Expression of Multipotent and Retinal Markers in Pigment Epithelium of Adult Human *in Vitro*

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Immunoperoxidase and molecular genetic analysis showed that retinal pigment epithelial cells from adult human eye undergo morphogenetic changes *in vitro*. They lose expression of tissue-specific protein *RPE65* and start to express stem cell markers: *Oct4 (POU5F1), Nanog, Prox1, Musashi 1*, and *Pax6,* which indicates their differentiation. Expression of *Musashi 1* and *Pax6* attest to neural differentiation, which is also confirmed by the expression of *βIII-tubulin*, a neuroblast marker, and markers of differentiated neuronal cells, tyrosine hydroxylase and neurofilament proteins. These findings attest to the capacity of retinal pigment epithelium from adult human eye to transdifferentiation into neural lineage cells, which makes them an interesting object for cell therapy in neurodegeneration.

Key Words: *retinal pigment epithelium of human eye; transdifferentiation; cell culture; immunohistochemistry; polymerase chain reaction*

Recent studies showed that mammalian and human differentiated somatic cells can be reprogrammed into induced pluripotent SC (IPSC) by using four genes *Oct4, Sox2, c-myc, Klf4* [15] or the corresponding mRNA [17], while reprogramming of human neural stem/progenitor cells (SPC) into IPSC requires only one gene *Oct4* [8]. These discoveries raised new interest to the problem of SC plasticity and provided the basis for understanding of the processes of neural cell de- and transdifferentiation.

Brain neural SPC-like cells were recently detected in the retina; these are Muller glial cells and retinal pigment epithelial cells (RPE) [5].

RPE seem to be more interesting, because of their documented potential to de- and transdifferentiation into other cell types in various vertebrates [11,18,19]. RPE and neural retina originate from the anterior neural plate [10]. Probably due to their common origin, embryonic RPE in many vertebrates can transdifferentiate into neural retina, but only tailless amphibians retain this feature in the adult state [11]. Moreover, ciliary body pigment cells exhibit multipotent properties *in vivo* [16], while they are not stem cells in mammals [6].

Various pathologies of human eye are associated with proliferation of RPE cells, migration, depigmentation, and expression of untypical markers [9]. Functional and phenotypic heterogeneity (mosaicism) of RPE [4] suggests that these phenomena are underlain by activation of population of pigment cells exhibiting multipotent properties and undergoing properties similar to transdifferentiation in lower vertebrates.

RPE cells from adult human eye demonstrate heterogeneity in culture: some cells exhibit multipotency and start to produce marker proteins of neural SC and neurons at early and late differentiation stages [1-3]. These findings indicate that human RPE cells under certain *in vivo* and *in vitro* conditions can exhibit multipotency and transdifferentiate into other cell types [3,13]. However, the mechanisms of transdifferentiation of human RPE are poorly studied, because the studies were primarily performed on amphibian, rodent, and chicken cells.

This study is aimed at elucidation of molecular and genetic mechanisms of plasticity of RPE cells

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from adult human eye in vitro, which will extend our knowledge on their differentiation and induction into cells of stable neural pool.

MATERIALS AND METHODS

Experiments were performed on RPE cells from adult human eye; autopsy material was taken within 12-48 h postmortem or in 5 h after surgeries for traumatic eye damages. For preparing cell culture, RPE cells were isolated from the eyeballs under a binocular lens. To this end, the posterior sector of the eye was separated, the vitreous body and the retina were removed, and cold Hanks saline with EDTA (Sigma) was poured in the eyeball and incubated for 10-30 min at 4°C. The cells detached from the choroid were thoroughly collected with a pipette. Enzyme treatment of RPE cells was not used.

The following methods were used: cell culturing, PCR, qualitative real-time PCR and immunoperoxidase staining.

RPE cells were cultured at 37°C and 5% CO_2 in DMEM/F12 (1:1; Invitrogen) containing 2 mM L-glutamine (Biolot) and 5-10% FCS (Sigma) and supplemented with B27 (1:100; Invitrogen) or N2 (Invitrogen) with 20 ng/ml bFGF (Invitrogen).

The cells grew in the form of adherent cultures. After attaining dense confluence, the cells were subcultured with trypsin (Biolot).

For molecular analysis, total RNA from RPE cell cultures was isolated using TRI-Reagent (Sigma). cDNA was synthesized using RevertAid H Minus Kit (Fermentas) and 1 μg total RNA treated with DNase (Fermentas). PCR analysis of genes encoding transcription factors and differentiation markers *Oct4 (POU5F1), Nanog, Pax6, Musashi 1, Prox1, βIII-tubulin,* and *RPE65* (Table 1) was performed using kits from the same company. Amplification conditions were as follows: 45 sec at 94°C, 45 sec at 56°C, and 1 sec at 72°C (30-40 cycles). cDNA libraries were standardized by RPL19, human ribosomal protein with a molecular weight of 19 kDa. Gene expression was evaluated by the intensity of fluorescence of bands obtained after electrophoretic separation of PCR products in 1% agarose gel on a gene analyzer (BIO-RAD™XR).

Quantitative real-time PCR was performed on a ABI Prizm 7500 cycler using glyceraldehyde-3-phoshate dehydrogenase (GAPDH) as endogenous control. The reaction mixture (25 μl) contained ROX dye allowing sample volume inaccuracy leveling. Reaction conditions were as follows: 3 min at 95°C, 15 sec at 95°C, and 60 sec at 60°C (45 cycles). Each experiment was repeated 3 times. Expression of the studied genes was evaluated by $\Delta\Delta C_t$ method. The expression of *βIII-tubulin* and *Pax6* genes was evaluated. Primers with a fluorescent label were selected and synthesized by DNA-Sintez Company: *βIII-tubulin*

TABLE 1. Functions of Genes and Primer Sequences for PCR

Gene	Function	Primer sequence	Fragment length, b.p.
Oct4 (POU5F1)	Transcription factor, pluripotent status	Forward 5'atgtgtaagctgcggcccttg3' Reverse 5'gtttgaatgcatgggagagcc3'	491
Nanog	Transcription factor, pluripotent status	Forward 5'gtgtggatccagcttgtccc3' Reverse 5'ctgcgtcacaccattgctattc 3'	497
Musashi1	RNA-binding protein, regulates self-renewal of neural SC	Forward 5'tgaaggagtgtctggtgatgcg3' Reverse 5'tgccggttggtggttttgtc3'	310
Pax6	Transcription factor of neural differentiation	Forward 5'gtcatcaataaacagagttcttc3' Reverse 5'cgattagaaaaccatacctgtat3'	424
Prox1	Transcription factor, controls proliferation of neural progenitor cells	Forward 5'agtctgaggaccaagatgtcat3' Reverse 5'cttcactatccagcttgcagat3'	595
III-tubulin	Neuroblast microtubule protein	Forward 5'cagtgcggcaaccagatcgg3' Reverse 5'caggtcagcgttgagctggc3'	720
RPE65	RPE protein controlling interaction with retinal photoreceptor	Forward 5'ctctttgaagttggatctgagcc3' Reverse 5'agggcattgtcagtaacctctac3'	254
RPL19	Human ribosomal protein for standardization of cDNA libraries	Forward 5'agggtacagccaatgcccga3' Reverse 5'ccttggataaagtcttgatgatc3'	326

F=5'-ggg cca agt tct ggg aag tc-3', probe=5'-FAM-atg agc atg gca tcg acc cca gc-BHQ1-3', R=5'-cga gtc gcc cac gta gtt g-3'; *Pax6* F=5'-caa ttc cac aac cca cca cac-3', probe=5'-FAM-tcc tcc ttc aca tct ggc tcc atg t-BHQ1-3', R=5'-ctg tag gtg ttt gtg agg gct gt-3'.

For immunoperoxidase analysis, the cultured RPE cells from adult human eye were fixed in cold acetone or 4% paraformaldehyde (Sigma) in 0.01 M PBS (pH 7.4) (Biolot). Cell differentiation was evaluated using antibodies to βIII-tubulin (Abcam), tyrosine hydroxylase (Abcam), and 68- and 200-kDa neurofilaments (Abcam) dissolved in 0.1% BSA. The preparations were stained by the standard method using EnVision™ Gj2 Doublestain System, Rabbit/Mouse (DAB+Permanent Red; Dako). The preparations were then stained with hematoxylin and embedded in aqueous non-fluorescent medium Shandon Immu-Mount™. The results of immunocytochemical reactions and photographed were evaluated using an Olympus AH3 microscope.

RESULTS

RPE cells grew in the form of adherent cultures, actively proliferated, and lost pigmentation. In the heterogeneous cell population, two cell subtypes differing by adhesion capacity, migration, and phenotypic changeability were distinguished, which agreed with our previous findings [1,2]. Cells of the first subtype (subpopulation I) rapidly acquired fibroblast-like morphology, started actively migrate and proliferate, possessed weak adhesion capacity, and rapidly acquired round shape and detached from plastic during trypsin treatment, due to which they can be separated from cells of the second subtype. Cells of the second subtype (subpopulation II) retained epithelial morphology, weakly migrated, and were highly adherent to plastic.

RPE65 protein is one of the main markers of RPE cells. It actively participates in the phototransduction cycle during interaction of RPE with photoreceptors. The expression of gene encoding this protein in native RPE of adult human eye is high and is determined at the major level (Fig. 1). However, the cells lost *RPE65* expression under conditions of cell culture; it is detected at low level in few cultures (Fig. 1). The loss of *RPE65* expression during culturing attests to possible dedifferentiation.

PCR assay of native RPE of adult human eye revealed mRNA of *Oct4 (POU5F1)* gene at the minor level (Fig. 1). This suggests the presence of a multipotent subpopulation in total RPE. During culturing, the expression of *Oct4 (POU5F1)* gene in RPE cells sharply increased in comparison with native tissue. The level of expression practically did not depend on culturing conditions. It can be hypothesized that activation of *Oct4* expression in RPE *in vitro* reflects increased cell plasticity in culture.

Apart from expression of *Oct4* gene, we detected transcripts of *Nanog* gene, which are absent in native RPE (Fig. 1). Culturing conditions little affected the level of the studies gene. In media of the same composition, the expression of *Nanog* was lower in fibroblast-like cells (subpopulation I) than in epithelium-like cells (subpopulation II; Fig. 1).

The presence of transcripts of *Oct4* and *Nanog* genes in cultured RPE cells from adult human eye suggests that the cell acquired the properties of SPC *in vitro*.

Expression of *Musashi 1* gene was detected in native and cultured RPE of adult human eye (Fig. 1). The expression in cell culture was higher and did not depend on the duration of culturing and medium composition. Expression of *Musashi 1* gene was similar in different subpopulations (Fig. 1). It is known that transdifferentiation of RPE of adult newts is associated with synthesis of Musashi 1, an RNA-binding protein

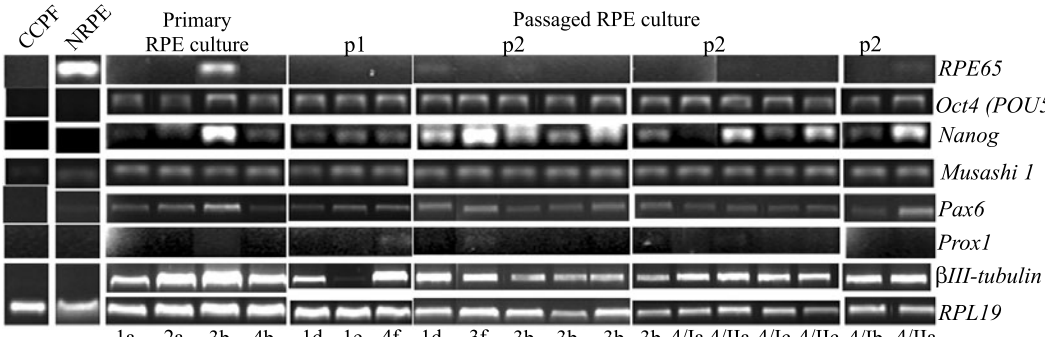

Fig. 1. The results of PCR analysis illustrating the pattern of *Oct4 (POU5F1), Nanog, Musashi 1, Pax6, Prox1, bIII-tubulin, RPE65* gene expression in primary and passaged adherent cultures of RPE cells of human adult eye and in the control: cultures of postnatal fibroblasts (CCPF) and native RPE (NRPE). *RPL19* gene was used for standardization. P: passage. Here and in Fig. 2: composition of culture media for RPE cells: *a*) 10% FBS+N2+bFGF; *b*) 10% FBS+B27; *c*) 10% FBS; *d*) 5% FBS+N2+bFGF; *e*) 5% FBS+B27; *f*) 5% FBS. 1-4) donors of cell cultures; I: subpopulation I; II: subpopulation II. 3b: culture obtained from donor 3 was subcultured in 3 flasks.

playing an important role in the maintenance of stem functions of neural cells, differentiation, and tumor growth [12,14]. Expression of *Musashi 1* gene in native RPE cells of adult human eye attests to the presence of a subpopulation of low-differentiated cells exhibiting properties of neural SPC. High level of *Musashi 1* expression in cell culture can be related to active differentiation of cells.

A similar picture is observed during the analysis of the expression of *Pax6* gene encoding the key transcription factor of neural SPC. In native RPE cells its level is lower than in cell culture (Fig. 1). Under *in vitro* conditions, *Pax6* expression varies insignificantly. In some cases, *Pax6* expression in cells of epithelial morphology (subpopulation II) was higher than in fibroblast-like cells (subpopulation I). The presence of this transcription factor suggests the presence of cells exhibiting properties of neural SPC.

Expression of *Prox1* gene encoding transcription factor, a marker of subpopulation of neural progenitor cells, was detected by PCR at a low level in some RPE cultures (Fig. 1). In native RPE this gene was not expressed. Expression of *Prox1* gene can indicate initial stages of neural differentiation in cultured RPE cells.

Expression of *βIII-tubulin* gene was detected by PCR in all studied cultures, but was absent in native RPE. The amount of mRNA in cells slightly decreased during culturing (Fig. 1). The presence of *βIII-tubulin* gene transcripts implies the capacity of RPE cells to neuronal differentiation.

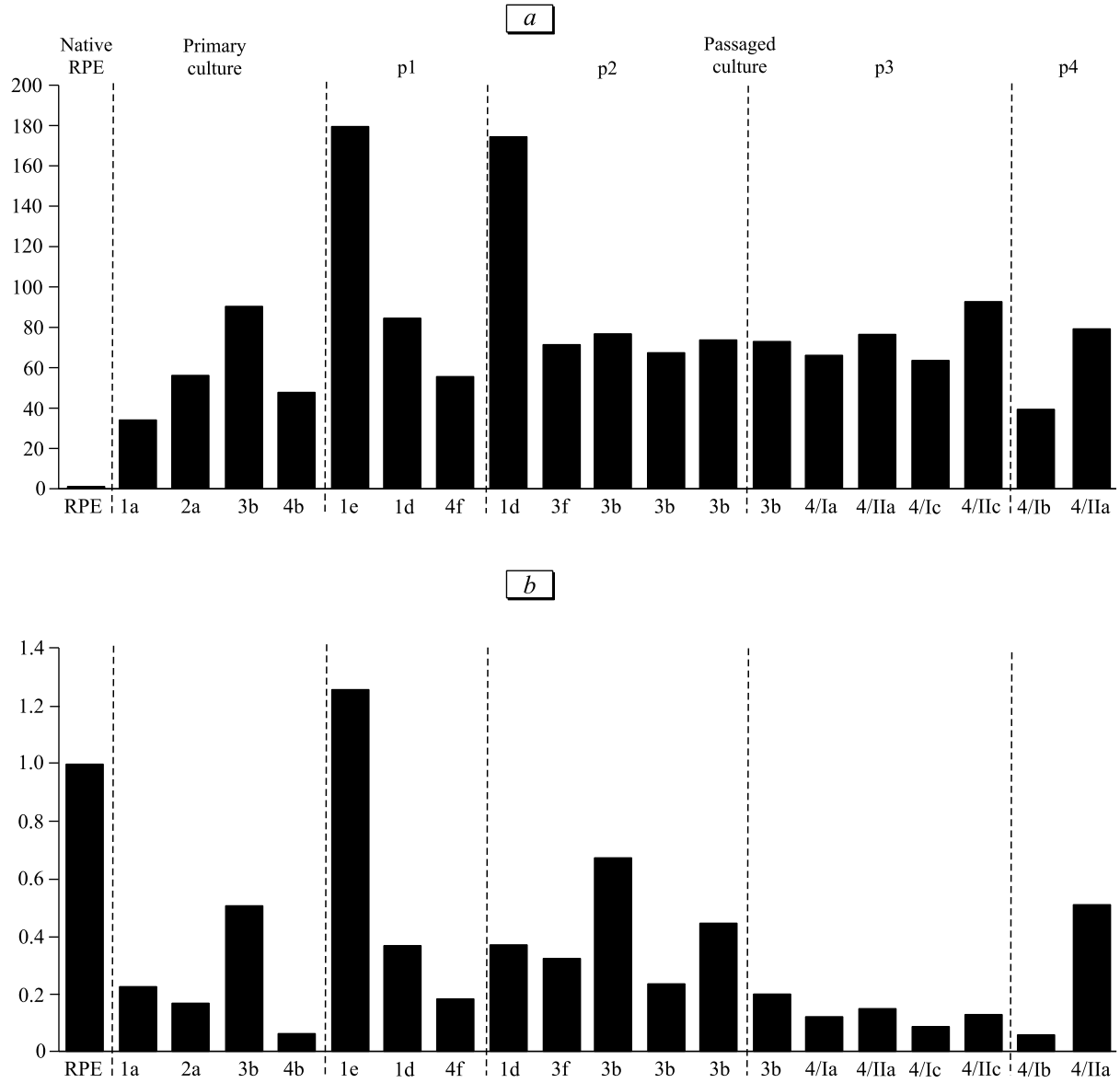

Fig. 2. Results of PCR analysis illustrating the pattern of β*III-tubulin* (*a*) and *Pax6* (*b*) gene expression in primary and passaged adherent cultures of RPE cells of human adult eye and in native RPE (control). Ordinate: relative expression level.

Thus, PCR analysis of native RPE of adult human eye revealed minor expression of pluripotent status gene *Oct4 (POU5F1)* and markers of neural SC *Musashi 1* and *Pax6*, which suggests the existence of a small population of cells exhibiting properties of neural SPC.

A sharp decrease in *RPE65* gene expression in RPE cells from adult human eye *in vitro* indicates the start of dedifferentiation. In parallel, expression of *Oct4 (POU5F1), Nanog, Musashi 1, Prox1 u Pax6* genes, markers of SPC, was detected. The expression of neural cell marker *βIII-tubulin*, *Pax6*, and *Musashi 1* attests to neural potential of RPE cell cultures.

PCR analysis revealed differences in the expression of some genes in RPE populations *in vitro*. For instance, fibroblast-like cells (subpopulation I) characterized by higher reactivity to environmental changes demonstrated lower expression of *Nanog* and *Pax6* genes. These differences are probably related to different differentiation status of RPE cells under conditions of *in vitro* culturing. Subpopulation I cells have already passed dedifferentiation stage, which might had been characterized by elevated expression of these genes, in contrast to cells of epithelial morphology that have not yet entered or have just entered this stage.

Quantitative real-time PCR analysis confirmed the presence of mRNA of neural differentiation markers *βIII-tubulin* and *Pax6* in native and cultured RPE from adult human eye. In RPE cells, the expression of *βIII-tubulin* gene sharply increased under *in vitro* conditions (by 35-175 times in different samples; Fig. 2). The maximum expression of *βIII-tubulin* gene was detected in passage 1-2 cultures in media with 5% serum containing B27 or N2 with bFGF.

Expression of pax6 gene was detected in native RPE and decreased during *in vitro* culturing. The discrepancy with PCR data is determined by inhibition of polymerase reaction by melanin in native cells. Real-time PCR analysis of *Pax6* expression in cultured RPE cells showed that it depended on the duration and conditions of culturing. Cells growing in media containing B27 supplement were characterized by enhanced expression of *Pax6*. However, the content of the corresponding mRNA gradually decreased during culturing. Comparison of real-time PCR data in different subpopulations of RPE cells confirmed enhanced

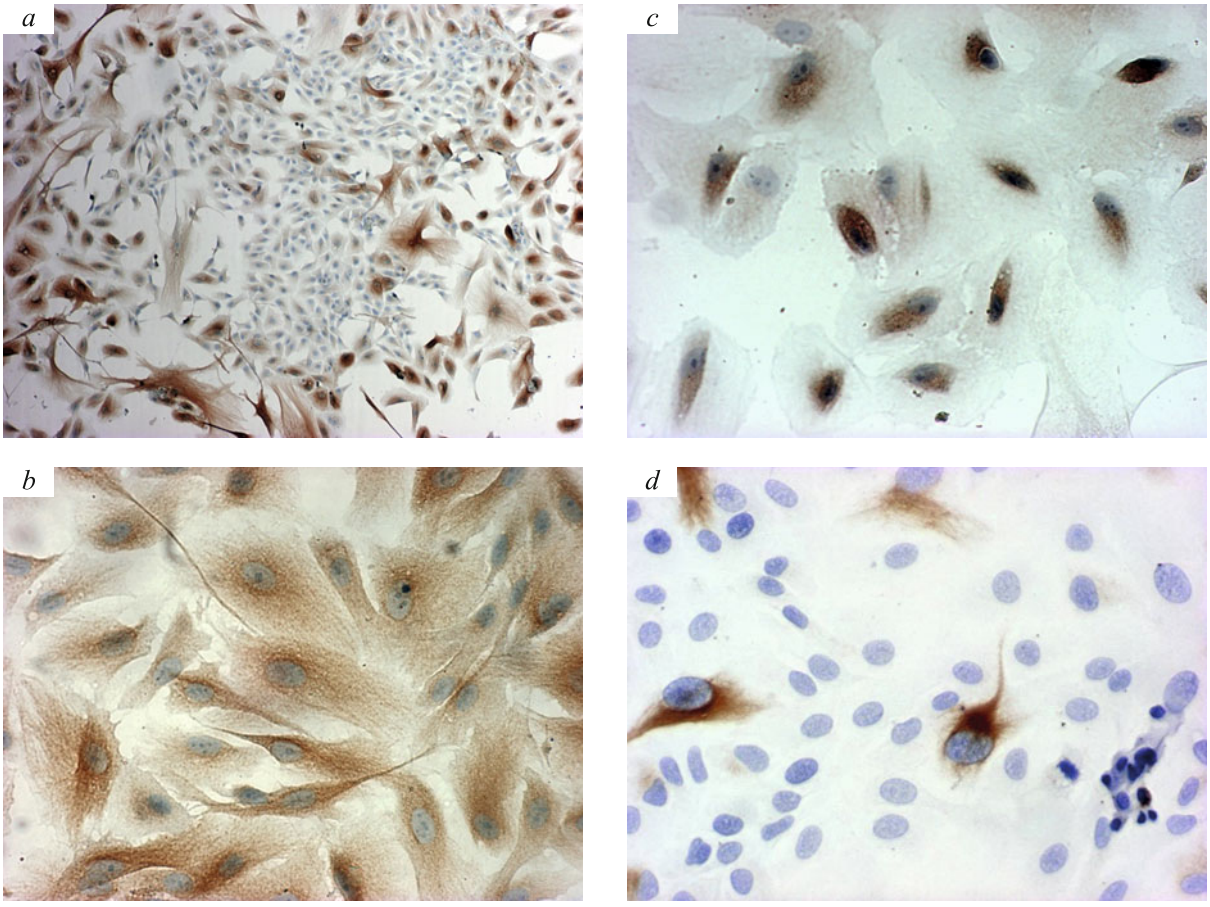

Fig. 3. Immunoperoxidase analysis of passage 2 adherent RPE cell culture from adult human eye grown on DMEM/F12 medium containing 10% serum and N2 with bFGF. *a*) βIII-tubulin (×40); *b*) βIII-tubulin (×200); *c*) tyrosine hydroxylase (×200); *d*) 68- and 200-kDa neurofilament proteins (×200). The nuclei were poststained with hematoxylin.

expression of *βIII-tubulin* and *Pax6* gene in cells of epithelial morphology (Fig. 2).

Immunoperoxidase method showed the presence of βIII-tubulin, tyrosine hydroxylase, and 68- and 200-kDa neuronfilament proteins, markers of neuronal differentiation in RPE cells irrespective of culturing conditions (Fig. 3). βIII-Tubulin in RPE cultures was detected in cells of epithelial (subpopulation II) and fibroblast-like (subpopulation I) morphology. The most pronounced staining was observed in large dendritic cells. Some colonies of small cells retaining epithelial morphology demonstrated weak or background staining as did small fibroblast cells (Fig. 3, *a*, *b*). Tyrosine hydroxylase, a marker of dopaminergic neurons, was found in perinuclear area of few cells of RPE culture (Fig. 3, *c*), primarily in cells of epithelial morphology. Immunoperoxidase staining for 68- and 200-kDa neurofilament proteins was detected in solitary small fibroblast-like cells (Fig. 3, *d*).

Thus, immunoperoxidase staining and molecular genetic analysis revealed considerable morphogenetic changes in RPE cells from adult human eye during culturing. These cells enter dedifferentiation process associated with the loss of specific markers (*RPE65),* and expression of SC markers *Oct4* (*POU5F1), Nanog, Prox1, Musashi 1,* and *Pax6*. Expression of neural SPC markers *Musashi 1* and *Pax6,* neuroblast marker *βIII-tubulin*, and markers of differentiated neuronal cells tyrosine hydroxylase and 68- and 200-kDa neurofilament proteins attest to neural differentiation. These results confirmed previous data on heterogeneity of native RPE cells in human adult eye and their capacity to dedifferentiation and development to neural lineage cells [1,2].

The detected expression of SC marker genes supports the hypothesis on the presence of subpopulation of cells exhibiting the properties of neural SPC. Hence, cultured RPE cells of adult human eye can be considered as the source of neural SPC for cell therapy [7].

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