In Vitro Phenotypic Modification of Pigmented Epithelium Cells from Human Eye at Early Stages of Development

L. A. Milyushina, R. A. Poltavtseva, M. V. Marei*, O. V. Podgornyi, G. T. Sukhikh*, and M. A. Aleksandrova

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Multipotent characteristics of human fetal (9-11.5 weeks) pigmented epithelial retinal cells and capacity to transdifferentiation in neuronal direction were studied *in vitro* under different culturing conditions. The cultures were analyzed using a wide spectrum of antibodies. It was found that pigmented epithelium of human eye is a heterogeneous cell population with three subtypes differing by adhesion characteristics, migration, transdifferentiation potential, and reaction to microenvironmental factors. Subtype 1 cells steadily retain their epithelial characteristics, subtype 2 cells change their morphotype and produce neuroblast and photoreceptor cell proteins, and subtype 3 cells form free floating spheres and are capable to multipotent differentiation.

Key Words: *human ocular pigmented epithelium; differentiation; proliferation; tissue culture; immunohistochemistry*

Stem/progenitor cells (SPC) of mammalian and human central nervous system are capable of self-maintenance and multipotent differentiation with the formation of neurons and glia *in vivo* and *in vitro* [1,12,21].

Cells with progenitor characteristics were detected in the retina, which, similarly to the entire forebrain, develops from neuroepithelial cells of the prosencephalon. It is hypothesized that Muller glial cells from the visual part of the retina and pigmented cells from the ciliary area and pigmented epithelium (PE) possess progenitor properties [6,7,8,22]. PE cells capable of plastic modification and transdifferentiation [5] attract special interest.

Neuroepithelial cells of presumptive midbrain form the eye vesicle during the early embryonic development of the brain in vertebrates. The cells in this vesicle are morphologically and molecularly indiscernible and co-express transcription factors Otx2, Pax6, Rx, Six3, and other regulatory genes essential for the formation of the eye. Inductive signals from the ectoderm (various FGFs) and from mesenchymal cells (transforming growth factor-β family members), transcription factors Mitf, Otx1/Otx2, and Pax6, morphogenes BMP, Shh, and cell cycle regulators are involved in PE differentiation. A cell-cycle inhibitor marker protein Cx43 mediating gap-junctional intercellular communication, is produced in PE cells from the start of their differentiation [9]. Neuroepithelial cells differentiating into PE express transcription factors and some specific molecules, differing them from the neural retina, during the very first stages of morphogenesis [8,11]. Normally, PE in differentiated eye of vertebrates is a monolayer of intensively pigmented hexagonal polarized nonproliferating cells involved in extremely important defense and physiological functions providing vision [18].

PE of adult lower vertebrates is capable of de- and transdifferentiation into other cell types. In triton eye, PE cells after proliferation, depigmentation, and multipotent differentiation transform into nerve and glial cells, thus completely reconstructing damaged or re-

N. K. Koltsov Institute of Developmental Biology, Russian Academy of Sciences; *V. I. Kulakov Center of Obstetrics, Gynecology, and Perinatology, Federal Agency for Health and Social Development of Russia, Moscow, Russia. ***Address for correspondence:*** mariaaleks@inbox.ru. M. A. Aleksandrova

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moved retina [13]. RNA-binding protein Musashi-1 is expressed in PE cells of adult tritons and mice during transdifferentiation. This protein plays an important role in the realization of the stem functions, differentiation, and tumor growth [16,20].

In birds and mammals, PE transdifferentiation is observed only during the early embryonic development and can be stimulated by FGF [28] and *Pax6* gene insertion [3]. In adult specimens, transdifferentiation of PE cell can be *in vitro* stimulated by FGF and EGF (which are essential for culturing cerebral SPC) and some morphogens [17,25,27].

In human PE cells, the processes similar to transdifferentiation were first detected in eye diseases involving active proliferation, migration, and depigmentation of cells, which start expressing atypical markers [2]. Mosaicism of PE [4] suggests that these pathological events are mediated by activation of cells with multipotent characteristics; changes in these cells are similar to transdifferentiation processes in lower vertebrates.

Cultured PE cells from adult human eye demonstrate heterogeneity: some retain epithelial status, others demonstrate active behavior and phenotypical variability, proliferate, and loose pigmentation and differentiation markers characteristic of PE. Some cells exhibit multipotency and start producing marker proteins intrinsic of the neural SC of the early and late stages of neuronal differentiation, though the major part of PE cells do not undergo transdifferentiation, but are just modified [11]. At the same time, differentiation into lenticular cells is possible [19]. The results of these studies indicate that under certain conditions human PE cells exhibit multipotency and transdifferentiate into other cell types *in vivo* and *in vitro*. Studies in this direction are interesting for detection of other sources of neural progenitor cells and are important for deciphering the basic mechanisms of cell modification, which can underlie many retinal diseases.

We studied the capacity of human fetal PE cells to transdifferentiation in the neuronal direction *in vitro*. The behavior and phenotypical changes in PE cell from human fetal eye were studied under different conditions of culturing and the resultant cultures were analyzed using a spectrum of antibodies, neural differentiation markers.

MATERIALS AND METHODS

The study was carried out on the eyes of nonviable aborted human fetuses of 9, 9-10, 10.5, 11.5, 12 weeks of development, delivered from licensed institutions, functioning within the framework of legislation of the Russian Federation on public health protection in accordance with authorized list of medical indications with approval of the local academic council.

Enucleated eyes were put into cold Hanks EDTA solution (Sigma), the anterior segment was separated from the posterior one under a binocular microscope. After removal of the vitreous body and retina, PE fragments were isolated and cultured in DMEM/F12 (Sigma) with 10% fetal calf serum (FCS; Sigma) without factors at 37°C and 5% CO_2 for 14-15 days in order to obtain adherent cultures. In parallel, fetal (11.5 weeks) PE cells were cultured in a special medium consisting of DMEM/F12 factors (Sigma) with 2% N2 (Ivitrogen), 1% FCS (Sigma), 20 ng/ml bFGF (Sigma), and 20 ng/ml EGF (Sigma) (this medium is used for the maintenance and accumulation of neural SPC) at 37°C and 5% CO_2 for 14-15 days. The medium was partially replaced with a fresh portion every 3 days in all cultures.

In the medium with growth factors PE cells formed adherent culture and small floating spheres. After 14 days in culture, the spheres were transferred into different media containing 10% FCS or 10% FCS with bFGF and EGF factors. After 2 days, some cultures were fixed for immunochemical analysis. The remaining cells were cultured for 1 week more in a medium with 10% FCS or containing medium conditioned by human embryonic neural stem cells (NSC). For immunohistochemical analysis, the cell cultures were fixed in 4% paraformaldehyde in 0.01 M PSB (pH 7.4).

Immunohistochemical study of human fetal eyes was carried out at the stages of gestation weeks 9-10 and 12. The eyes were fixed in 4% paraformaldehyde in PSB, washed 3 times in PSB, successively treated with 5, 10, 20% sucrose, placed into Tissue Freezing Medium, and frozen in liquid nitrogen. Serial sections of the eyes (12 μ) were mounted on gelatin chromium-coated slides.

After washout, the samples (eye sections and cell cultures) were treated for 30 min with 0.1% Triton X-100, 0.25% Twin in PSB, after which the cells were left for 30 min in 3% BSA, 0.25% Twin in PSB. The samples were then incubated for 1 h at 37°C in 3% BSA solution, 0.25% Twin in PSB with first antibodies to the following proteins: connexin-43 (Cx43; gap junction marker, Sigma, 1:200), nestin (NSC marker, Chemicon, 1:200), β-III-tubulin (early neuroblast marker, Abcam, 1:200), recoverin (photoreceptor marker; kind gift from Prof. P. P. Filippov), Ki-67 (proliferating cell marker, Abcam, 1:50), Oct4 transcription marker (pluripotent cell marker, Abcam, 1:200), and Pax6 (neural progenitor cell marker, Chemicon, 1:100). Double immunohistochemical staining was used in some cases. After washout, the cells were treated with second antibodies with fluorescent stains. The nuclei were stained with Hoechst-33342. Stained preparations were embedded in glycerol and analyzed under OLIMPUS-DP70 fluorescent microscope with a digital

camera. Quantitative analysis of cell cultures stained with antibodies was carried out using CellsCount3 software.

RESULTS

According to the data of immunohistochemical analysis of human fetal eyes at gestation weeks 9-10 and 12 carried out on frozen sections, PE at these terms was presented by a layer of cubical or slightly cylindrical pigmented cells. Cx43 protein (gap junction marker) responsible for the integrity of the epithelial layer and barrier functions of PE was detected by the immunohistochemical method at all stages of the study (Fig. 1, *a*, *b*). The PE cells, stained with antibodies to Ki-67 (proliferation marker), exhibited a low level of proliferative activity and were not stained with antibodies to neural differentiation markers. However, in a parallel series on the retina these antibodies detected nestin-positive radial glia SC, β-III-tubulin-positive neuroblasts, and recoverin-positive photoreceptor cells at 10 weeks of development (Fig. 1, *a-c*).

Human fetal PE cells (gestation weeks 9, 10.5, and 11.5) cultured in DMEM/F12 with 10% FCS adhered to the bottom of culture flask in all cases. Two main cell types were detected in these cultures: flat polygonal epithelial and elongated or stretched fibroblast-like slightly pigmented or pigment-free cells (Fig. 1, *d*, *e*). The Cx43 gap junction protein served as a characteristic marker for the majority of cells of epithelial morphology. This protein was distributed over the entire contour of the cell (Fig. 2, *a*), while just solitary fibroblast-like cells produced it. The content of Cx43-positive cells in PE cultures of 10.5 and 11.5 weeks of development was 65.42 and 74.26%, respectively (Fig. 3). Many cells were stained with antibodies to Ki-67 protein (proliferation marker; Fig. 2, *a*); their percentage was higher among fibroblast-like cells. The total content of proliferating cells in cultures was 7.19% at 9 weeks of development, 10.9% at 10.5 weeks, and 10.56% at 11.5 weeks of development (Fig. 3). These data indicate that proliferation in human PE cell cultures derived from eyes at the early stages of development virtually does not depend on the term of fetal development.

Cultured PE cells differed from those in intact eye: they actively proliferated, lost pigmentation, and were virtually depigmented by day 14 of culturing. Immunohistochemical analysis with neural markers showed significant changes in cells. Few nestin-positive cells (nestin is the marker of neural SPC) were detected in the culture derived from 9-week fetuses; these cells were strongly elongated and bipolar. Double staining with antibodies to Ki-57 revealed proliferation of nestin-positive cells. Antibodies to β-III-tubulin detected solitary and small groups of cells with fibroblast-like morphology in PE cultures originating from fetuses of 9 and 10.5 weeks of development (Fig. 2, *b*, *c*), their number being much higher (54.20%) in cultures derived from fetuses of 11.5 weeks of development

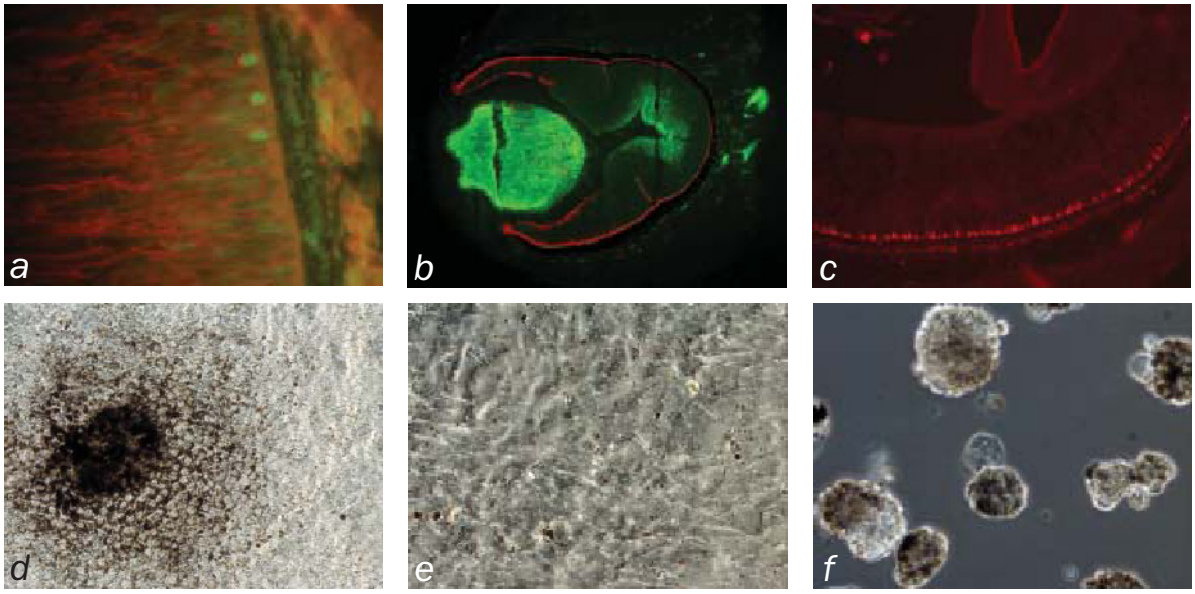

Fig. 1. Immunohistochemical analysis of retinal PE cells of 9-10 and 12 weeks of development (*a-c*) and phase contrast microscopy of PE cultures of 11.5 weeks of development (*d-f*). *a*) proliferating cells in external neuroblastic layer of the retina (green) and in PE layer, nestin-positive cells in the thickness of the retina (red; ×200); *b*) β-III-tubulin-positive cells in the forming layer of nervous fibers (green) and Cx43-positive PE cells (red; ×100); *c*) forming photoreceptors in external neuroblastic layer of eye retina at 12 weeks, antibodies to recoverin (×100); *d*) cells of epithelial morphology (×100); *e*) cells of fibroblast-like morphology (×200); *f*) free confluent spherical aggregations (×100).

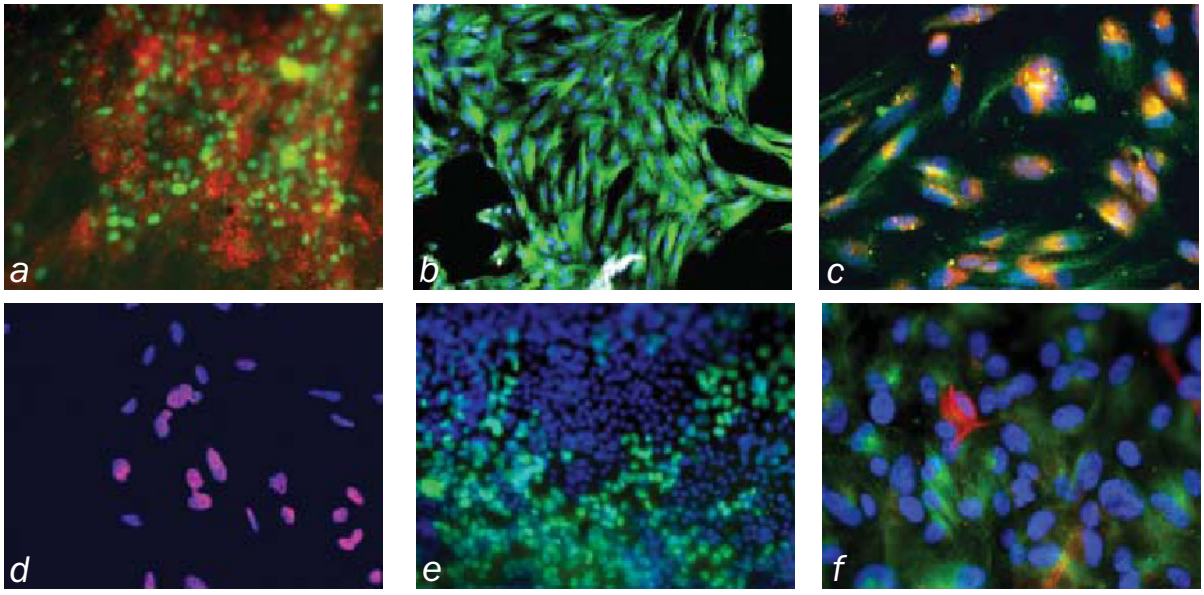

Fig. 2. Phenotypes of PE cells of 9, 10.5, 11.5 weeks of development, cultured in DMEM/F12 with 10% FCS and in DMEM/F12 with 1% FCS and with bFGF and EGF. *a*) proliferating PE cells of epithelial and fibroblast-like morphology, staining with antibodies to Cx43 (red) and Ki-67 (green; ×100); *b*) group of β-III-tubulin-positive cells in culture of PE cells of 9 weeks of development (×100); *c*) recoverin-positive cells in cultured PE cells of 10.5 weeks of development: double staining for recoverin (red) and β-III-tubulin (green; ×200); *d*) solitary Oct4-positive cells in PE cell culture of 10.5 weeks of development (×200); *e*) Pax6-positive cells in PE cells of 11.5 weeks of development, cultured in medium with growth factors (×40); *f*) neuroblasts (green) and photoreceptor cells (red) in PE cells of 11.5 weeks of development, cultured in medium with growth factors: double staining for recoverin and β-III-tubulin (×200).

(Fig. 3). Double immunohistochemical staining of cultures of 10.5 and 11.5 weeks of development showed that some of β-III-tubulin-positive cells were also stained with antibodies to recoverin. Recoverin was seen as small and large granules in the perinuclear and peripheral cytoplasm, and the cells differed by the density of its distribution (Fig. 2, *c*). The percentage of recoverin-positive cells in cultures of 9, 10.5, and 11.5 weeks of development, growing in media with 10% FCS, was 0, 14.21, and 1.31%, respectively (Fig. 3). Solitary fibroblast-like cells in PE culture starting from 10.5 weeks of development were stained for Oct4 protein (marker of pluripotent status; Fig. 2, *d*), which can indicate the possibility of their dedifferentiation. In none of the studied cultures Pax6-positive cells were detected.

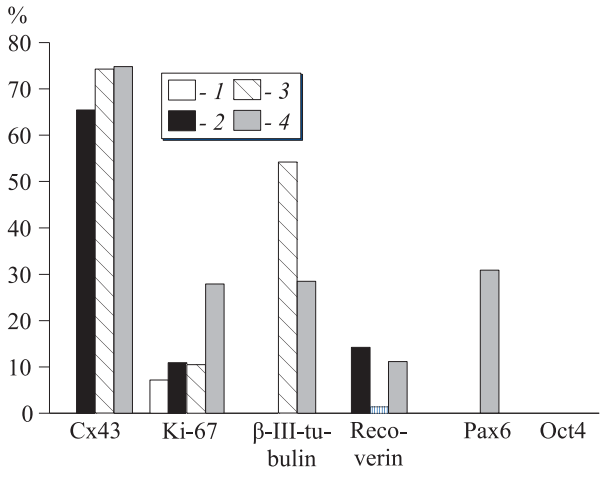

Fig. 3. Content of cells of different phenotypes in PE cultures of 9, 10.5, and 11.5 weeks of development, cultured in DMEM/F12 with 10% FCS. *1*) 9 weeks of development, 10% FCS; *2*) 10.5 weeks of development, 10% FCS; *3*) 11.5 weeks of development, 10.5% FCS; *4*) 11.5 weeks of development, FGF+EGF.

Human fetal PE cells of 9, 10.5, and 11.5 weeks of development, cultured in DMEM/F12 with 10% FCS adhered to the bottom of flasks and formed two morphological cell types: epithelial and fibroblast-like cells. Both subpopulations contained proliferating cells, their number being much higher among fibroblast-like cells than among epithelial ones. The number of proliferating cells virtually did not change in cultures originating from human fetal PE of different stages of development, though the number of fibroblast-like cells obviously increased. It seems that the subpopulation of cells of epithelial morphology not only decreases with age, but proliferative capacity of these cells decreases, which was previously detected in chicken PE cultures [23]. With proliferation, PE cells are depigmented and start expressing differentiation markers not intrinsic of adult PE *in vivo*, which is confirmed by the results of other studies [17]. Production of β-III-tubulin protein indicates that the cells are capable of differentiation in the neuronal direction. Recoverin detected in fibroblast-like cells of human

PE culture indicates the possibility of their differentiation into photoreceptors.

In order to evaluate the impact of medium composition for cell behavior, PE cells of 11.5 weeks of development were cultured in DMEM/F12 with bFGF and EGF and with 1% FCS; this medium is used for accumulation of SPC from the brain. Some PE cells in this medium adhered to the bottom of the culture flask and formed adherent cultures (Fig. 1, *d*, *e*), while the other part did not adhere and formed free spherical aggregations similar to cerebral neurospheres (Fig. 1, *f*).

After 14 days the adherent cultures contained poorly and depigmented cells of epithelial and fibroblast-like morphology. Both cell types proliferated, their number being 3-fold higher than in media without growth factors (Fig. 2, *g*). Growth factors used in our study stimulated cell proliferation in culture, which was also noted by other authors [17,25,27]. Epithelial cells and some fibroblast-like cells produced Cx43 (Fig. 3). However, a more important difference was a numerous population of cells immunohistochemically stained for Pax6 (30.86%) detected under these conditions (Fig. 2, *e*, *g*). Antibodies to Pax6 revealed cells of epithelial and fibroblast-like morphology. Since Pax6 transcription factor is expressed in neuroepithelial SC [15] and in the neural retina, but not in PE [10,11], these data could indicate dedifferentiation of PE cells. Presumably, Pax6-positive cells are capable of dedifferentiation in the neuronal direction, as the culture also contained a numerous population of β-III-tubulin-positive cells (Fig. 2, *f*). The morphology of fibroblast-like β-III-tubulin-positive cells was different; there were cells of spread and strongly elongated bipolar shape. Solitary β-III-tubulin-positive cells were small, round with few long thin processes. Double staining showed that some cells stained for β-III-tubulin were recoverin-positive, recoverin distributing in the cytoplasm as separate granules. In parallel, there were β-III-tubulin-negative cells with the entire cytoplasm evenly stained with antibodies to recoverin; these cells looked like differentiated photoreceptors (Fig. 2, *f*). The percentage of β-III-tubulin- and recoverin-positive cells was 28.5 and 11.5, respectively (Fig. 3). Immunohistochemical staining revealed no Oct4-positive cells in this cell culture.

Some PE cells exhibited a trend to differentiation in the neuronal direction irrespective of the composition of culture medium. On the other hand, bFGF and EGF stimulated the proliferative activity and expression of Pax6, which intensified the plastic changes in the cell. The PE cells transdifferentiated in the neuronal (retinal) direction, which was seen from emergence of Pax6-positive cells and a drastic increase in the number of recoverin-positive cells.

Spherical cell aggregations, obtained by culturing of PE cells of 11.5 weeks of development in medium with bFGF, EGF, and 1% FCS, consisted of pigmented and partially depigmented cells (Fig. 1, *f*). According to the data of immunohistochemical analysis, the cells in the spheres differed from the initial PE cells by the absence of Cx43 gap junction marker protein and hence, had no epithelial characteristics. In addition, the spheres contained numerous multipotent cells detected by staining with antibodies to Oct4 (Fig. 4, *c*; Fig. 5).

Study of differentiation of cells in these spheres was carried out in several experiments. In experiment 1, the spheres were transferred into the so-called differentiation medium (without growth factors with 10% FCS), similarly as the neurospheres from the cerebral SPC for differentiation stimulation. In experiment 2, the spheres were put into medium with bFGF and EGF and 10% FCS, to which medium, conditioned by fetal brain SPC (neurospheres), was added.

The spheres adhered to the bottom of the culture flask in both experiments; undifferentiated cells started active migration and proliferation (Figs. 4, 5), while on day 9 the proliferation dropped sharply (Fig. 5) and active differentiation started. Presumably, Oct4 multipotent cells from spheres developed by the neural trend, because some migrating cells were stained with antibodies to Pax6 transcription factor (Fig. 4, *f*; Fig. 5). In turn, Pax6-positive cells developed in the neural (retinal) and epithelial directions. Some cells were stained for β-III-tubulin and/or recoverin in all cultures, while other cells acquired epithelial characteristics and were stained with antibodies to Cx43 gap junction protein over the entire polygonal surface. The numerical proportion of these cell types changed significantly under the effect of medium composition: in experiment 1 neuronal differentiation of cells predominated (63.5% recoverin-positive and 17.95% Cx43-positive cells; Fig. 5), while in experiment 2 epithelial differentiation was more pronounced (52.34% Cx43-stained cells and 8.95% recoverin-positive cells; Fig. 4, *d*, *e*; Fig. 5). In addition, a small population of Oct4-positive cells was retained in this culture.

Results of studies of the spheres obtained by culturing of PE cells in medium with bFGF and EGF indicate that the spheres consisted of pigmented cells which lost the epithelial characteristics, with many Oct4-positive cells among them. Spheres from PE were similar to the spheres from the ciliary region cells [6], but differed from the cerebral neurospheres by structure and re-inoculation potential [1,14,26]. The expression of Oct4 and Pax6 indicated dedifferentiation of the cells from the spheres under the effect of factors of the medium. Presumably, some of them possessed properties of poorly differentiated cells, characterized

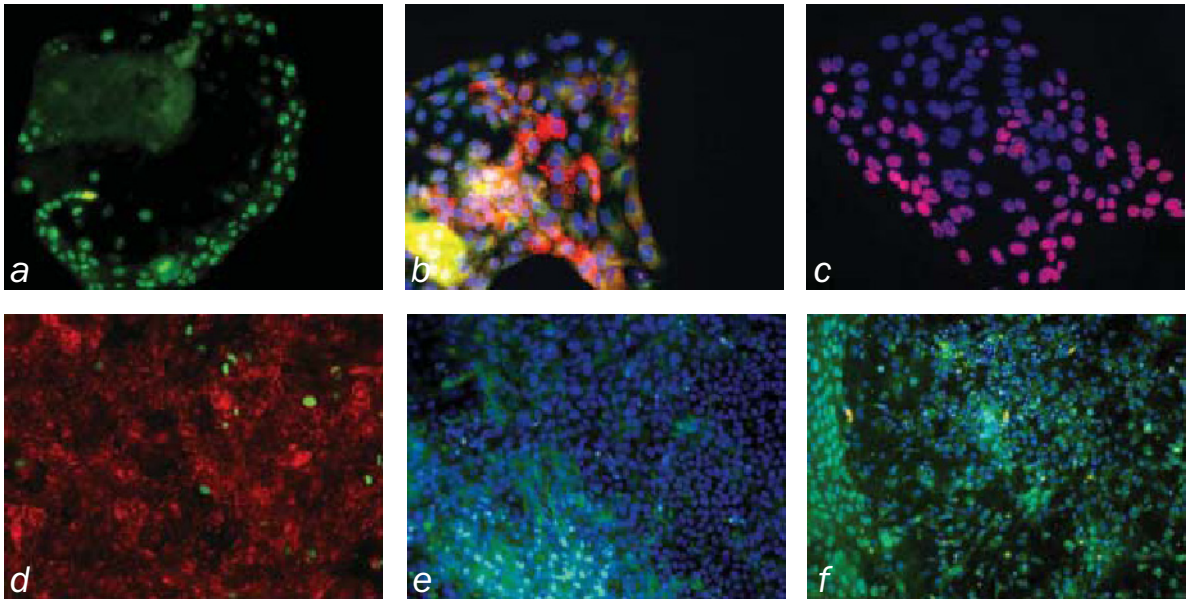

Fig. 4. Immunohistochemical analysis of cultured PE spheres of 11.5 weeks of development on days 2 (*a-c*) and 9 (*d-f*). *a*) proliferating cells: staining for Ki-67 (×100); *b*) recoverin- (red) and β-III-tubulin-positive (green) cells: double staining (×100); *c*) Oct4-positive cells (×100); *d*) proliferating cells (green) of epithelial (red) and fibroblast-like morphology: staining for Ki-67 and Cx43 proteins (×100); *e*) neuroblasts cultured with addition of culture medium, stained with antibodies to β-III-tubulin (×40); *f*) cultured Pax6-positive cells in medium with addition of culture medium (×40).

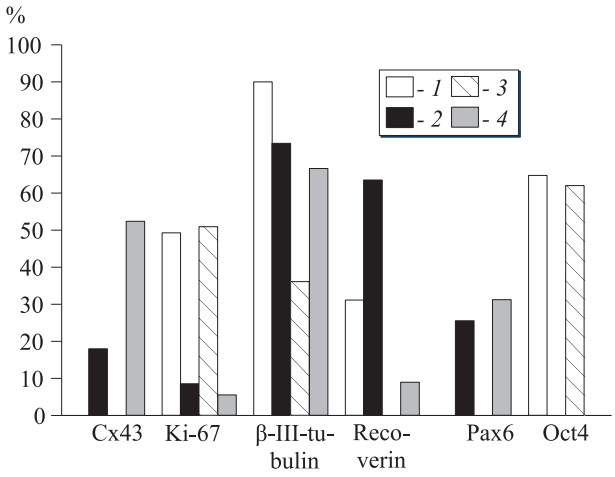

Fig. 5. Content of cells of different phenotypes in PE spheres of 11.5 weeks of development on days 2 and 9 after adhesion in different media. *1*) 10% FCS, day 2; *2*) 10% FCS, day 9; *3*) FCS+bFGF and EGF, day 2; *4*) 10% FCS+conditioned medium, day 9.

by expression of Pax6, essential for maintaining the proliferation and differentiation [15,24]. Multipotency of cells from the spheres manifested by their development in the neuronal and epithelial direction.

Hence, our findings prove that human ocular PE at early stages of development (9-11.5 weeks) are a heterogeneous population of cells with at least three types of PE cells differing by adhesion characteristics, migration, transdifferentiation potential, and response to microenvironmental factors. In culture some of PE cells steadily retain the epithelial characteristics, others demonstrate phenotypical plasticity (manifesting by modification of the morphotype and expression of neuroblast and photoreceptor cell proteins). Type 3 PE cells, cultured in medium with factors, form free floating spheres exhibiting multipotent differentiation.

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REFERENCES

1. M. A. Aleksandrova, O. V. Podgornyi, and M. V. Marei, *Kletochn. Tekhnol. Biol. Med.*, No. 1, 13-19 (2005).
2. K. Amemiya, M. Haruta, and M. Takahashi, *Biochem. Biophys. Res. Commun.*, **316**, No. 1, 1-5 (2004).
3. N. Asuma, K. Tadokoro, A. Asaka, *et al.*, *Hum. Mol. Genet.*, **14**, No. 8, 1059-1068 (2005).
4. J. M. Burke and L. M. Hjelmeland, *Mol. Interv.*, **5**, No. 4, 241-249 (2005).
5. Ch. Chiba and V. Mitashov, *Strategies for Retinal Tissue Repair and Regeneration in Vertebrates: from Fish to Human*, Ed. Ch. Chiba, India: Trivandrum Res. Singpost (2008).
6. B. L. Coles, B. Angenieux, T. Inoue, *et al.*, *Proc. Natl. Acad. Sci. USA*, **101**, No. 44, 15,772-15,777 (2004).
7. M. Engelhardt, N. Bogdahn, and L. Aigner, *Brain Res.*, **1040**, Nos. 1-2, 98-111 (2005).
8. H. Klassen, D. S. Sakaguchi, and M. J. Young, *Prog. Retin. Eye Res.*, **23**, No. 2, 149-181 (2004).
9. A. Kojima, K. Nakahama, K. Ohno-Matsui, *et al.*, *Biochem. Biophys. Res. Commun.*, **366**, No. 2, 532-538 (2008).

10. T. Marquardt, R. Ashery-Padan, N. Andrejewski, *et al.*, *Cell*, **105**, No. 1, 43-55 (2001).
11. J. R. Martinez-Morales, I. Rodrigo, and P. Bovolenta, *Bioessays*, **26**, No. 7, 766-777 (2004).
12. F. T. Merkle and A. Alvarez-Buylla, *Curr. Opin. Cell. Biol.*, **18**, No. 6, 704-709 (2006).
13. V. I. Mitashov, *Int. J. Develop. Biol.*, **41**, No. 6, 893-905 (1997).
14. M. C. Moe, R. S. Kolberg, and C. Sandberg, *Exp. Eye Res.*, **88**, No. 1, 30-38 (2009).
15. N. Osumi, H. Shinohara, K. Numayama-Tsuruta, and M. Maekawa, *Stem Cells*, **26**, No. 7, 1663-1672 (2008).
16. B. Raji, A. Dansault, J. Leemput, *et al.*, *Mol. Vis.*, **13**, 1412-1427 (2007).
17. J. S. Schwegler, M. C. Knorz, I. Akkoyun, and H. Liesenhoff, *Ibid.*, **3**, 10 (1997).
18. O. Strauss, *Physiol. Rev.*, **85**, No. 3, 845-881 (2005).
19. O. G. Stroeva and V. I. Mitashov, *Int. Rev. Cytol.*, **83**, 221-293 (1983).
20. K. Susaki, J. Kaneko, Y. Yamano, *et al.*, *Exp. Eye Res.*, **88**, No. 3, 347-355 (2009).
21. A. D. Tramontin, J. M. Garcia-Verdugo, D. A. Lim, and A. Anarez-Buylla, *Cereb. Cortex*, **13**, No. 6, 580-587 (2003).
22. V. Tropepe, B. L. Coles, B. J. Chiasson, *et al.*, *Science*, **287**, No. 5460, 2032-2036 (2000).
23. U. Vielkind and B. J. Crawford, *Develop. Growth*, **30**, No. 6, 727-736 (1988).
24. H. Xu, D. D. Sta Iglesia, J. L. Kielczewski, *et al.*, *Invest. Ophthalmol. Vis. Sci.*, **48**, No. 4, 1674-1682 (2007).
25. F. Yan, Y. N. Hui, Y. Li, *et al.*, *Ophthalmologica*, **221**, No. 4, 244-250 (2007).
26. Y. Yanagi, Y. Inoue, Y. Kawase, *et al.*, *Exp. Eye Res.*, **82**, No. 3, 471-478 (2006).
27. S. Zhao, L. J. Rizzolo, and C. J. Barnstable, *Int. Rev. Cytol.*, **171**, 225-266 (1997).
28. S. Zhao, S. C. Thornquist, and C. J. Barnstable, *Brain Res.*, **677**, No. 2, 300-310 (1995).