the moment of visualization of the first proliferating cells and further every day. Cells in Petri dishes and slide flasks were cultivated up to 90% confluence.

Fluorescent immunocytochemistry

The cells were stained by the following protocol: the cells in cultural slide flasks were washed with phosphate buffer solution (PBS) (B-60201, PanEco) three times, 5 minutes each. Then they were fixed in 10% neutral formalin (141328, AppliChem) for 10 minutes. Permeabilization was carried out with a 0.3% solution of X-100 triton (X100, Sigma Aldrich) in PBS for 15 minutes. The blocking solution containing 5% fetal bovine serum (SH30109.03, HyClone Laboratories) and 0.1% saponin (84510, Sigma Aldrich) in PBS for 1 hour was used to block nonspecific binding sites. After that, primary antibodies diluted in the blocking solution were added to the wells to such markers as p63 (stemness, proliferation) [20] (1:300, ab124762, Abcam), vimentin (intermediate filaments) [21] (1:250, ab8978, Abcam), ZO-1 (Zonula occludens-1, dense intercellular contact protein type 1) [22] (1:100, ab216880, Abcam). Primary antibodies were not added to the control wells, and the same blocking solution volume was used. The slides were incubated for 18 hours at +4 °C. After washing in the blocking solution, secondary fluorescent antibodies Alexa Fluor 488 (1:250, ab150077, Goat Anti-Rabbit IgG, Abcam) or Alexa Fluor 594 (1:250, ab150116, Goat Anti-Mouse IgG, Abcam)

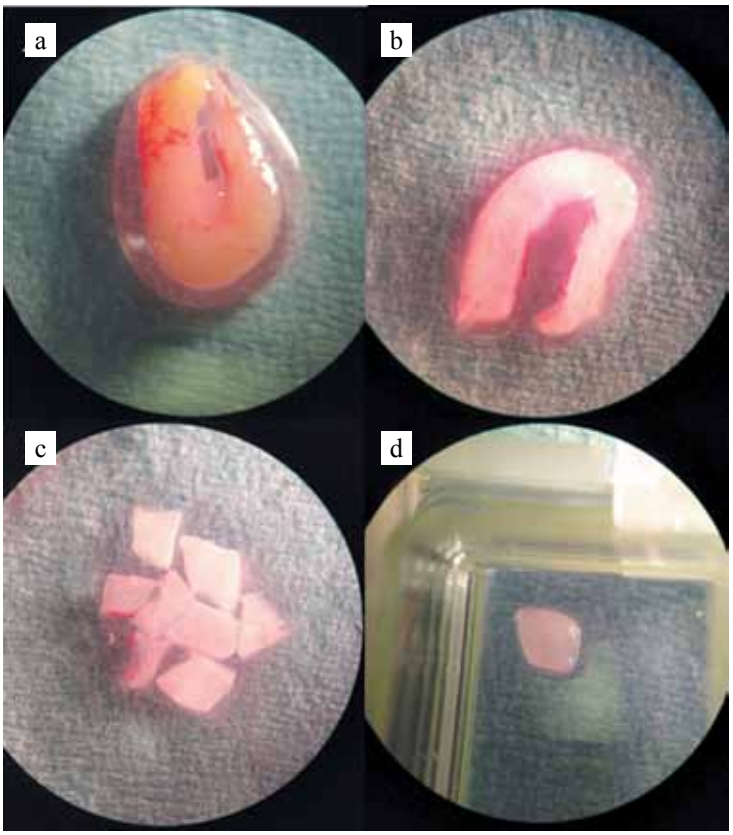

Fig. 1. Stages of processing and seeding of human labial mucosal explants, macro photography: a – overview of the strip of a labial mucosa in a drop of the Borzenk–Moroz medium, a strip of tissue facing the epithelial side down, and visible fatty submucosal layer anteriorly; b – a strip of the labial mucosa after removal of the submucosa, visible whitish layer, with underlying the epithelium; c – labial mucosa explants prepared for seeding; d – explant on the surface of the well of a glass culture slide with the epithelial side facing up

diluted in the blocking solution were added to all wells. The cells were then incubated for 1 hour at +4 °C under darkroom conditions. Secondary antibodies were then removed and stained with Hoechst (O150, PanEco) for 2 minutes in all wells. Slide flasks were then dismantled and mounted under the coverslip with the medium to enclose the histological preparations VitroGel (12-001, BioVitrum). Immunofluorescence detection was performed with the Fluoview FV10i (Olympus) confocal laser scanning microscope.

Image analysis

The images were initially prepared in the internal software environment of the Fluoview FV10i microscope. The obtained photographs were then analyzed by the Fiji (ImageJ 2.0.0.0-rc69/1.52) program [23]. Nuclei by Hoechst stain, as well as the nuclei positive for p63 marker were counted with the CellCounter plugin. Internal Set Scale, Segmented line, and Measure basic tools were used to determine the length of areas of the expressing protein ZO-1. To do this, the ratio was first set of μm to pixels in the image (Set Scale), then a line (Segmented line) was manually drawn coinciding with the ZO-1expression, and finally, the area length was calculated (Measure). The total lengths were divided by the number of nuclei in the photo.

RESULTS

Primary culture behavior *in vitro*

When observing the primary tissue attachment and the beginning of the growth of epithelial cell culture, a number of manipulations contributing to the adhesion of pieces and the growth of cell culture were empirically determined, namely drying of the samples on the cultural plastic before adding the complete culture medium provides their better attachment, the samples do not float, there is less debris and fallen off differentiated cells in the culture. At first, before the cell growth initiation, it is necessary to close the incubator doors very carefully to prevent the shaking of the pieces. A separation in the first few days slows down their readhesion. This leads to the fact that the pieces floating in the medium begin to discard the debris and cells, clogging the culture. The pieces prepared for seeding should be placed on the cultural surface with the epithelial side upwards. Otherwise, they were not attached and there was no growth. The epithelial side of the explant is distinguished by the absence of whitish stripes, by relative transparency and the presence of a smooth surface with a glare. When the first signs of growth appear, it is necessary to carefully wash the culture from the detached cells and then use the nominal volume of the complete culture medium: 1 ml for a 4-well slide flask well, and 2 ml for a 35 mm Petri dish.

In compliance with the methodological conditions described above, the primary culture of the labial mucosa epithelial cells was obtained from all donors in both groups. Cell morphology in cultures corresponded to the classical “cobblestone” type; however, variability in the cell area was noted (Fig. 2, a, b), which was more pronounced in the samples cultivated in DMEM/F12 medium. In general, the specific morphology of epithelial cells and their relative size uniformity remained for the whole period of primary cultivation.

When cultivated on a complete culture medium on the basis of DMEM/F12 with 1.05 mM Ca+++, the cells were observed to leave the slice on day 3–5. As the culture grew, the zones with cells becoming larger were formed in the culture. These zones further increased in size, and large cells began to detach, forming cavities, which then were not filled with new cells. In the process of cultivation on the specified medium, many rounded unattached cells were formed. In some cases, it was noted that fibroblasts with specific morphology were distributed from a piece. In the course of observation of the primary culture on the basis of DMEM F12, the “growth wave” phenomenon, which can be described as a wave of dividing cell conglomerate propagating from a piece of tissue, was detected. At the same time, radial cell traction was formed from a piece of tissue to a “growth wave” (Fig. 2, c, e).

When cultivated on a complete culture medium on the basis of EpiLife with 0.06 mM Ca++, a later (day 5–8) release of epithelium cells from a piece was observed. In general, cell growth was slower compared to the first group. The phenomenon of “growth wave” and the cell conglomerates traces were not noted (Fig. 2, d). However, the culture obtained on the low Ca base medium was featured by the best cell morphology. Small polygonal cells with large nuclei were mainly present. The smaller areas with larger cells and a relatively smaller nucleus were found, which is typical for ripening cells. The number of detached cells was minimal. The fibroblast growth was recorded in only one case.

Immunophenotypic characterization of cells

The fluorescent immunocytochemistry detected positive expression by p63 marker in cells, which was colocated with Hoechst nuclear stain. Namely, 34.7% of such cells were detected in cells cultivated in DMEM/F12 medium (1:1) using the median (n = 3), whereas in EpiLife medium – 39.2% (median, n = 3). In both groups of cells, a weakly positive expression of vimentin was observed with some predominance in EpiLife samples. The expression of ZO-1 protein was presented along the edge of large cells. According to the data of the program analysis, the length of ZO-1 protein expression sites in terms of the number of nuclei per cell was 17.05 μm

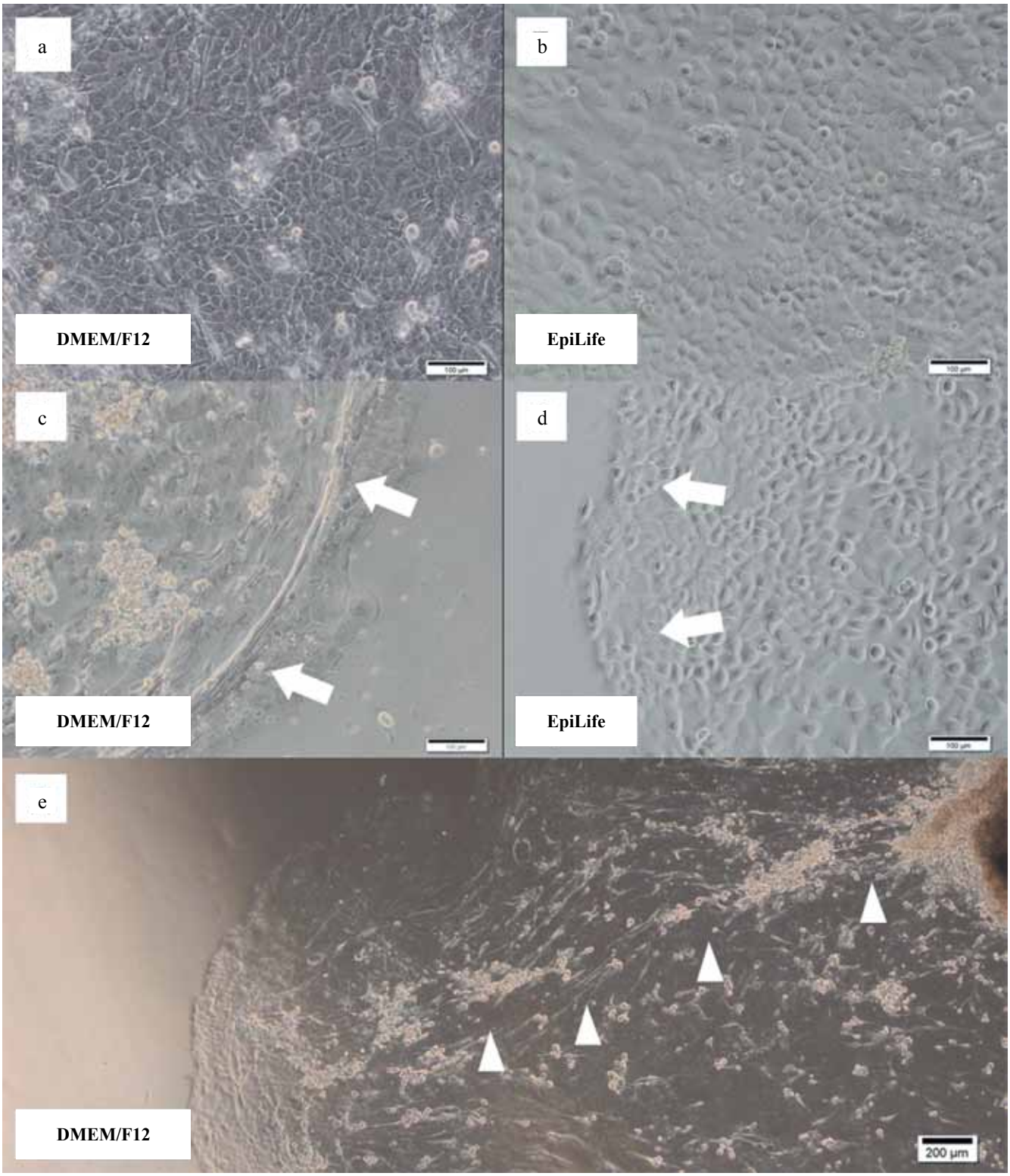

Fig. 2. Morphology of the primary culture of human labial mucosal epithelial cells obtained in DMEM/F12 (1.05 mM Ca) or EpiLife (0.06 mM Ca) media: a, b – primary cell culture had the classical "cobblestone" phenotype. Areas with large and small cells are visible; c – micrograph of the phenomenon of "growth wave" (marked by arrows); d – the absence of a "growth wave" in the epithelial cell culture on the EpiLife medium (marked by arrows); e – micrograph of the trace of conglomerates of cells from the explant (indicated by arrows); a–d – phase-contrast microscopy; e – combined photo; a–d – ×100, e – ×40

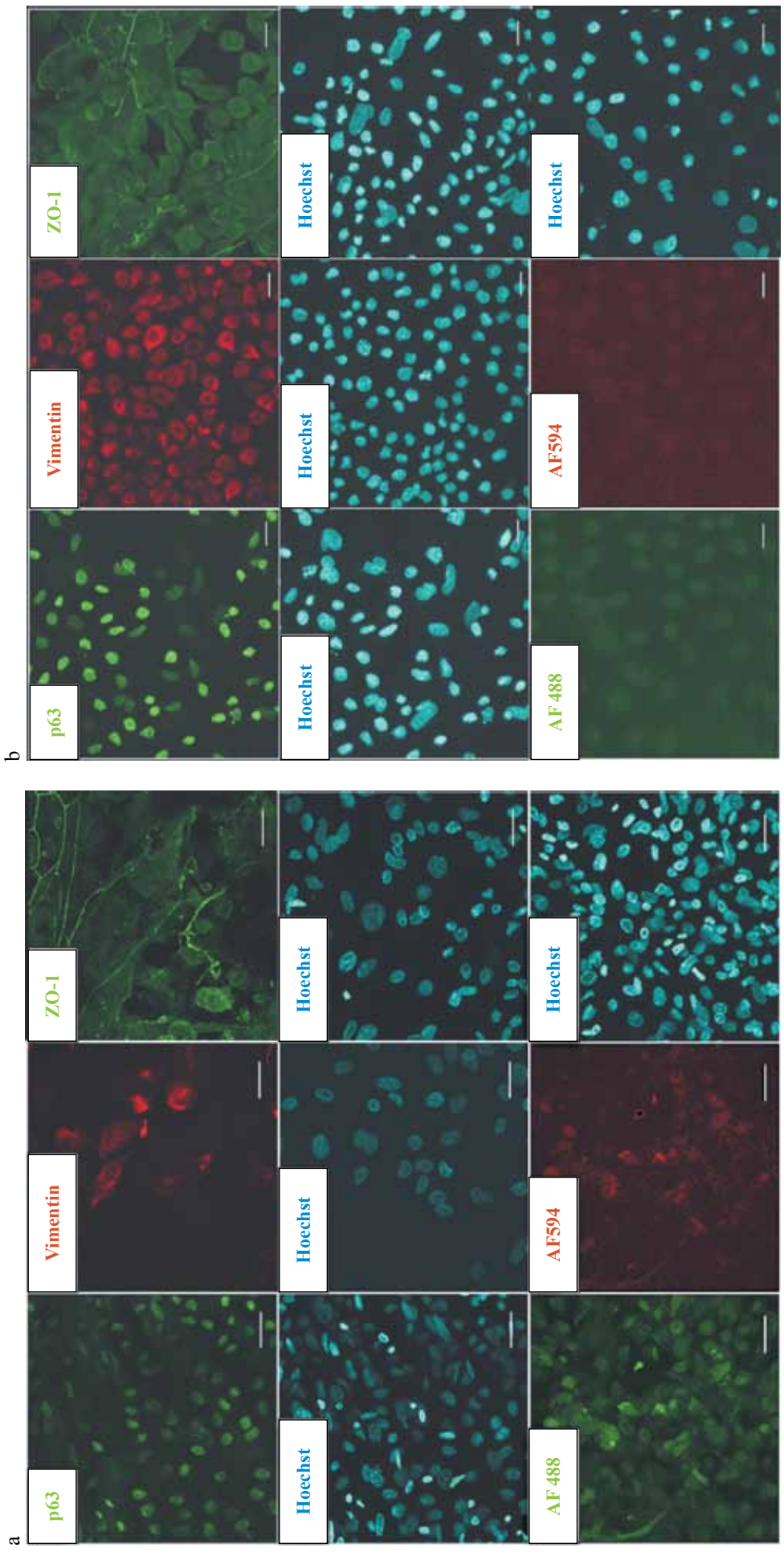

Fig. 3. Expression of immunocytochemical markers in the primary culture of human labial mucosal epithelial cells cultured in DMEM/F12 (1.05 mM Ca) (a) and EpiLife (0.06 mM Ca) media (b). The p63 marker (AlexaFluor 488), co-localized with the Hoechst nuclear dye. Vimentin (AlexaFluor 594), represented in the cytoplasm of the stained cells. ZO-1 marker (AlexaFluor 488), expressed on the edge of large cells. Control samples of fluorescent dyes Alexa Fluor 488, Alexa Fluor 594 and Hoechst (bottom row, left to right). Confocal laser scanning microscopy, ×600

per median (n = 3) for DMEM/F12 (1:1) and 5.18 μm for EpiLife.

DISCUSSION

Obtaining a stable culture of epithelial cells is a complex methodological task in general. Maintenance of epithelial cells proliferation and inhibition of fibroblast excess growth allows achieving a certain culture purity regardless of the method of cell isolation [15]. In particular, such specialized growth factors as insulin, transferrin, hydrocortisone, cholera toxin, epidermal growth factor, triiodothyronine, and Bovine Pituitary Extract can be used for the cultivation of human cheek epithelium [14]. However, to obtain a cellular construct for use in the clinic, xenobiotic components should be absent in the formulation of the medium, and the growth factors must be produced according to the rules of good clinical practice [24]. Notably, according to M. Formanek et al. [17], cholera toxin and triiodothyronine do not have a significant positive effect on the proliferation of oral cavity epithelium, in contrast to the combination of hydrocortisone, epidermal growth factor, and insulin. Therefore, these three factors were examined in the present study, and the insulin and hydrocortisone used in the formulation were released by pharmaceutical companies for use in patients.

In the present study, a low Ca (0.06 mM) medium related to selective media was used to compare the results of cultivation with a standard culture medium containing 1.05 mM Ca (DMEM/F12). This made it possible to determine the tendencies of an increase in proliferation and a decrease in the level of differentiation of cultivated epithelial cells in the low Ca medium. The feeder layers were not used in the study either, which will allow excluding feeder cells transfer during autologous cell structure transplantation to patients.

The method of cultivating explants applied in the present study has some advantages over the method of enzymatic isolation; it allows to preserve the extracellular matrix and use small tissue samples. In our opinion, the cultivation of explants in combination with a low-calcium selective medium limits the yield of resident fibroblast cells from the explant, and the presence of the best combination of specialized growth factors allows the epithelium to proliferate and occupy the cultural surface.

The results of immunofluorescent coloring in the process of labial mucosal epithelium cultivation showed high activity by p63 marker in both medium formulations. The obtained values were close to those published on the cultivation of cheek epithelial cell layers for transplantation, where it was shown that the cellular construct contained 30.7 ± 7.6% (mean ± SD) p63 positive cells [25]. The expression of vimentin, a protein of intermediate filaments, is most often associated with the mesenchymal phenotype of cultivated cells or with the phenomenon of epithelial-mesenchymal plasticity [26]. The weak expression of vimentin noted in cultures confirms that the protocol of cultivation and the formulation of media used in the work allow preserving the epithelial phenotype of cells. The expression of the dense intercellular contact protein (ZO-1) is an important marker of epithelial cell differentiation associated with the physiological process of cell attachment to each other for the formation of a flat epithelial layer [27]. In the present study, a decrease in the expression of the ZO-1 marker in the medium with 0.06 mM of calcium was noted, which is consistent with the recommendations to maintain the undifferentiated state of epithelial cells in the culture [15].

CONCLUSION

In the present study, the protocol of cultivation of human labial mucosal epithelium without feeder layer is discussed, which considerably simplifies the methodology of autologous cellular construction preparation and allows to avoid its contamination by feeder cells. The protocol allows obtaining cells with high proliferative potential using a relatively small biopsy sample. A simplified recipe of the culture medium in two variants was also tested in the work. The use of DMEM/F12 as a basic medium allows activating cell maturation, which can be used for modeling epithelium differentiation in vitro. The formulation of the medium with 0.06 mM of Ca supports a reduced level of ripening cells in the culture, and, consequently, is promising for obtaining a cellular construct and its transplantation to patients in case of corneal epithelial stem cells failure. It should be noted that these formulations of media can be improved. Thus, to completely eliminate xenogenic components, the fetal bovine serum can be replaced by autologous one, and such components as insulin, hydrocortisone, and EGF are currently produced in compliance with GMP rules.

Thus, media formulations, source, and method of cell culture described in this study may be used for the transplantation of the autologous labial mucosal epithelium in patients with bilateral limbal insufficiency.

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The authors declare no conflict of interest.

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