Molecular Genetic and Immunophenotypical Analysis of *Pax6* Transcription Factor and Neural Differentiation Markers in Human Fetal Neocortex and Retina *In Vivo* and *In Vitro*

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Neurotransplantation of various cells, including heterotransplantation of fetal cerebral stem/progenitor cells into the eye is used in experimental studies of central nervous tissue repair during neurodegeneration. For evaluation of this approach, human fetal (weeks 9-20) stem/progenitor cells of the neocortex and retina were studied *in vivo* and *in vitro* by quantitative PCR and immunohistochemical staining. Native tissues and cultures were characterized by expression of Pax6 transcription factor (critical for the development of the retina and neocortex) and differentiation markers (nestin, βIII-tubulin, glial fibrillary acidic protein, recoverin, NeuN, neurofilaments, Ki-67). The expression of *Pax6* gene in the retina during active neurogenesis was stable and much higher than in the neocortex. In primary cultures, the pattern of *Pax6* gene expression is retained and repeats that in native tissues. Immunohistochemical analysis revealed similarity of nestin and βIII-tubulin expression in the neocortex and retina during the early (9-10 weeks) and later (20 weeks) periods and differences in cell phenotypes and their distribution. Culture studies showed that neocortical and retinal stem/progenitor cells are determined and exhibit specific differentiation characteristic of the corresponding native tissues. It can be hypothesized that heterotransplantation of the cerebral progenitor cells into the retina of experimental animals can lead to realization of their neurotrophic effect, but not to their functional integration.

Key Words: *human neural stem/progenitor cells; retina; neocortex; Pax6; immunohistochemistry; cell cultures*

Cell therapy with poorly differentiated stem/progenitor cells (SPC) is among the most promising approaches to the treatment of neurodegenerative diseases of the central nervous system (CNS). Methods of growing and culturing of SPC originating from human fetal brain were recently developed [6]. Cultured human cells from the presumptive cortex after transplantation into the brain, spinal cord, or retina caused significant recovery of functions, which was shown on various models of neurodegenerative diseases in experimental animals [1,3,7,13,22]. For realization of specific potential in cell therapy, neural SPC during culturing should express transcription factors regulating their proper development and differentiation.

During the development of the CNS, the stem/progenitor neuroepithelial cells of the anterior encephalic vesicle form all structures of the forebrain and

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the retina. The morphogenesis of the neocortex and retina is similar by the mechanisms of heterochronous appearance of neuroblasts, their migration along the radial glial processes, and by specific differentiation in certain cell layers [15]. These processes are provided for by one of the most important transcription factors for both the retina and the brain, highly conservative *Pax6* factor, a member of the *Pax* transcription factor family [12,19]. Changes in *Pax6* expression during the development cause significant disorders in the morphogenesis [4,5,9]. No obvious behavioral disorders were detected in mutant rodents with insufficient expression of *Pax6*, but in humans this deficiency is associated with deviations in mental development and cognitive functions [10]. Hyperexpression of *Pax6* in PAX77 mice leads to (in addition to microphthalmia and retinal dysplasia) disorders in the growth and navigation of axons of ganglionic cells [18]. Results of experiments and clinical observations indicate that normal morphogenesis in the neocortex and retina is maintained at the expense of a certain and stable level of transcription factor *Pax6* expression.

The expression of *Pax6* in the primordial neocortex of humans and rodents is located in the ventricular and subventricular zones mainly in SPC of the radial glia. It regulates cell exit from the proliferation cycle, thus controlling the production of neocortical cells [21]. The expression of *Pax6* in human fetal forebrain is observed in all cells of the radial glia and in solitary neuronal precursors [19]. Similar processes accompany the development of the retina, in which *Pax6* is expressed in the neuroepithelial cells and in the radial glia from the stage of presumptive eye primordium in the anterior encephalic vesicle [9]. Its expression is retained in all proliferating neuronal precursors. In differentiated retinal cells, the expression of *Pax6* is detected in the amacrine, horizontal, and ganglionic cells [8]. Hence, *Pax6* plays a similar role in the development of the neocortex and retina: it regulates proliferation of neuroepithelial cells, radial glia, and precursor cells, exit from the cell cycle, direction of differentiation, neuroblast adhesion and migration [9, 20,21].

Studies on cultured retinal and neocortical cells are scanty. Their results are contradictory. Expression of *Pax6* gene was detected in human cerebral SPC culture; its suppression led to a reduction of cell proliferation and to disorders of neuron differentiation [19]. However, in another study *Pax6* expression was detected only in the retinal culture, but not in the neocortex [16]. Pathologically enhanced proliferation of radial glial cells was demonstrated in SPC culture from the brain of mice with homozygotic mutation in *Pax6* gene [14]. On the other hand, hyperexpression of *Pax6* was paralleled by reduced proliferation of cells, which determined their reduced capacity to form secondary spheres [9]. Though the results of these studies are contradictory, in general they indicate that the expression of *Pax6* can be disordered during culturing and can lead to changes in cell proliferation and differentiation.

We carried out a molecular genetic and immunophenotypical analysis of Pax6 transcription factor and neural differentiation markers in human fetal neocortical and retinal SPC *in vivo* and *in vitro*.

MATERIALS AND METHODS

The retina and brain of human fetuses of weeks 8-22 were obtained by medical abortions, which were carried out at licensed medical institutions of the Federal Agency for Health Care and Social Development within the framework of Legislation of the Russian Federation on public health protection and in accordance with approved list of medical indications.

The following methods were used in the study: tissue culturing, PCR, real-time PCR, and immunohistochemical staining.

The retina and forebrain were isolated from the fetuses and dissociated in Accutase solution (Sigma) to single-cell suspension. Suspensions with viability of at least 60% were cultured. Two culturing protocols were used. Adherent cultures were obtained in DMEM/F12 (1:1) medium with 10% FCS. Suspension cultures (neuro- and retinospheres) were obtained in DMEM/F12 (1:1) with fibroblast growth factor 2 (FGF-2; 20 ng/ml), epidermal growth factor (EGF; 20 ng/ml), leukemia inhibition factor (LIF; 10 ng/ml), heparin (8 μg/ml), and penicillin/streptomycin (10 μg/ml). The cells were cultured at 37°C and 5% CO_2.

For immunohistochemical analysis, the eyeballs and the forebrain were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C. The specimens were incubated in 30% sucrose for 24 h at 4°C, embedded in OCT medium (Leica Microsystems), and frozen at -40°C. The sections (15 μ) were sliced on a cryostat (Leica Microsystems). Retinal and brain cell cultures were fixed in the same fixative for 20 min at ambient temperature. Cerebral and retinal cell cultures and sections were incubated with primary antibodies for 24 h at 4°Cand then with second antibodies labeled with Texas Red and Cy-2 fluorochromes (Jackson) and poststained with Hoechst 33342 fluorescent dye (Sigma). The preparations were examined using a mercuric lamp with a combination of filters under an Opton-3 microscope. The photographs were taken with an Olympus ST-310 digital camera.

The following antibodies were used: to Pax6 (murine monoclonal; Chemicon, 1:100), nestin (rabbit polyclonal; Chemicon, 1:100, and murine monoclonal; Abcam, 1:100), to glial fibrillary acidic pro-

tein (GFAP; rabbit polyclonal; Chemicon, 1:200), to βIII-tubulin (mouse monoclonal; Chemicon, 1:400), to recoverin (rabbit polyclonal; kind gift from Prof. P. P. Filippov from the Moscow University), to vimentin (mouse monoclonal; Chemicon, 1:4), Ki-67 (mouse monoclonal; Abcam, 1:50), NeuN (mouse monoclonal; Chemicon, 1:1000), and 68 and 200 kDa neurofilaments (mouse monoclonal; ICN, 1:10).

Total RNA from the native retina and human fetal forebrain and from cell culture was isolated using TRI^R Reagent (Sigma). All procedures were carried out in accordance with the instruction. The cDNA libraries were synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) on the total RNA pretreated with DNase (Fermentas) using oligo(dT) primer. PCR was carried out on an Eppendorf Mastercycler personal amplifier using Taq DNA polymerase (recombinant; Fermentas) with deoxynucleotide triphosphates and reaction buffer from the same firm. Large ribosomal protein (RPL19, 19 kDa) was used for cDNA standardization. Band intensities in 1.0% agarose gel were compared.

The PCR was carried out with primers constructed by means of DNAStar software and NCBI (National Center of Biotechnological Information) BLAST database:

RPL19 F=5'-agggtacagccaatgcccga-3', R=5'-ccttggataaagtcttgatgatc-3';

Pax6 F=5'-gtcatcaataaacagagttcttc-3', R=5'-cgattagaaaaccatacctgtat-3';

nestin F=5'-agcgttggaacagaggttg-3', R=5'-tgggagcaaagatccaagac-3';

βIII-tubulin F=5'-cagtgcggcaaccagatcgg-3', R=5'-caggtcagcgttgagctggc-3';

recoverin F=5'-gccctgtccaaggagatcct-3', R=5'-ggccagtgtcccctcaatga-3';

GFAP F=5'-atgaggcaatggcgtccagcaa-3', R=5'-ccacgatgttcctcttgaggtg-3'.

Quantitative real-time PCR was carried out with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for endogenous control. The expression of Pax6 transcription factor was analyzed during the period of active neurogenesis in fetal retinas of weeks 9, 11, 12, and 18 and in the neocortex of weeks 9, 11, 12, and 20 and in their cultures. The following primers (DNA synthesis) were used:

Pax6 F=5'-caattccacaacccaccacac-3', probe=5'-FAM-tcctccttcacatctggctccatgt-BHQ1-3', R=5'-ctgtaggtgtttgtgagggctgt-3' and GAPDH.

RESULTS

The *Pax6* gene mRNA was detected by PCR in developing human fetal retina and cerebral neocortex at all studied stages of development (Fig. 1, *a*). Quantitative real-time PCR analysis revealed a high and stable level of *Pax6* expression in human retinal cells of weeks 8, 9, 11, 12, and 18 of development (Fig. 1, *b*). This suggests that the percentage of cells expressing *Pax6* is changing negligibly during active neurogenesis.

The expression of *Pax6* in the neocortex at weeks 9-20 is significantly lower than in the retina (Fig. 1, *b*). Presumably, this is due to the fact that in the brain it is expressed mainly by radial glial cells, while in the retina by these cells and by ganglionic cells and then by amacrine and horizontal cells [11,19]. An increase of *Pax6* gene mRNA expression was detected in the neocortex during week 11, after which it decreased to the initial level. This could be explained by transition from embryonic to fetal period of development.

Cell differentiation was studied on sections of native retinal and brain tissues by the immunohistochemical method. Analysis at the early term (9 weeks) showed similar organization of these structures. Radial glial cells stained with antibodies to nestin were clearly seen in the retinal and neocortical sections (Fig. 2, *b*, *d*); their processes protruded through the entire thickness of the tissue from the proliferative zone to the surface. In the brain, radial glial cells not yet stained for GFAP actively divided in the ventricular zone (Fig.

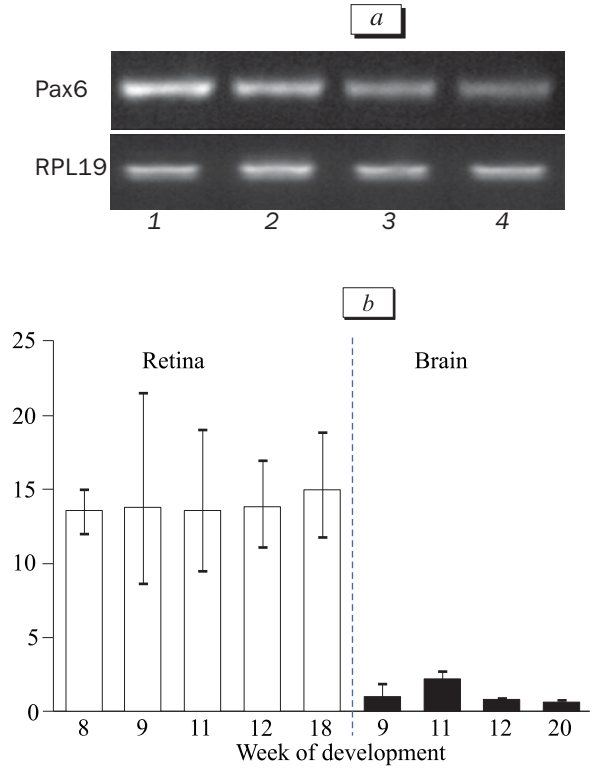

Fig. 1. Expression of *Pax6* transcription factor in human native tissues, developing neocortex, and developing retina. *a*) PCR: *1*) retina, 9 weeks; *2*) brain, 9 weeks; *3*) retina, 20 weeks; *4*) brain, 20 weeks. *b*) quantitative PCR of the native retina (light bars) and brain (dark bars). Ordinate: level of expression.

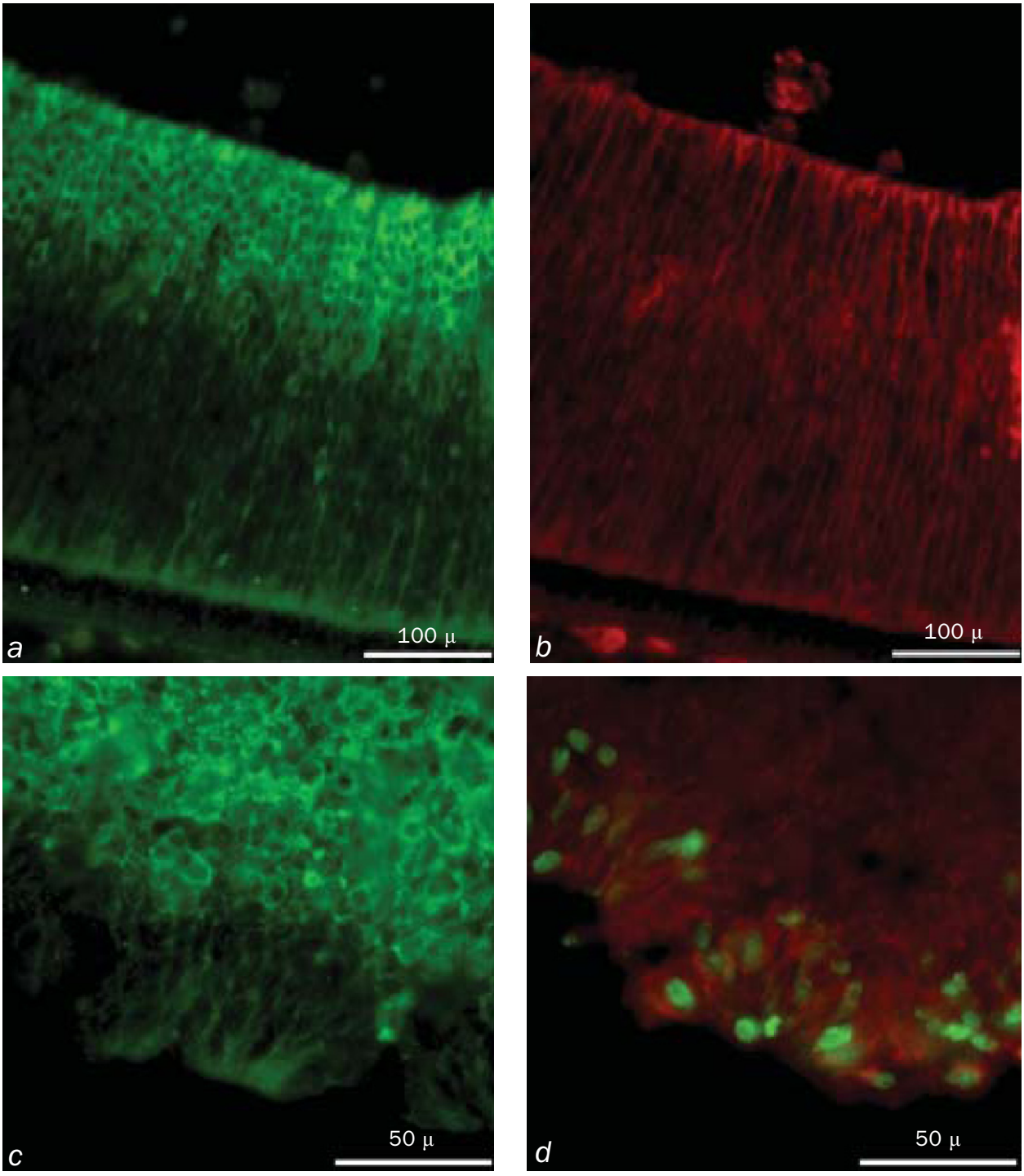

Fig. 2. Human fetal retina, week 9 of development. *a*) distribution of early neuroblasts in the retina (staining with antibodies to βIII-tubulin); *b*) radial nestin-positive filaments in the retina; *c*) early neuroblasts in the brain (staining with antibodies to βIII-tubulin); *d*) proliferation in ventricular zone of the brain (Ki-67, green) and nestin-positive filaments in the radial glia (red).

2, *d*). Their descendants (βIII-tubulin-positive neuroblasts) were detected outside the ventricular zone; they migrated along the processes of the radial glial cells (Fig. 2, *c*). The retina was organized similarly: βIII-tubulin-positive cells formed a compact inner and loose outer neuroblast layers (Fig. 2, *a*). Staining for recoverin showed no immunopositive cells in the retinal sections, because photoreceptor cells had virtually not yet differentiated. The results of PCR analysis confirmed expression of mRNA of proteins detected by the immunohistochemical methods in the cerebral and retinal tissue, respectively.

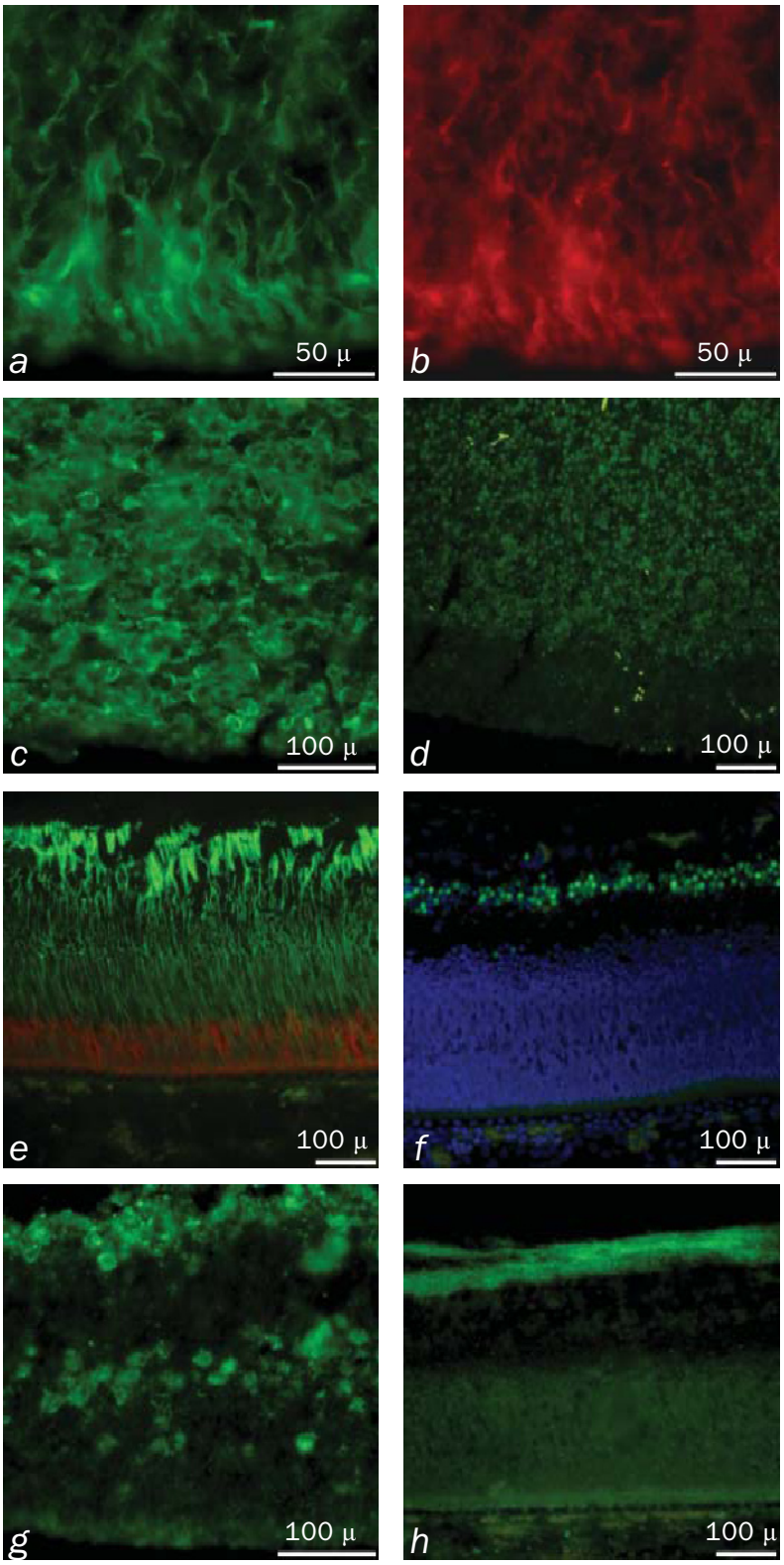

Fig. 3. Human fetal retina and cerebral neocortical compartments, week 20. *a*) nestin-positive filaments in the radial glia in the brain; *b*) filaments in the brain stained with antibodies to GFAP; *c*) early neuroblasts in the brain stained for βIII-tubulin; *d*) differentiated neurons occupy all zones except the subventricular (staining for NeuN); *e*) nestin-positive filaments (green) and a layer of photoreceptors (recoverin, red) in the retina; *f*) differentiated neurons in a layer of ganglionic cells in the retina (staining for NeuN); *g*) distribution of early neuroblasts in two layers of the retina (staining for βIII-tubulin); *h*) processes of ganglionic cells forming a layer of nervous fibers in the retina (staining for neurofilaments).

Cells of the radial glia in the neocortex and retina during weeks 20-22 of development still expressed nestin (Fig. 3, *a*, *e*). The βIII-tubulin-positive neuroblasts in the neocortex were located less compactly than during week 9 (Fig. 3, *c*). On the other hand, many cells were positively stained for NeuN (marker of differentiating neurons; Fig. 3, *d*). Positive staining for GFAP was detected in the neocortex (Fig. 3, *b*). The pattern of staining suggested that during this stage GFAP was expressed by radial glial cells. In the retina, βIII-tubulin-positive neuroblasts were located less compactly than during week 9 and formed two layers (Fig. 3, *g*). NeuN-positive cells were detected in the ganglionic cell layer (Fig. 3, *f*). Staining for neurofilaments showed processes of ganglionic cells, forming a layer of nerve fibers (Fig. 3, *h*). Staining for GFAP was detected in layer of nerve fibers. In contrast to the brain, retinal SPC formed no astrocytes, and hence, GFAP was expressed by astrocytes migrating into the retina from the optic nerve [23]. Staining for recoverin detected a well-formed layer of photoreceptors (Fig. 3, *e*). Study by the PCR method confirmed the presence of mRNA of specific differentiation marker proteins in the neocortical and retinal tissues.

The expression of *Pax6* gene was detected by PCR in all studied cultures, in adherent and floating neuro- and retinospheres (Fig. 4, *a*). Quantitative real-time PCR showed that the expression of *Pax6* gene in cultured retinal cells was higher than in cultured

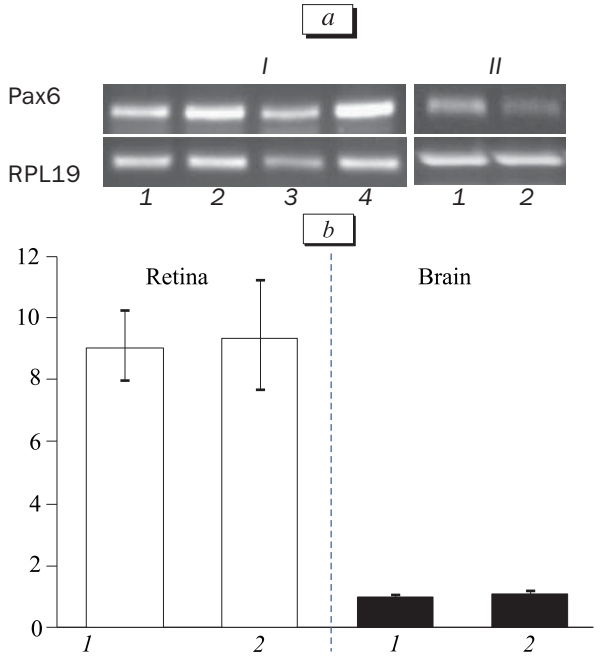

Fig. 4. Expression of *Pax6* transcription factor in neocortical and retinal cell cultures. *a*) PCR: *I*: retinal cultures; *II*: brain cultures; *1-4*) specimens of cultures. *b*) quantitative PCR analysis of retinal and brain cultures in two samples (*1*, *2*). Ordinate: level of expression (%).

brain cells (Fig. 4, *b*), and virtually coincided with our results obtained on native tissues. It seems that conditions of the neocortical and retinal SPC culturing used in our study did not suppress their regional determination and these cells retained their differentiation potential, similar to that of native tissue.

Cell differentiation in cultures was studied by immunohistochemical staining and PCR with the same markers as those used for native tissues. Fetal retinal and brain cell cultures in the form of floating spheres have been partially characterized previously [1,2], and hence, here we describe them in brief. Cell proliferation in neurospheres and retinospheres was high during the first passages and decreased with cell growth and number of passages. Phenotypical analysis of neuro- and retinospheres revealed their heterogeneous cell composition. Neurospheres consisted of undifferentiated cells stained with antibodies to nestin (SPC) and vimentin (progenitor cells). In addition, there were cells labeled with antibodies to βIII-tubulin (early neuroblasts) and GFAP (radial glia, astrocytes), this indicating rapid differentiation of glial cells. In contrast to neurospheres, retinospheres were characterized by specific organization, formation of cell rosettes. Retinospheres consisted of progenitor cells, neuroblasts with phenotypes of ganglionic, bipolar, and horizontal cells (photoreceptors) differentiating more rapidly than normally. GFAP-immunopositive cells were detected in cultures originating from the retina of 20-22-week (no earlier) fetuses.

Adherent cultures of brain cells formed a monolayer from large spread cells with large nuclei and smaller cells above them. Active cell proliferation in the cultures was detected by staining with antibodies to Ki-67 (Fig. 5, *a*). Positive staining for nestin and vimentin was observed mainly in large spread cells (Fig. 5, *b*, *c*). GFAP-positive glial cells often formed a sublayer for βIII-tubulin-positive neuroblasts above them (Fig. 5, *b*, *c*).

Retinal cells in adherent cultures formed a monolayer with rare compact accumulations. Cell rosettes were seen only in compact groups or nearby. Staining with antibodies to KI-67 showed a significant number of proliferating cells in the monolayer (Fig. 5, *d*) and solitary cells in compact groups. Antibodies to *Pax6* stained the nuclei of cells scattered diffusely or in compact groups, in which they constituted the greater portion (Fig. 5, *g*). Apart from nuclear location of *Pax6*, the cytoplasm was stained in some cells. The greater part of the monolayer was formed from poorly differentiated cells stained for nestin (Fig. 5, *e*). Solitary and groups of βIII-tubulin-immunopositive neuroblasts were detected (Fig. 5, *f*). GFAP-positive glial cells detected only in cultures of 20 weeks of development formed compact foci in the monolayer or clusters

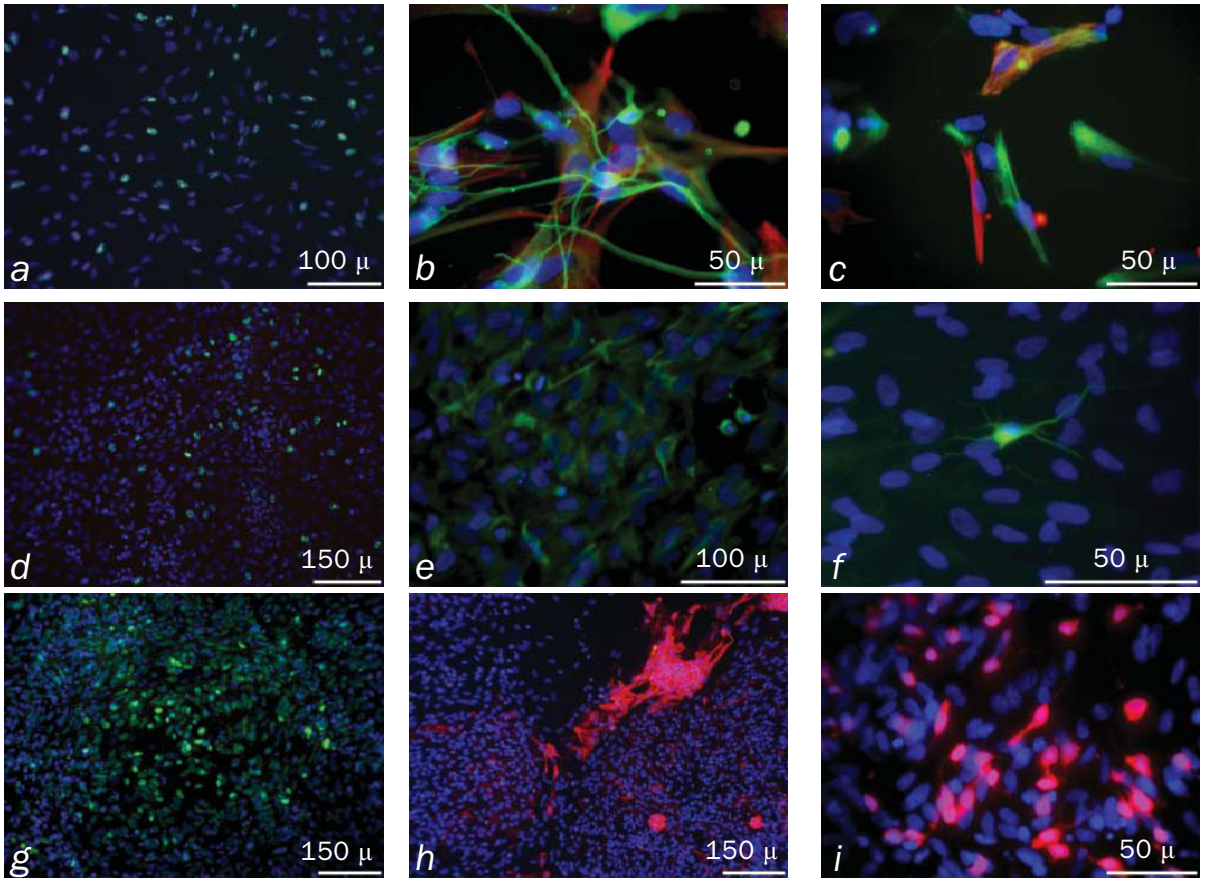

Fig. 5. Immunohistochemical analysis of adherent cultures of human fetal retina and anterior compartments of the brain. *a*) cell proliferation in brain culture (staining for Ki-67); *b*) neuroblasts: βIII-tubulin- (green) and nestin-positive (red) cells in brain culture; *c*) cells stained for GFAP (red) and nestin (green) in brain culture; *d*) cell proliferation in retinal culture (staining for Ki-67); *e*) nestin-positive cells in retinal culture; *f*) cell stained for βIII-tubulin in retinal culture; *g*) Pax6-positive cells in retinal culture; *h*) accumulations of GFAP-positive cells in retinal culture; *i*) photoreceptor differentiation of cells in retinal culture (recoverin staining).

of different size (Fig. 5, *h*). Recoverin-positive cells differentiating into photoreceptors were present in all retinal cultures (Fig. 5, *i*). The presence of mRNA of differentiation marker proteins in adherent cultures of the neocortex and retina was confirmed by PCR.

The results of our molecular genetic and immunophenotypical comparative analysis of expression of *Pax6* transcription factor and neural differentiation in human fetal neocortex and retina *in vivo* and *in vitro* indicate that *Pax6* gene mRNA is detected by PCR in the developing human fetal retina and neocortex during active neurogenesis. According to the results of quantitative real-time PCR, the level of *Pax6* gene expression in the retina is significantly higher than in the neocortex, this being an important difference between these tissues. Similar results were obtained in the analysis of cell cultures. The expression of *Pax6* in retinal SPC cultures was higher than in cultured brain cells, which is in line with the results obtained on native tissues. Immunohistochemical analysis confirmed that the conditions of retinal and neocortical SPC culturing used in our study did not block local specificity of these cells. Differentiation in the primary cultures was associated with realization of the genetically determined cell potentials intrinsic of the native tissues. Despite some experimental data indicate integration of the cerebral progenitor cells into the retina [22], we think that, due to specific features of differentiation of these cells, only the neurotrophic effect of heterotopically transplanted progenitor cells of the brain on the retina is possible. The prospects of this approach are confirmed by experimental animal studies [13]. Functional replacement is most probable in experiments with homotopic cell neurotransplantation [17].

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