

Retinal Pigment Epithelium Culture; a Potential Source of Retinal Stem Cells

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Purpose: To establish human retinal pigment epithelial (RPE) cell culture as a source for cell replacement therapy in ocular diseases.

Methods: Human cadaver globes were used to isolate RPE cells. Each globe was cut into several pieces of a few millimeters in size. After removing the sclera and choroid, remaining tissues were washed in phosphate buffer saline and RPE cells were isolated using dispase enzyme solution and cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 supplemented with 10% fetal calf serum.

Results: Primary cultures of RPE cells were established and spheroid colonies related to progenitor/stem cells developed in a number of cultures. The colonies included purely pigmented or mixed pigmented and non-pigmented cells. After multiple cellular passages, several types of photoreceptors and neural-like cells were detected morphologically.

Conclusion: Cellular plasticity in RPE cell cultures revealed promising results in terms of generation of stem/progenitor cells from human RPE cells. Whether the spheroids and neural-like retinal cells were directly derived from retinal stem cells or offspring of trans-differentiating or de-differentiating RPE cells remains to be answered.

Key words: Retinal Pigment Epithelium; Stem Cells

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in elderly subjects in developed countries; its prevalence has also increased in developing countries. Pathologic changes affect retinal pigment epithelial (RPE) cells, Bruch's membrane and the choriocapillaris.¹ RPE cells play an important protective

role for the neurosensory retina; photoreceptors in particular degenerate and die due to RPE dysfunction in patients with AMD.²

Despite the large volume of studies on AMD, the exact pathogenesis of the disease is not clearly known and therapeutic options remain limited. Many attempts have been made to halt the destructive process in the neurosensory retina and to reestablish normal func-

tion in the outer retina including RPE cells and Bruch's membrane. Currently available therapies include photodynamic therapy (PDT), laser therapy and anti-vascular endothelial growth factor (anti-VEGF) therapy.³ PDT entails adverse effects on normal tissues and laser therapy inevitably destroys the outer retina which may result in loss of central vision. Main drawbacks to anti-VEGF therapy are short half-life, intraocular route of administration and limited efficacy.^{4,5} These features have forced researchers to investigate other therapeutic modalities such as retinal cell transplantation for the treatment of end-stage retinal diseases.⁶ RPE cells are a main source for replacing destroyed cells.^{7,8} Many attempts have also been made to generate RPE cells from various sources of stem cells.⁹⁻¹¹ The purpose of this study was to establish the role of primary culture of human RPE cells as a source of retinal cell replacement therapy.

METHODS

Isolation and Cultivation of RPE Cells

Human globes were obtained from cadavers earlier than 24 hours after death at the Eye Bank of I.R. Iran and sent to the biochemistry laboratory at the National Institute of Genetic Engineering and Biochemistry (NIGEB) for isolation of RPE cells. To prepare the globe for this purpose, the optic nerve was cut and peribulbar tissues were removed using scissors. The globe was emptied from its contents and the neurosensory retina was removed using a forceps. The inner surface of the globe was irrigated with phosphate buffered saline (PBS) and the RPE layer was separated by a forceps and cut into small sections. Thereafter, segments of the RPE layer were incubated for 90 minutes in 2 u/ml dispase at 37°C. The mixture was then centrifuged for 5 min at 300 g and 4°C. The resulting cellular preparation from each globe was cultured in 25 cm² flasks previously coated with fetal calf serum (FCS).

The culture media included Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM: F12), FCS (20% at the beginning of culture), penicillin (120 µg/ml), streptomycin

(220 µg/ml), gentamicin (50 µg/ml) and amphotericin B (2.5 µg/ml). The medium was changed twice a week (DMEM: F12 supplemented with 10% FCS) until the cultures reached confluence which usually occurred by 2-3 weeks. Then the cultures were passaged using 3×10⁵ cells per 25 cm² culture flask. Confluent cultures from passages 3 to 7 were used for all experiments.

Immunocytochemistry

Immunocytochemistry (ICC) was performed on slides prepared from harvested RPE cells. Cultured RPE cells were first fixed by paraformaldehyde 4% in PBS (V/V) for 20 minutes at room temperature. The cells were made permeable using Teriton x-100 (0.25%) and cultures were blocked in 1% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Rabbit anti-human RPE 65 polyclonal antibody (1:1000; Santa Cruz, USA) against the specific protein of RPE microsomal membranes was used to confirm the RPE characteristics of isolated cells. Cytokeratins are intermediate keratin filaments present in the cytoskeleton of epithelial cells, therefore it was essential to check RPE cells for existence of these filaments. We utilized specific murine anti-human cytokeratine 8/18 monoclonal antibody (1:1000; Santa Cruz, USA) for reconfirmation of RPE characteristics of isolated cells. To identify the origin of the cells generated within the spheroid colonies, we examined the cultures with rabbit polyclonal anti-human Nestin, goat polyclonal anti-human Pax-6 and goat polyclonal anti-human Chx-10 (1:100; Santa Cruz, USA) antibodies.

Fluorescein isothiocyanate (FITC) conjugated antibody (goat anti-mouse IgG, goat anti-rabbit IgG and donkey anti-goat IgG, Santa Cruz, USA) against the above-mentioned antibodies were diluted (1:400 to 1:1000) and used to detect immunoreactivity of cultures for primary antibodies. After final washing, the slides were incubated in 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 mg/ml, Santa Cruz, USA) for 10 minutes to counter-stain nuclear DNA. The slides were examined by Axiophot Zeiss fluorescence microscope (Zeiss, Germany)

equipped with a 460 nm filter for DAPI dye and a 520 nm filter for fluorescein isothiocyanate (FITC) conjugated antibodies.

RNA Extraction

RNA extraction from RPE cells was performed using Tripure RNA isolation reagent (Roche, Germany) according to the manufacturer's protocol. cDNA pools were made using a reverse primer and superscript III reverse transcriptase (Invitrogen, Belgium). Resulting cDNAs were amplified using iQ SYBER Green supermix kit (BioRad, Italy).

Quantitative Real-Time RT-PCR

Real-time PCR was performed to assess the expression of VEGF and pigment epithelium derived factor (PEDF) genes in harvested RPE cells. Since RPE cells are the only source of production and secretion of VEGF and PEDF in the human eye; detection of their expression in isolated cells would be a valuable marker for confirming the presence of RPE in cultures. The length of the amplicon was 141 base-pairs for VEGF and 342 base-pairs for PEDF. The primer sequences were as follows:

VEGF forward:

5'ACTTCTCTGGGCTGTTCTCG3'

VEGF reverse:

5'TCCTCTTCTTCTCTCTTCC3'

PEDF forward:

5'TGCAGGCCAGATGAAAGGG3'

PEDF reverse:

5'TGAACTCAGAGGTGAGGCTC3'

Specific primers for glyceraldehyde phosphate dehydrogenase (GAPDH) were used as intracellular controls beside the above mentioned genes. The length of this amplicon was 219 base-pairs and the primer sequences were:

GAPDH forward:

5'GCAGGGGGGAGCCAAAAGGGT3'

GAPDH reverse:

5'TGGGTGGCAGTGATGGCATGG3'

The PCR thermal profile was as follows:

Cycle 1: (1X)

Step 1: 95.0°C for 5 minutes

Cycle 2: (35X)

Step 1: 95.0°C for 1 minute

Step 2: 61.5°C for 1 minute

Step 3: 72.0°C for 1 minute

Step 4: 80.0°C for 10 seconds

To draw the melting curve and confirm the precision of the reactions, the following steps were performed:

Cycle 3: (1X)

Step 1: 95.0°C for 1 minute

Cycle 4: (1X)

Step 1: 65.0°C for 1 minute

Cycle 5: (60X)

Step 1: 65.0°C for 10 seconds

At this step, the temperature of the system was increased 0.5°C every 10 seconds until a final temperature of 95°C was reached.

For amplification of VEGF cDNA the above-mentioned protocol was performed at annealing temperature of 61.5°C. Amplification of PEDF cDNA and GAPDH cDNA was performed in a similar fashion with annealing temperature of 53.8°C and 62.8°C, respectively.

RESULTS

Cultivation of RPE Cells

Early stages of RPE cell culture were successful (Fig. 1) and spheroid colonies pertaining to stem cells/progenitor cells were observed in the cultures (Fig. 1D). When RPE cells were obtained from a fetus or newborn, as compared to those obtained from adult globes, the spheroids appeared earlier and in greater number.

Serum Starvation in Culture

When confluent cultures were deprived from FCS, the cells that had grown as a monolayer began to establish floating spheroids. It appeared that serum starvation per se, without addition of growth factor supplements, increased the number of spheroids and accelerated the process (Fig. 2).

Reculturing Spheroids

To demonstrate the self renewal potential of colony forming cells, the spheroids were collected from the supernatant of the cultures and recultured in complete media supplemented with

10% FCS after dissociation using trypsin or without any treatment. New secondary spheroids were generated from reculturing the dissociated primary colonies (Fig. 3A). When the primary spheres were not treated with trypsin, they reattached to the surface of the culture vessels and began to grow in a mono-layer leading to new free floating colonies (Figures

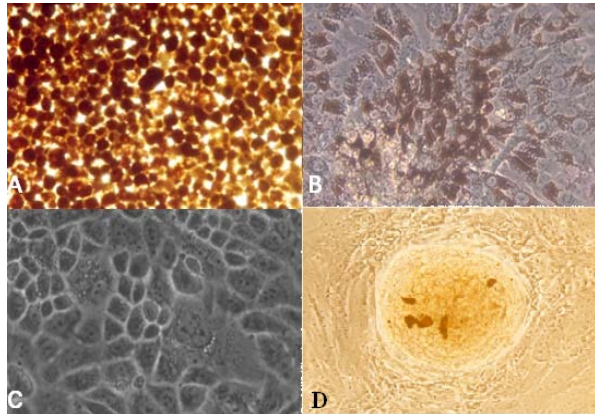


Figure 1 (A) Retinal pigment epithelial (RPE) cells immediately after separation from a globe. (B) RPE cell culture after one week shows pigment dispersed on the cell surface. (C) Culture after 3 weeks revealed that RPE cells completely covered the floor of the container, retained the appearance of epithelial tissue and lost their pigment to some extent. (D) The appearance of a spheroid colony. The cells were visualized using Zeiss Axiovert 405M inverted microscope (magnification: 10x20 for A, B and D and 10x32 for C).

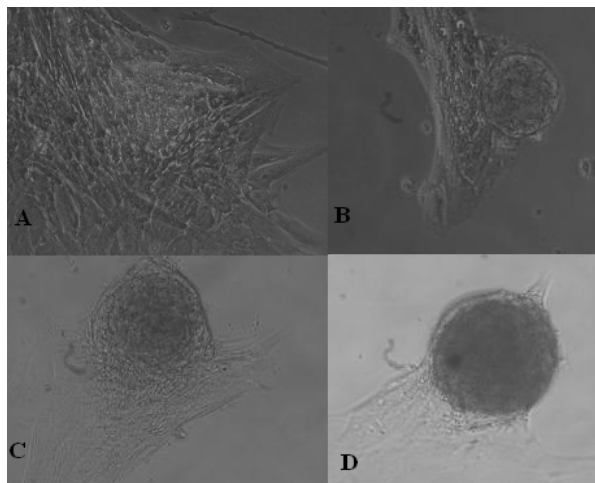


Figure 2 Culture of retinal pigment epithelial cells after deprivation from fetal calf serum. Cells which had grown in a monolayer began to establish spheroid colonies floating in the supernatant in a few days. A through D shows a typical process of spheroid formation (magnification: 10x20).

3B, 3C and 3D).

Appearance of Photoreceptors and Neural-like Cells in High Passage Cell Cultures

Photoreceptors and neural-like cells were morphologically detected very late, usually after 8 passages in cultures (Fig. 4).

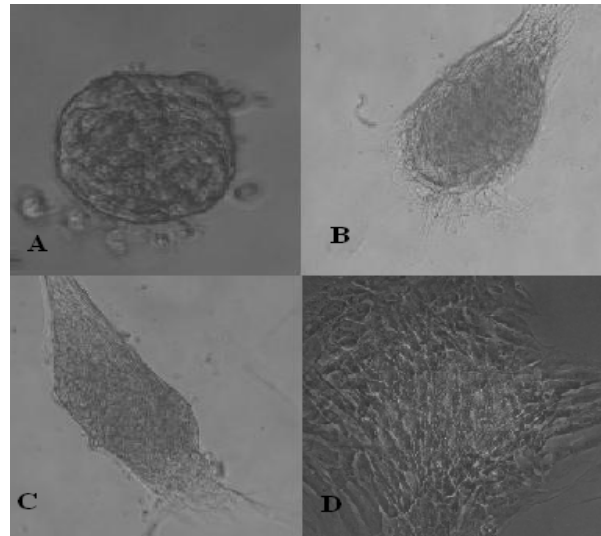


Figure 3 (A) A non-pigmented colony derived from dis-sociated spheroid colonies. (B and C) Recultured colonies derived from retinal pigment epithelial cell cultures. (D) They mostly reattached to the culture vessel surface and grew in a monolayer (magnification: 10x20).

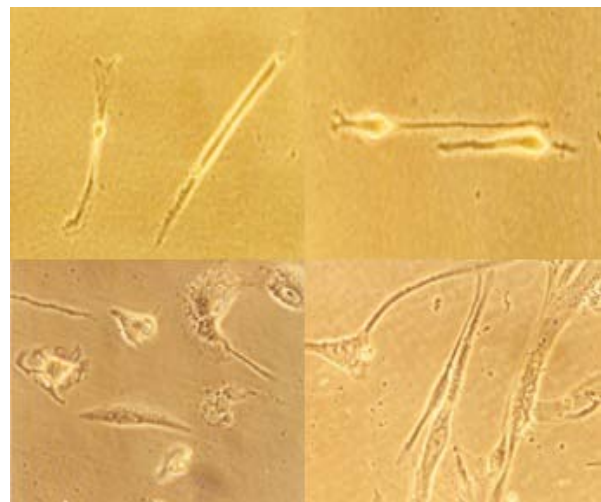


Figure 4 Photoreceptors and neural-like cells observed within the cultures (magnification: 10x32).

Characterization of Cultures

RPE-Specific Markers

Results of evaluation for RPE-specific markers including RPE 65 and cytokeratine 8/18 are presented in Figure 5 demonstrating RPE cells in green. All cells expressed RPE 65 and 30% reacted with antibody against cytokeratine 8/18.

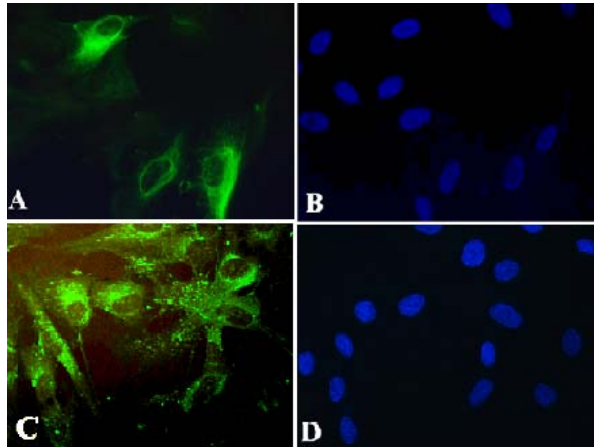


Figure 5 (A) Retinal pigment epithelial (RPE) cells stained green with cytokeratine antibody. (B) Nuclei of RPE cells are stained blue using 4,6-diamidino-2-phenyindole dihydrochloride (DAPI). (C) RPE cells are stained green using RPE 65 antibody. (D) Nuclei of the RPE cells stained blue with DAPI (magnification: 8x40).

Retinal Stem/Progenitor Cell Markers

Immunostaining for Pax-6, Nestin and Chx-10 revealed that cells and/or colonies within the cultures were positive for Chx-10 in 11.1%, for Pax-6 in 8.6% and for Nestin in almost all colonies (Fig. 6). Cultures were simultaneously processed without primary and secondary antibodies served as controls which were negative for these markers.

Cultured RPE Cells Express Transcripts for VEGF and PEDF

Results of real-time PCR for VEGF and PEDF are summarized in Figure 7. Quantitative comparison of threshold cycles for PEDF and VEGF amplification in multiple independent tests revealed that the expression ratio for PEDF/VEGF was 1.37 in cultured RPE cells.

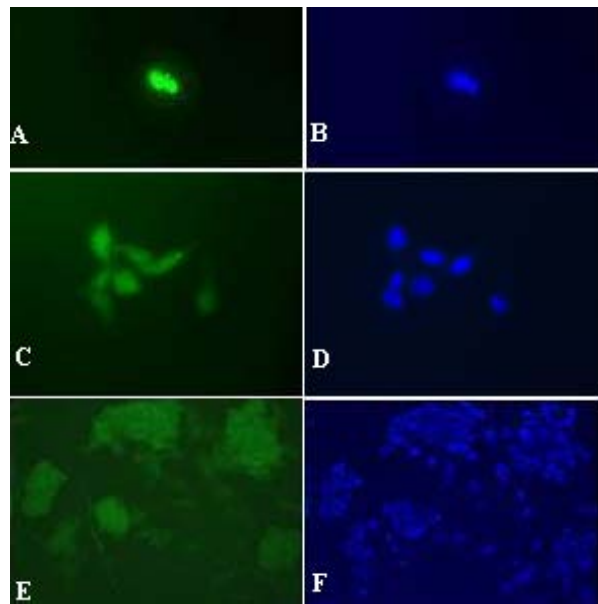
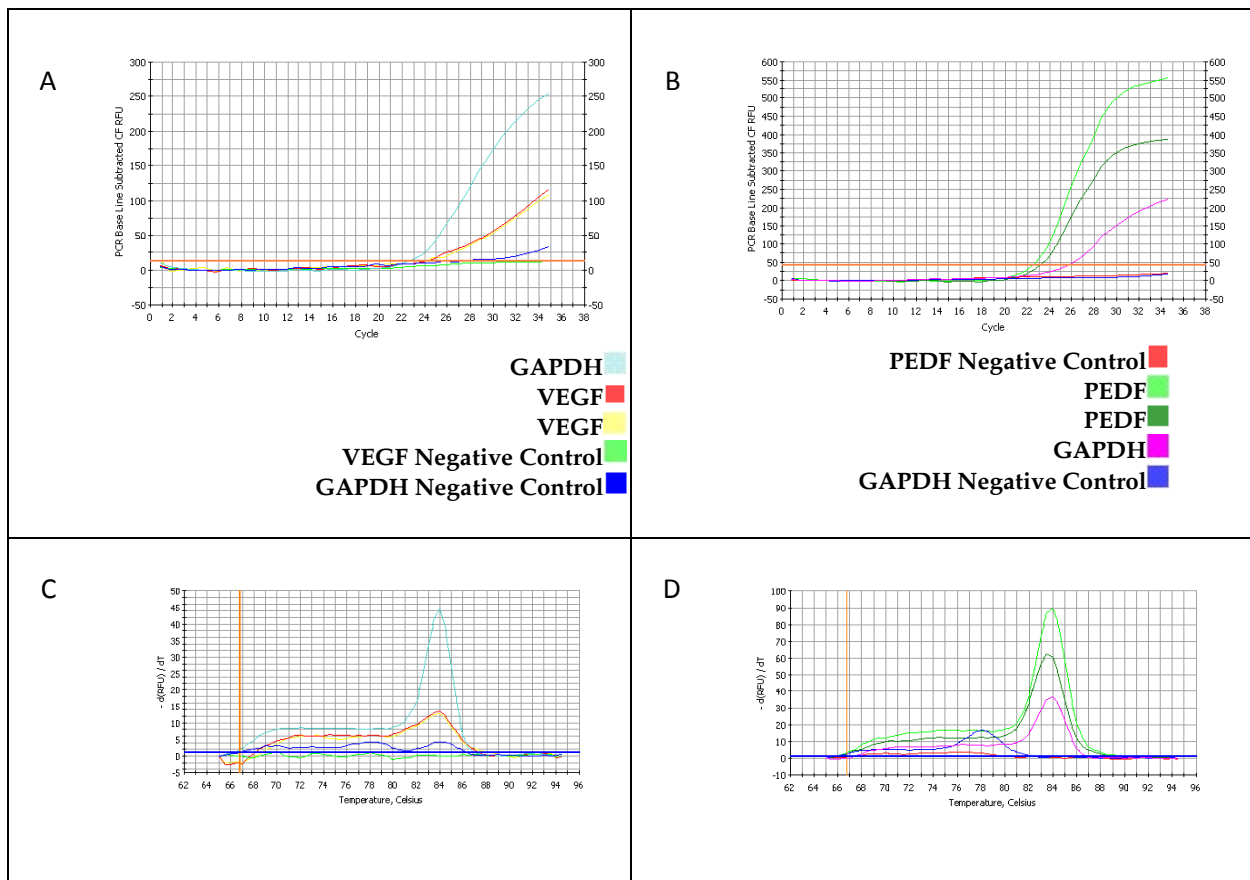


Figure 6 Immunostaining for retinal stem/progenitor cell markers including Chx-10 (A), Pax-6 (C) and Nestin (E). Slides (B), (D) and (F) show nuclei stained with 4,6-diamidino-2-phenyindole dihydrochloride and correspond to slides (A), (C) and (E), respectively (magnification: 8x40 for A-D and 8x 20 for E-F).

DISCUSSION

It was long believed that mature stem cells were limited to the bone marrow, muscle, skin and gastrointestinal tract, however it is now recognized that stem cells may also be derived from brain, heart, bone, and adipose tissues as well as from various parts of the eye including the limbus, conjunctiva, ciliary epithelium and retina.^{12,13} Cell replacement therapy using human embryonic stem cells has had encouraging preliminary results in many conditions. These cells possess the unique ability to differentiate into all 3 embryonic germ cell layer derivatives.¹⁴⁻¹⁶ Studies have revealed that in the absence of development inducing agents, embryonic cells pass through neural pathways by default.¹⁷ RPE cells obtained from human embryonic stem cells (hESC) can be used as an invaluable source for replacing destroyed cells in degenerative retinal disorders.^{18,19} In animal models of AMD and retinitis pigmentosa (RP), stem cell transplantation has been able to reduce the severity of the condition and transplantation in the fovea has led to visual improvement.⁷



GAPDH, glyceraldehydes-3-phosphate dehydrogenase

Figure 7 (A) Quantitative amplification of cDNA for vascular endothelial growth factor (VEGF). (B) Quantitative amplification of cDNA for pigmented epithelial derived factor (PEDF). (C) Melting curve analysis of VEGF specific amplification. (D) Melting curve analysis of PEDF specific amplification.

In recent years it has been shown that RPE cells can dedifferentiate into stem cells.²⁰ RPE cell transplantation has been investigated in animal and human studies for treatment of disorders such as AMD and RP.^{21,22} Mannagh et al²³ evaluated RPE cell behavior, and Fischer and Reh²⁰ were able to isolate and culture human RPE cells using an enzymatic method. The method of separation of RPE cells from the globe is one of the most important factors in the establishment of successful RPE cell cultures. We were able to successfully separate RPE cells from globe using an enzymatic method, resulting in separation of intact and highly pure RPE cells leading to rapid induction of cell growth and proliferation in culture. When the ratio of PEDF/VEGF expression was utilized as a marker of functional and healthy RPE cells, our findings were comparable to *in vivo* studies.²⁵

In addition to successful primary culture of RPE cells, spheroid colonies pertaining to stem/progenitor cells were observed. After several cellular passages, some cell types morphologically resembled photoreceptors and neural cells were detected on some cultures. Immunocytochemical analysis of cultured RPE cells showed co-expression of a considerable amount of stem cell markers including Chx-10, Nestin and Pax-6 involved in ocular development.

Chen and colleagues²⁶ compared serum containing media with serum free media for hESC proliferation. They reported that three hESC lines were established which grew at similar speed in both media but the serum-containing medium yielded significantly higher percentages of morphologically qualified colonies and cells expressing stem cell markers.

Mannello and Tonti²⁷ aimed to define the optimal conditions for stem cell cultures in order to minimize the theoretical risk of using xenogenic compounds and to reduce immunological reactions once stem cells are transplanted but at the same time maintain characteristics such as multipotentiality, self-renewal and transplantability. There are published clinical trials on human stem cells using FCS for supplementing the culture medium without any significant side effects.^{28,29} FCS includes valuable bioactive factors with neuroprotective properties which play a crucial role in attenuating the cytotoxic consequences of necrotic and apoptotic factors.³⁰ Human mesenchymal stem cells (hMSC) expanded in FCS-supplemented medium displayed comparable morphology, phenotype and differentiation capacities to hMSC expanded in medium supplemented with platelet lysate.²⁷ Furthermore, it has been shown that FCS supplemented culture media is more convenient in terms of preventing alloreactivity-related immune complications.³¹

In summary, the generation of primitive retinal cells from cultured RPE cells in serum containing media was in line with recently published data.^{22,25} Our data showed that serum starvation caused a great increase in the number of formed spheroids without need for exogenous growth factors. Due to the observed plasticity in cell cultures, RPE cells seems to be an encouraging source of stem cells for retinal cell replacement therapy.

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