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Mouse Retinal Progenitor Cell (RPC) Cocultivation with Retinal Pigment Epithelial Cell Culture Affects Features of RPC Differentiation

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Abstract—We provide evidence that coculturing of retinal progenitor cells (RPC) with retinal pigment epithelial cells significantly biases the standard *in vitro* RPC differentiation patterns. In particular, in cocultivation experiments RPCs lost the ability to differentiate spontaneously and displayed ~2.1-2.4-fold increase in immunoreactivity to the neural stem cell marker nestin and ~1.6-1.7-fold increase in rod photoreceptor cell rhodopsin marker immunoreactivity. The data suggest the influence of the intercellular interaction networks on RPC differentiation.

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Key words: retinal progenitor cell, retinal pigment epithelium, stem cell differentiation

In mammals, retinogenesis occurs mostly during embryo development and stops few days after birth. Cell differentiation in the retina is initiated in the inner layer of the central optic cup and progresses concentrically in a wave-like fashion until reaching the peripheral edges of the retina. There are seven known retinal cell types: rod and cone photoreceptor cells required for black-white and color vision, respectively; Muller glia cells; bipolar, ganglion, horizontal, and amacrine neurons, which perceive signals from photoreceptor cells, transform them, and transduce to the brain. The important characteristic feature of mammalian retinogenesis is the relatively fixed chronological sequence after which the different retinal cell types are generated [1]. Retinal ganglion cells and horizontal cells differentiate first, followed in overlapping phases by cone-photoreceptors, amacrine cells, rod-photoreceptors, bipolar cells, and, finally, Muller glia cells.

Although neuronal stem cell niches were found in brains of several mammalian species [2], adult organisms are thought to lack any kind of retinal cell population renewal [3]. Therefore, the treatment of various retinal disorders would require the use of neuronal stem cells to replace damaged retinal neurons or glia [4]. Indeed, in animal models such an approach was rather efficient for the treatment of diverse neurodegeneration-associated illnesses, such as Huntington disease and Parkinson disease [5-7], as well as for curing some retinal disorders [8-10]. Retinal progenitor cells (RPCs) would probably be very useful for diverse retinal disease treatment because of their natural program to differentiate into any of the above mentioned retinal cell classes. Several retinopathies are often associated with the loss of particular retinal cell types [11]. For instance, during cytomegalovirus infection of the inner retina, many horizontal and bipolar cells are infected, in contrast to rare amacrine and ganglion cell infection events [12].

Abbreviations: bFGF) basic fibroblast growth factor; DIL) 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; EGF) epidermal growth factor; FCS) fetal calf serum; GFAP) glial fibrillary acidic protein; NGF) nerve growth factor; NT3) neurotrophin-3; PDGF) platelet-derived growth factor; RPC) retinal progenitor cell; RPE) retinal pigment epithelial cell; RA) all-*trans* retinoic acid; TGFα) transforming growth factor α.

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Inherited retinal diseases, associated with death of photoreceptors, which include *retinitis pigmentosa*, form the largest single cause of blindness in the developed world [13]. The directed differentiation of RPCs into the desired retinal cell type(s) would be relevant not only to retinopathy treatment [14], but would be also extremely useful for better understanding the complex processes of retinal development, including intercellular interactions. There is a great difference between *in vivo* and *in vitro* stem cell differentiation and survival [15]; however, it is very likely that the knowledge obtained in *in vitro* experiments will be necessary for the development of new *in vivo* assays and curing strategies.

Many research teams are now focused on directed *in vitro* stem cell differentiation. To our knowledge, two mainstream approaches are generally used separately or in combination: (i) stem cell culturing in the presence of various growth/differentiation factors and (ii) coculturing of cells derived from different tissues. Both strategies have been applied to retinal precursor cells and have given some interesting results. It was demonstrated that RPCs can be isolated from prenatal or few days postnatal mouse [16], rat [17], and human [18, 19] retinas and can be cultured *in vitro*. Qiu and coauthors recently reported that the serum-free media may be superior for preventing rat RPC differentiation while culturing [20]. Ezeonu and colleagues [21] demonstrated that cell commitment in human retinal precursor cells can be modified in response to exogenous growth factors, basic fibroblast growth factor (bFGF), and transforming growth factor α (TGFα). Moreover, RPC differentiation was density-dependant: a larger proportion of multipotential precursors plated at a density of 10^4 cells/cm^2 differentiated into the cells displaying photoreceptor phenotypes compared to cells plated at $(3\text{-}5)\cdot10^4$ and 10^5 cells/cm^2 and the effects were amplified 7- to 8-fold in response to growth factors.

Kelley et al. [22] used two photoreceptor-specific antibody markers, anti-recoverin (which is expressed in all photoreceptors) and anti-rhodopsin (which is specific for rod photoreceptors) to assess the effects of all-*trans* retinoic acid (RA). They found an increase in the number of cells expressing these markers after 2-8 days *in vitro*. The effect on photoreceptor differentiation was specific, since the other major cell type produced at this point in development, amacrine cells, did not increase with RA treatment, but were inhibited instead [23].

In 1995, Sheedlo and Turner reported that conditioned medium from cultures of transformed neonatal rat retinal pigment epithelial cells (RPE) "promoted the production, survival, proliferation and maturation" of rat retinal progenitor cells [17]. More recently, Sheedlo and colleagues isolated a 67 kD protein secreted by cultured RPE cells, which was shown to promote the survival of RPCs [24]. Finally, Chiou et al. recently demonstrated that the coculturing of human bone marrow stem cells (BMSC) with RPE cells resulted in BMSC differentiation into cell culture displaying retinal neuron-like phenotypes, including photoreceptor-like phenotype [25].

The present study was aimed at the investigation of the effects of mouse RPC cocultivation with RPE on RPC phenotype and differentiation. We report here that coculturing of mouse RPCs with RPE feeder layer ~2.0-2.5-fold increases the number of neuroepithelial stem cell marker nestin expressing cells and results in ~1.5-2.0-fold increase in rod photoreceptor cell marker protein rhodopsin production. Another finding was the loss of RPC ability to differentiate spontaneously into mature retinal-like neurons. We also investigated the effects on mouse RPC differentiation of the growth/differentiation factors bFGF, TGFα, NGF, EGF, PDGF, NT3, and RA and of cultivation with RPE conditioned media.

MATERIALS AND METHODS

Animals. All the experiments with mice were performed in accordance with both the Russian and the European Communities Council Directive (86/609/EEC) and institutional guidelines, and followed the tenets of the Declaration of Helsinki. In all cases, efforts were made to minimize the sufferings of the animals. CBA strain mice were used throughout the study. The plug date of timed pregnant mice was defined as embryonic day 0.

Retinal progenitor cell and retinal pigment epithelium cell isolation. Embryonic retinal tissues were isolated from day 14 mouse embryos. Eyes from 15 embryos were isolated and washed twice in 1× PBS standard buffer solution (ICN, USA) with added antibiotics Claforan (10 μg/ml) and lincomycin (50 μg/ml). Neural retinas were isolated by removing the retinal pigment epithelium and vitreous body. The retinas were then dissected and approximately 1 mm^2 flat fragments were incubated in 0.25% trypsin solution (PanEko, Russia) in PBS buffer for 30 min at 37°C to homogenize the tissue. The trypsinolysis was then stopped by the addition of fetal calf serum (FCS) (HyClone, USA) to the concentration of 10%. Cells were washed twice in PBS and transferred to DMEM/F12 medium (Sigma, USA) supplemented with 10% FCS. Cells were plated in 6-well polystyrene culture plates (Corning Costar, USA) at the density $5\cdot10^5$ cells/cm^2. Approximately 90% of the cells were viable. For viable cell counts, an aliquot was counted by trypan blue (Sigma) exclusion assay in a hemocytometer. Retinal pigment epithelium (RPE) cells were isolated from two seven days after birth mice killed by cervical dislocation. The eyeballs were isolated and washed twice in 1× PBS with added antibiotics Claforan (10 μg/ml) and lincomycin (50 μg/ml). RPE cells were isolated exactly according to Mayerson et al. [26]. Then 1 mm^2-sized flat pieces of pig-

ment epithelium were treated as described above for RPC isolation.

Media and culturing conditions. Media for cell culturing were prepared on the basis of DMEM/F12 medium (Sigma) (0.2% $NaHCO_3$, pH 7.4) with the addition of 10% FCS, 2 mM L-glutamine (ICN), and 25 μg/ml lincomycin. Cultures were maintained at 37°C in an incubator with 96% air, 4% CO_2. Cells were cultivated in multiwell plates or in cell culture flasks at the densities $5 \cdot 10^5$, 10^5, $5 \cdot 10^4$, and 10^4 cells/cm^2. Cell passaging was performed every 2-6 days, depending on the cultured cell proliferation rate, at 75% confluence level. Cells were harvested with trypsin/versene solution (0.25% trypsin, 0.02% EDTA) in PBS by washing two times and then centrifuged at 300*g*.

Preparation of RPE-cultured media supernatant. RPE cells were grown until 90% confluence level, and then RPE-cultured media was collected and centrifuged twice for 20 min at 300*g* and for 20 min for 600*g*, respectively. The final supernatant was added to the RPC culturing media to the concentration of 50% of the total volume.

Growth and differentiation factors. To determine the effects of growth factors on cell differentiation, RPCs in passage 8 were plated at different densities at plastic culture dishes. Transforming growth factor α (TGFα), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), neurotrophin-3 (NT3), and all-*trans* retinoic acid (RA) were purchased from Sigma. For cell culture assays, DMEM/F12-solubilized protein factors and DMSO-solubilized RA were added separately to the culturing RPCs to the final concentration 100 ng/ml for each factor. The culture medium was changed, and the growth factors replenished every 2 days. The cells were monitored by dark-field and phase-contrast microscopy over a period of 1-7 days, and finally (after 7-day incubation) analyzed in immunocytochemistry experiments.

Vital cell staining. To fluorescently label living cells, RPE cell suspension (~10^6 cells/ml) was incubated with 2 μg/ml DIL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) (Sigma) solution. Cells were incubated for 4 h at 37°C while mixing every 30 min. Afterwards cells were washed three times with 1× PBS, transferred to the culturing medium, and plated.

RPE/RPC cocultivation. DIL-labeled RPE cells were grown in 6-well plates until 75% confluence level, UV-irradiated for 15 min to stop further proliferation, and washed twice with culturing medium. The resulting RPE cell layer was overlaid with retinal precursor cells at the density $5 \cdot 10^4$ cells/cm^2, and cells were cocultured for 7 days.

Light microscopy. Cell cultures were investigated using an Axio Vert 200 inverted light microscope (Carl Zeiss, Germany). Samples were photographed with a AxioCam HRm digital camera (Carl Zeiss) at 10-80× enlargement. For imaging and data analysis, KS400 3.0 software was used.

Immunocytochemistry. Cells designated to immunocytochemical and flow cytometry analyses were harvested with trypsin/versene solution (0.25% trypsin), washed twice with PBS, followed by 30-min fixation with phosphate-buffered 2% aqueous paraformaldehyde solution (PanEko) at 4°C. Fixed samples were washed twice in PBS and permeabilized in 0.6% saponin (Sigma) PBS solution for 30 min at room temperature. Samples were then washed twice with PBS containing 1% FCS, followed by 1-h incubation at 4°C with the following primary anti-mouse antibodies (Santa Cruz, USA): anti-rhodopsin, mixed anti-green/blue opsins, anti-nestin, anti-β-arrestin, anti-calbindin, anti-calretinin, anti-recoverin, anti-Ki-67, anti-GFAP (glial fibrillary acidic protein), and anti-protein kinase C α. Each representative sample including $5 \cdot 10^5$ cells was incubated with 2 μg of primary antibodies in 100 μl of 1× PBS/10% FCS. Samples were washed twice with 1× PBS/1% FCS, and further incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Santa Cruz) at 4°C. Samples were then washed and fixed in 2% aqueous paraformaldehyde solution as described above. Immunoreactivity analyses with a minimum of 5000 events per sample acquired were performed on an EPICS Coulter XL-MCL flow cytometer. For fluorescent microscopy, cells were grown on glass slides in eight-well culturing plates. Staining with antibodies was performed as described above with the following modifications: phycoerythrin (PE)-conjugated secondary antibodies (Santa Cruz) were used. Nuclear stains were performed by using 4′,6′-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, USA) at 1 : 5000 dilution in PBS. After antibody staining, cell nuclei were labeled with DAPI (1 μg/ml, 5-min incubation) and washed twice with PBS.

RESULTS AND DISCUSSION

Cultured retinal progenitors are capable of differentiation into cells with retinal neuronal phenotypes. For our experiments, we aimed to investigate the multipotential retinal neuronal stem cell culture capable of differentiation into any of the adult retinal cell types. To this end we used day 14 mouse embryos as tissue donor source, as the differentiation of RPCs into any of the retinal neuronal cell types has not yet completed at this time point [1]. To characterize the primary cell culture obtained from prenatal mouse retina, we have tried to investigate its ability to display typical retinal cell phenotypes in differentiation conditions, either by expressing marker proteins, or by demonstrating characteristic cell shapes. Ezeonu and colleagues have previously reported that the differentiation

of human RPC-like cells is density dependant and occurs primarily in low-density (~10^4 cells/cm^2) cell cultures [21]. In our differentiation experiments, we used densities ranging from 10^4 to $5 \cdot 10^5$ cells/cm^2. Another factor was the serum concentration in the media, which varied from 2 to 10%. Low serum content resulted in poor cell survival after 4 days of cultivation, whereas 5 and 10% FCS concentrations were, respectively, suboptimal and optimal for both cell proliferation and differentiation. In good agreement with Ezeonu et al. [21], the major differentiation factor was the cell density: >70% of the cells plated at 10^4 or $5 \cdot 10^4$ densities differentiated into various retinal neuronal-like cell types, in contrast to high density-plated cells, which tended to retain their undifferentiated phenotype (Fig. 1a). In the latter case, only RPCs located at cell group periphery could migrate away and spontaneously differentiate (Fig. 1b). For both 10^4 and $5 \cdot 10^4$ densities, the observed cellular phenotypes were as follows: retinal ganglion cell-like phenotype (4-6% of the cells, Fig. 1, c and d), bipolar cell-like phenotype (5-10% of the cells, Fig. 1e), typical astrocyte and Muller glia-like cells (5-8% of the cells, Fig. 1, f and g), retinal photoreceptor-like cells (3-6% of the cells, Fig. 1, h-j), as well as other cells with typical neuronal morphology, which could be considered ganglion, bipolar, horizontal, and amacrine-like cells (40-50% of the cells), and undifferentiated cells (20-27%).

To further characterize the cell culture, medium density (10^5 cells/cm^2)-plated RPCs were antibody stained for retinal marker proteins: blue and green opsins, expressed in cones; rod marker rhodopsin; β-arrestin and recoverin, which are expressed in both photoreceptor cell types; calbindin, protein kinase C α isoform and calretinin, which are markers for horizontal cells, bipolar cells, and retinal amacrine interneurons, respectively; glial fibrillary acidic protein (GFAP), characteristic of Muller glia cells, as well as for proliferating cell marker Ki 67 and neuroepithelial stem cell marker nestin. Cell staining was analyzed both by flow cytometry (table) and visually using a fluorescence microscopy. Cells were found to be immunoreactive against all of the above marker protein-specific antibodies at very different levels ranging from 3.6% of the cells for GFAP to 40.1% for green/blue opsins.

Marker protein expression in RPCs

Marker protein*	Expressed cells, %**
GFAP	3.6 ± 1.11
Calbindin	33.6 ± 2.62
Calretinin	26.1 ± 1.91
β-Arrestin	27.6 ± 2.11
Recoverin	39.3 ± 2.12

* Marker protein expression in RPCs (passage 10). The data on rhodopsin, green/blue opsins, nestin, Ki 67, and protein kinase C α expression are shown on the Fig. 3.

** Percent values of the cells expressing the corresponding marker protein, as measured using flow cytometry.

These data strongly evidence RPC origin of the isolated cell culture. At least for the first 15 passages, the cell culture has entirely retained all of the mentioned morphological and marker protein expression patterns, as controlled visually and immunocytochemically for every fifth passage of RPC culturing. Like Ezeonu and colleagues [21], we have been able to passage these cells up to the 50th passage. However, in passage 20 RPCs have lost the ability to differentiate into cells with mature retinal neuronal morphology. Nor did the cells keep their marker protein expression features. The proliferation rate was also biased: in passages 1-15, cells divided every 30-38 h, in contrast to ~16-h cell division rate for passage 50.

All-*trans* retinoic acid influences RPC phenotype and viability. We further tried to investigate the growth/differentiation factor influence on the RPC phenotype. The following factors, previously reported to be relevant to neural stem cell differentiation, were used: TGFα, bFGF, NGF, EGF, PDGF, NT3, and RA. The assay was performed on in passage 10, plated at density $5 \cdot 10^4$. Growth/differentiation factors were added to the 5% FCS culture media, and the resulting phenotypes were analyzed 7 days after factor addition visually and by flow cytometry.

Notably, RA was the only factor which was found to alter RPC phenotype—the others did not display any effects on RPC survival, morphology, and marker protein expression, probably due to the presence of these factors in fetal serum (data not shown). RA-treated RPCs demonstrated an improved viability (~1.7-1.9 times greater cell yield after 7 days of culturing, as compared with control RPCs), as well as at ~50% decreased immunoreactivity to anti-nestin antibodies (whereas expression of the proliferative marker Ki 67 remained unchanged). In contrast, expression of another retinal neuronal marker, protein kinase C α isoform, was up regulated, as evidenced by ~2.5-fold increase in RA-treated cell immunoreactivity. However, RA treatment did not alter the differentiated RPC morphologies, and the above ratio of photoreceptor, amacrine, bipolar, ganglion, and Muller glia cell-like phenotypes was essentially the same.

This suggests that in this particular case RA mostly influences RPC survival rather than differentiation. Interestingly, in line with this the same RA effects (increased cell viability and protein kinase C α expression with decreased nestin production) were observed for RPCs in passage 50 as well, which were unable to develop adult retinal neuron-like morphologies.

Cocultivation with retinal pigment epithelial cells affects RPC differentiation features. We further addressed the question whether retinal pigment epithelial (RPE)

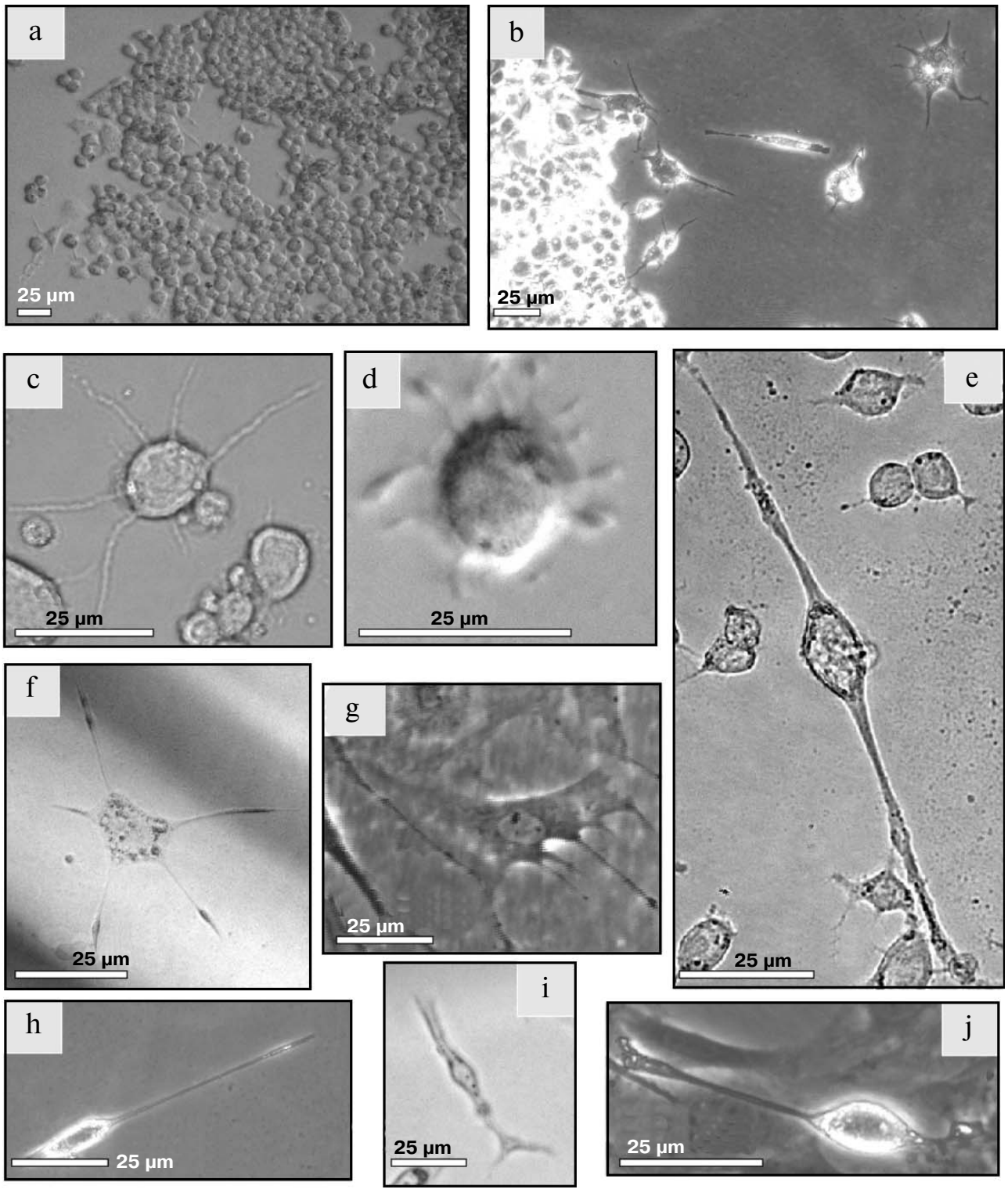

Fig. 1. Phase-contrast photographs of differentiated and undifferentiated RPC morphologies. a) Undifferentiated RPCs 7 days after plating at cell density $5 \cdot 10^5$ cells/cm^2; b) a colony of RPCs (plated at density 10^5 cells/cm^2) 5 days after plating; c, d) differentiated RPCs displaying ganglion cell-like morphology; e) bipolar neuron-like differentiated RPC; f, g) differentiated RPCs similar to astrocyte and Muller glia cells; h-j) photoreceptor-like morphologies of differentiated RPCs.

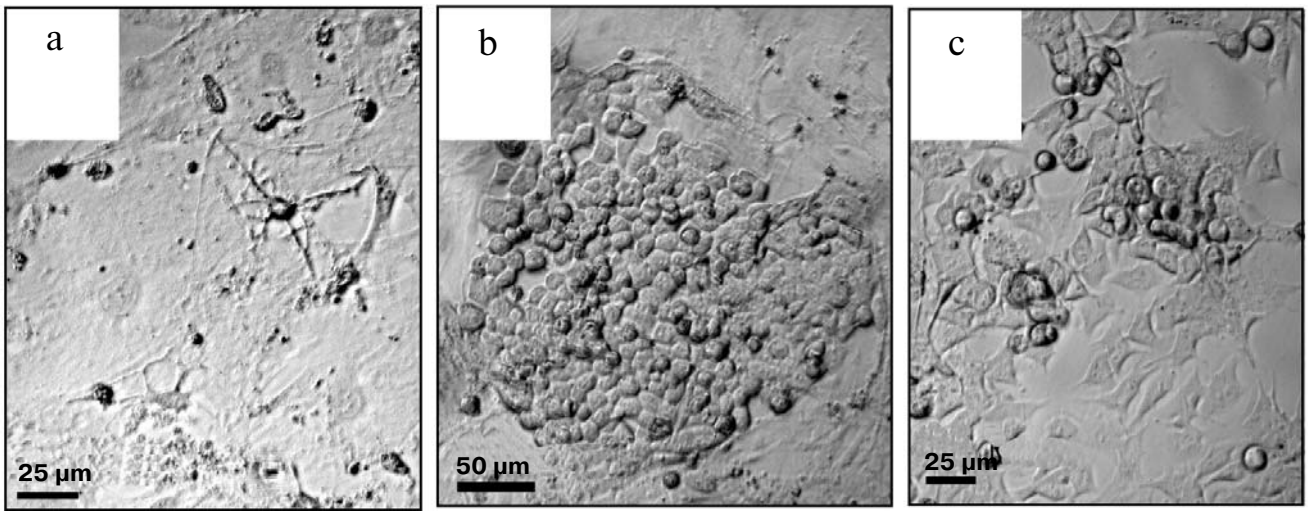

Fig. 2. Representative photographs of RPE/RPC cocultivation experiments. a) RPE cell culture (confluence level 90%); b) RPC colony grown for 7 days on RPE feeder monolayer; c) RPC in low density culture grown for 7 days on RPE feeder monolayer.

cells exert influence upon RPC differentiation. It was recently reported that RPE extracts and RPE conditioned media do contain factors which alter RPC phenotype and survival. However, to our knowledge no previous experiments on direct RPC/RPE coculturing have been done to date. In this study, we investigated both the influence of RPE conditioned medium and the effects of the cocultivation with RPE cells on the RPCs. RPE primary culture was obtained from 7-days after birth CBA black mice. In line with the data published by Engelhardt and coauthors [27], the cultured cells had characteristic RPE morphology: flat shape, lamellar structure, and inclusions of pigment granules, degraded with cell passaging (Fig. 2a). As RPE cells tend to alter their phenotype with passaging [27], we used RPE culture in passage 3 for all the experiments. To collect RPE-cultured medium, RPE cells were grown for 8 days, until 90% confluence level. The supernatant was then added to the RPCs plated at density $5 \cdot 10^4$ cells/cm^2 and cells were allowed to differentiate for 7 days.

However, neither visual inspection nor antibody staining with the subsequent flow cytometric assay was able to detect any significant difference from the control experiments (data not shown). In contrast, cocultivation with RPE cells did significantly alter RPC phenotype. For RPE/RPC cocultivation, RPE cell feeder layer was grown until confluence level of 75%, and RPCs were overlaid at the density $5 \cdot 10^4$ cells/cm^2. *Prior to* the essay, RPE cells were tagged using DIL as a vital stain. Such a labeling was necessary to distinguish RPC and RPE cells and to trace RPC phenotypes in further experiments. After 7 days of culturing, RPCs were analyzed by morphology and immunophenotyping methods. RPCs in passage 50 did not display any effects, whereas for cells in passage 10 the RPE influence on RPC phenotype was significant. Surprisingly, RPCs have totally lost their ability to differentiate into mature retinal neuron-like cells when growing on RPE cell layer (Fig. 2, b and c). Moreover, the expression of neuronal stem cell marker nestin has increased ~2.1-fold in cocultured RPCs as compared with control RPCs (Fig. 3). Another feature was that cocultured RPCs displayed ~1.6-fold stronger immunoreactivity for rhodopsin. Interestingly, the number of opsin-positive cells remained essentially the same (~40%) in both coculturing and control experiments.

Similar results (loss of mature neuron-like morphological types, 2.4-fold increase in immunoreactivity for nestin and 1.7-fold for rhodopsin) were observed when RA was added to the culturing media. The above mentioned RA-mediated effects, in turn, remained similar in cocultivation experiments: ~3.5-fold increase in anti-protein kinase C α immunoreactivity and ~1.4-fold decrease in nestin-positive cells. This implies RA-independent RPE "reprogramming" of the RPC differentiation towards the photoreceptor precursor formation (not surprisingly, as photoreceptors form the neighboring retinal cell layer relatively to the RPE). Complete RPC differentiation, however, is blocked by RPE cells, as no cells with differentiated morphologies appear in cocultivation experiments. The number of nestin positive RPCs in RPC/RPE coculturing experiments was increased ~2-fold, thus suggesting that retinal pigment epithelium promotes multipotential retinal neuronal stem cell production and survival. As no effect was observed with RPE supernatant, retinal pigment epithelium probably influences RPC differentiation via cell–cell contacts rather than by secreted soluble factor production. Although the mechanisms of such intercellular interactions are to be

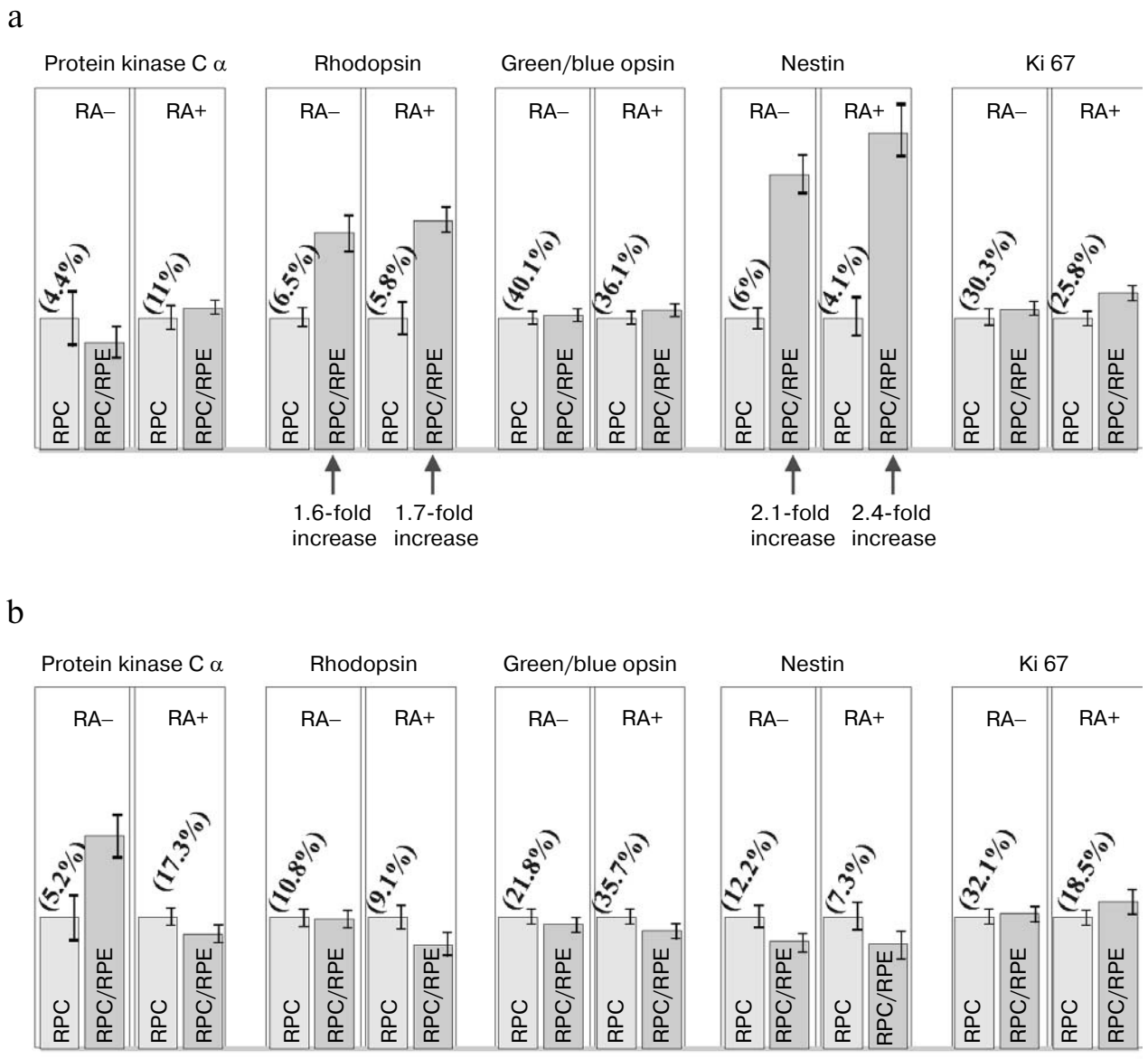

Fig. 3. Retinal neuronal marker expression in RPCs in RPC/RPE cocultivation experiments. Left columns, expression of marker proteins by RPCs in control experiments; right columns, expression of marker proteins by RPCs in RPC/RPE coculturing experiments. "RA –/+" means whether all-*trans* retinoic acid was added to the culturing media. a) RPCs in passage 10; b) RPCs in passage 50.

further investigated, the data obtained here might be helpful for the development of better approaches for stem cell production, culturing, and differentiation pathway programming.

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