

Behavior of a Spontaneously Arising Human Retinal Pigment Epithelial Cell Line Cultivated on Thin Alginate Film

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Abstract

Purpose: A cell line spontaneously derived from human retinal pigment epithelium (hRPE) was cultured on alginate film gelatinized with different concentrations of neurobasal cell culture medium (NCCM) to assess its growth and morphological behavior on this naturally occurring polysaccharide.

Methods: Neonatal human globes were used to isolate hRPE cells. They were cultured in Dulbecco's modified Eagle's-medium-and-Ham's-F12-medium-(DMEM/F12) supplemented with 10% fetal bovine serum (FBS). Cultures were continuously studied using phase contrast microscopy. After the ninth passage, cells were characterized through immunocytochemical analysis for Oct4, Chx10, and Pax6 and Ki67 markers. In each well of a 6-well microplate, 1 and 2% weight/volume (w/v) alginate in deionized water was added and gelatinized using 1× and 10× NCCM. hRPE cells were cultured at a density of 2×10^5 cells/well in alginate-coated microplates. After 5 days, hRPE colonies were harvested and re-plated on polystyrene substrates. Morphology and growth of hRPE cultures were determined during the next 2 weeks.

Results: The first few passages of the cultures were purely hRPE cells that revealed typical morphological features of the pigmented epithelium. They made spaces, devoid of cells, between hRPE cell monolayer and fill in the unoccupied spaces. They grew faster than native RPE cells and rapidly overgrew. Immunocytochemical test revealed that the founded cells expressed Chx10, Pax6, Ki67 and Oct4. The hRPE cells survived unlimitedly on alginate film and formed giant adjoining colonies. After re-plating, hRPE colonies adhered quickly on polystyrene and displayed native hRPE morphological features.

Conclusion: Alginate film can support the survival and growth of hRPE cells and induce the cells to re-organize in tissue-like structures.

Keywords: Alginate; Human RPE Cell; Neurobasal Cell Culture Medium; RPE Cell Line; Stem/Progenitor Cell Marker

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INTRODUCTION

Retinal pigment epithelium (RPE) is the monolayer of cells in the outer part of the retina between photoreceptors and the choroid. It is firmly attached to the photoreceptors and plays a key role in their nourishment and protection.^[1] In degenerative eye diseases, such as macular degeneration and retinitis pigmentosa, RPE cells are damaged. These

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cells can be a suitable source for transplantation and replacement therapy to treat degenerative eye diseases.^[2] With the advent of tissue engineering technology, it is now possible to fabricate living replacement tissue for damaged body parts.^[3,4]

Cells in the human body grow within organized three-dimensional (3D) matrices, but in a culture vessel, they grow in a two-dimensional (2D) pattern. Many recent studies have introduced different natural/artificial substrates that can provide a 3D environment similar to the extracellular matrix (ECM) of soft tissues and an appropriate condition for the exchange of gases and nutrients, and for the adhesion, proliferation and differentiation of cells.^[5]

In tissue engineering, different kinds of polymers are used for creating substrates, some of which are natural, such as collagen,^[6] chitosan,^[7] hyaluronic acid,^[8] matrigel,^[9] fibrin,^[10] gelatin^[11] and alginate,^[12] while others are synthetic, such as polylactic-co-glycolic acid (PLGA),^[13] polyglycolic acid (PGA)^[14] and polyethylene terephthalate (PET).^[15]

In this study, alginate was used as a substrate to culture RPE cells. Alginate has been widely used in tissue engineering because of its high biocompatibility, low cost, low immunogenicity and capacity of forming hydrogels under gentle conditions.^[16] Alginate can be used as an appropriate surface for growth, adhesion, proliferation and differentiation of cells.^[17] It is a natural polysaccharide that is extracted from brown marine algae and composed of guluronic (G) and mannuronic (M) acid residues.^[18] It can form hydrogels by cross-linking divalent cations such as calcium (Ca^{2+}), barium (Ba^{2+}), strontium (Sr^{2+}), copper (Cu^{2+}), nickel (Ni^{2+}), and zinc (Zn^{2+}) and trivalent cations such as aluminum (Al^{3+}) and ferric (Fe^{3+}). In this research, instead of divalent or trivalent cations, neurobasal cell culture medium (NCCM) that contains CaCl_2 , MgCl_2 , KCl, NaHCO_3 , and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was used. Matyash et al demonstrated that NCCM increases differentiation of neural stem cells to neural cells.^[19]

In the current study, human RPE cells, which were established spontaneously in routine culture of hRPE cells, were characterized. Isolated RPE cells were cultured on alginate gelatinized with NCCM, and the morphology, viability and growth of cultured cells were studied; furthermore the ideal conditions for culture and propagation of these valuable cells was developed.

METHODS

Isolation and Culture of Human RPE (hRPE) Cells

hRPE cells were isolated from human neonatal globes, up to 1 year of age, less than 24 h after death which were transferred from the Central Eye Bank of Iran to

the Basic Science Laboratory at the National Institute of Genetic Engineering and Biotechnology. Isolated hRPE cells were cultured in flasks (Nunc, Roskilde, Denmark) containing Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12) (Sigma-Aldrich, Munich, Germany) medium supplemented with 20% fetal bovine serum (FBS) (GIBCO-BRL, Eggenstein, Germany). The flasks were placed in an incubator at 37°C with a humidified atmosphere of 5% CO_2 . The used culture medium was replaced with fresh DMEM/F12 medium supplemented with 10% FBS every 3 days until the cells were 80% confluent. Then the cultures were passaged by routine cell culture methods. Isolated cells were assessed by immunocytochemical (ICC) tests for RPE 65 and cytokeratin as specific markers for RPE cells (data not shown).

ICC Analysis of Derived hRPE Cells

hRPE cells in 9th passage were cultured on pre-coated coverslips in a 24-well microplates (Nunc, Roskilde, Denmark) at a density of 1×10^5 cells/well and incubated for 24 h. Cultured cells were fixed using ice-cold methanol (-10°C) (Merck, Darmstadt, Germany) for 5 min at room temperature and then slides were blocked using 1% bovine serum albumin (BSA) (Merck, Darmstadt, Germany) in 1% PBST (1% Triton X-100 in PBS) (Sigma-Aldrich, Munich, Germany) for 20 min at room temperature. Coverslips were incubated in diluted solution of antibodies against Oct4, Chx10, Pax6, and Ki67 (Santa Cruz, Carlsbad, CA, USA) in 1.5% BSA and 1% PBST overnight at room temperature. Then the coverslips were incubated with the secondary antibody in 1.5% BSA and 1% PBST for 1 hour at room temperature in the dark. The secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100, Santa Cruz, Carlsbad, CA, USA), FITC-conjugated goat anti-mouse IgG (1:100, Santa Cruz, Carlsbad, CA, USA), and FITC-conjugated donkey anti-goat IgG. The nuclei of cells were stained using 4',6-diamidino-2-phenylindole (DAPI) (1 mg/ml, Santa Cruz, Carlsbad, CA, USA) for 1 min. Coverslips were mounted with a drop of mounting medium (90% glycerol, 10% PBS, and 10% (w/v) phenylenediamine). Samples were observed under fluorescence microscope (Axiophot, Zeiss, Germany).

Preparation of Alginate Substrate

A 1 mm thick alginate film was prepared by adding 940 μl of 1 and 2% (v/w) sodium alginate in deionized water in 6-well plates. Alginate films were created by drying alginate solution at room temperature for 12 h. The alginate substrate was gelatinized using 2 ml of $1 \times$ and $10 \times$ NCCM composed of 1.8 mM CaCl_2 , 0.814 mM MgCl_2 , 5.33 mM KCl, 26.19 mM NaHCO_3 and 0.906 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and keeping the emerged substrates at

room temperature for 12 h. Substrates were washed twice with sterile water and equilibrated with 2 ml of DMEM/F12 for 2 days in an incubator.

Culture of hRPE Cells on Alginate Film

hRPE cells were harvested from the 7th passage of cultures. The hRPE cells were seeded at a density of 2×10^5 cells/well in gelatinized alginate-substrate coated 6-well plates in DMEM/F12 medium supplemented with 10% FBS. Morphological changes of the cultures were followed for 2 weeks.

Re-plating hRPE Colonies to Assess Viability of the Constituent Cells

Five days after cultivating hRPE cells on alginate film, 50 μ l of the cultured medium from each well was harvested. The developed colonies were precipitated and re-plated on polystyrene under routine culture conditions. Viability and morphology of the re-plated colonies were studied in the next few days.

Defining Optimum Alginate Substrates for Culturing hRPE Cells

After 2 weeks of culturing hRPE cells on alginate films, 2 ml of sodium citrate (100 mM) was added in each well and incubated for 20 min until the colonies detached from the substrates. Finally, cell suspensions were centrifuged at $300 \times g$ for 5 min, and the total number of isolated cells was determined.

Cell Viability Assay

The proliferative capacity of hRPE cells on alginate hydrogel film, as compared to polystyrene, was assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Alginate films were prepared in 96-well microplates. The hRPE cells were seeded at a density of 10^4 cells/well. After 2 days of culture, the cells were incubated with 0.5 mg/ml MTT (Sigma, Deisenhofen, Germany) for 4 h at 37°C. The solution was then removed and the resulting formazan crystals were dissolved using 0.01 M dimethyl sulphoxide (DMSO), and the absorbance of the resulting solution was determined at 570 nm using a plate reader (BP800 Microplate Reader, Biohit Plc, Helsinki, Finland).

Statistical Analysis

ICC test, MTT assay and cell counts were performed in triplicates. MTT assay results and cell counts were compared between control and experimental conditions using one-way analysis of variance (ANOVA). To calculate the percentage of cells that were immunopositive for assessed markers, the number of cells in three

different fields of vision was counted under a fluorescent microscope and average counts of immunoreactive cells was reported. $P < 0.05$ was considered as statistically significant.

RESULTS

Morphological Characteristics of hRPE Cells

hRPE cells formed adherent, elongated and fusiform shapes in culture. Cells could passage up to 10 times before exhibiting hallmarks of senescence [Figure 1]. hRPE cells were routinely isolated and each sample was assessed by ICC tests for RPE 65 and cytokeratin as specific markers for RPE cells.^[20] hRPE cells were established in the 6th passage of general hRPE cultures. These cells made sizeable spaces, devoid of cells, between the monolayer of hRPE cells, and gradually invaded unoccupied spaces. They grew rapidly and occupied the whole surface of the culture vessel. Simultaneously, native hRPE cells disappeared. Morphology of these cells was quite different as compared to normal hRPE cells. They were smaller than hRPE cells and desired to make colony-like structures. After sequential passages, they got smaller and gained a more granular morphology than earlier passages. Derived hRPE cells were subcultured several times; they were passaged for more than 17 times and culture medium was needed to be changed daily [Figure 2].

Immunocytochemistry for Retinal Stem/Progenitor Cell Markers in hRPE Derived Cultures

ICC revealed that hRPE cells were positive for Oct4, Chx10, Ki67+, and Pax6+ markers. Expression of Chx10

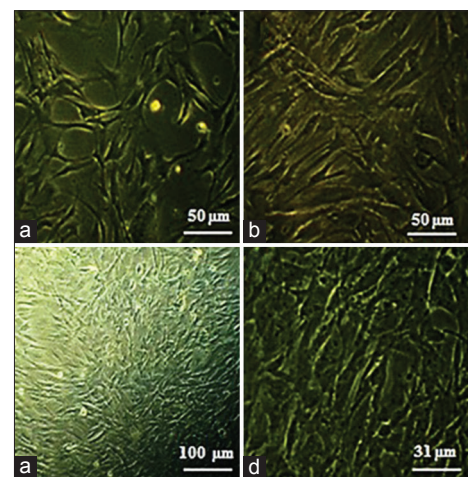


Figure 1. Morphology of human hRPE cells in culture at different magnifications, in the 4th passage of the culture. (a) Low confluency hRPE culture, magnification: $\times 200$. (b-d) High confluency cell cultures, $\times 200$, $\times 100$ and $\times 320$ magnifications, respectively. hRPE, human retinal pigment epithelial.

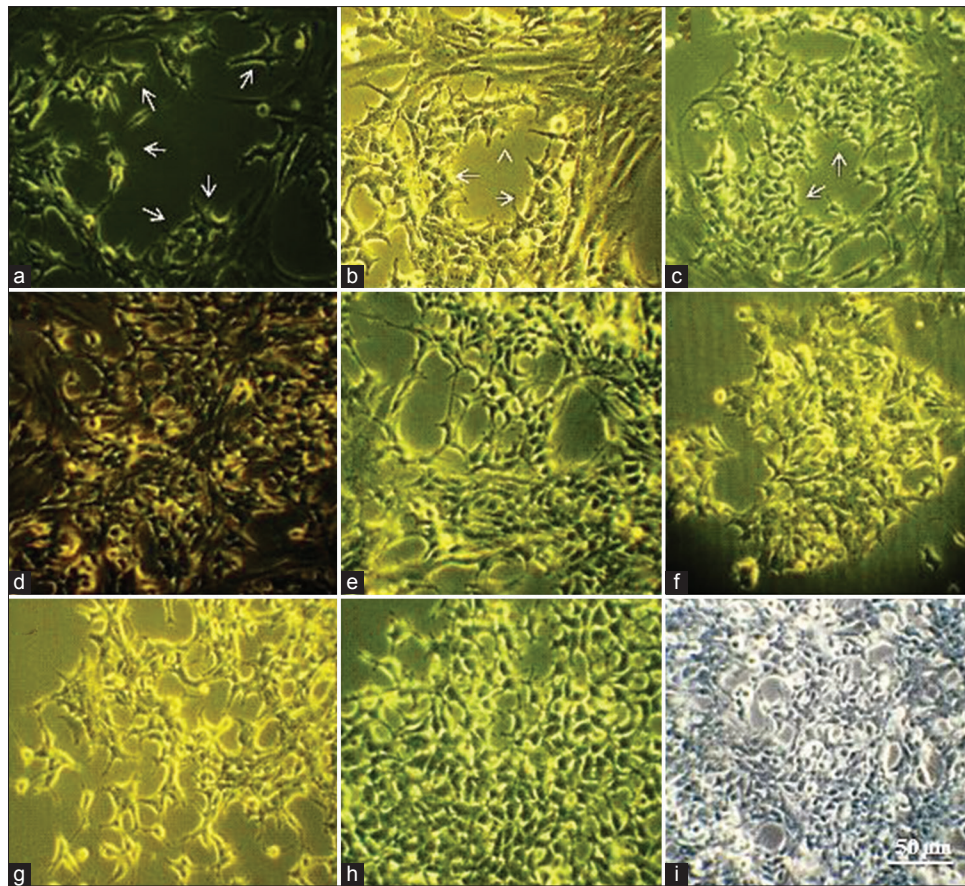


Figure 2. Manifestation and morphology of hRPE cell line. (a-e) Rising of hRPE cell line in unoccupied spaces between hRPE cell populations and its notable growth rate until the newly appeared cells completely populate the cultures. (f) hRPE cell line showed a great tendency to make colony-like structures. (g-i) Demonstrate hRPE cell lines in the 7th, 10th and 12th passages respectively, the cells got smaller size at higher passage numbers, magnifications $\times 200$. hRPE, human retinal pigment epithelial.

and Pax6+ proteins confirmed the identity of the isolated cells as retinal-like progenitor cells. Nearly 90% of cells expressed Chx 10 and more than 4% expressed Pax6+. In addition, more than 60% of the cells expressed nuclear Ki67 marker. This indicates that isolated cells retained their proliferative potential *in vitro*. Furthermore, Oct4 was detected in the nuclei of nearly 50% of the cells; this marker was expressed in embryonic stem cells (ESCs) as well [Figure 3]. Native hRPE cells did not express the aforementioned markers in a reasonable amount. The negative controls of hRPE cells were simultaneously immunostained without using primary antibodies or without using both primary and secondary antibodies [Figure 4].

Studying Morphology of hRPE Cells on Alginate Films

Morphology of hRPE cells was the same as that derived from 1 or 2% of alginate films, which were gelatinized with 1 or 10% NCCM. After 1 day *in vitro* (DIV) cells were observed as single cells adherent on alginate. After 2 to 6 DIV, single cells

started to make colonies with sustained contact to alginate film; these colonies became larger over time and eventually separated from the alginate film and got suspended in the medium. After 10 DIV, giant colonies were easily detected in the supernatant of the cultures. After 13 DIV, all colonies were floating in culture media [Figure 5].

Study that Examines Viability of hRPE Cells in Colonies

Human RPE colonies harvested from the supernatant of corresponding cultures and directly, without further manipulations, were re-plated on polystyrene plates without alginate coats. At first they adhered to the polystyrene surface and took morphological features of native RPE cells in cultures. They grew rapidly and reached full confluency until the 6th day in culture. They grew three dimensionally and made adjoining colonies and with time, disengaged from culture surfaces and became floating in the medium. Finally, dense colonies on polystyrene surface got suspended in DMEM/F12 medium in the 9th day [Figure 6].

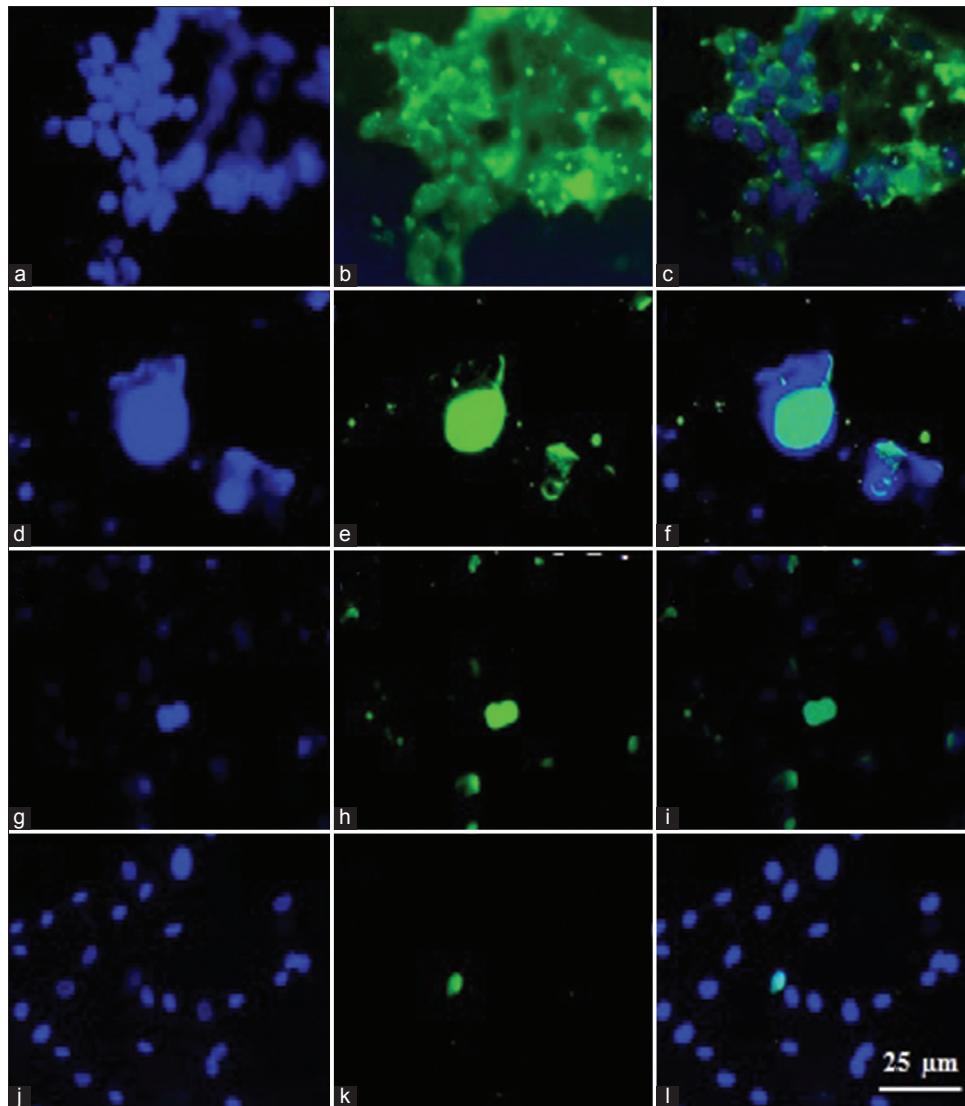


Figure 3. Immunocytochemistry results for Chx10, Ki67+, Oct4 and Pax6+ in established hRPE cell line. (a) Nuclei of the cells were stained blue using DAPI. (b) 90% of cells in established hRPE cell line expressed Chx10. (c) Merged a and b. (d) DAPI stained blue cell nuclei. (e) 60% of cells in established hRPE cell line expressed Ki67+. (f) Merged d and e. (g) Cell nuclei were stained blue using DAPI, (h) 50% of cells revealed Oct4 nuclei expression in established hRPE cell line. (i) Merged g and h. (j) Nuclei of cells were stained blue using DAPI. (k) 4% of established hRPE cells were positive for Pax6+ expression in culture. (l) Merged j and k (magnification, $\times 400$). hRPE, human retinal pigment epithelial; DAPI, diamidino-2-phenylindole.

Determination of the Best Condition for Culture of hRPE Cells

The number of hRPE cells on alginate substrate after 13 DIV demonstrated that hRPE cells showed better growth on 1% alginate film gelatinized with 1 \times NCCM ($P = 0.0003$), 1% alginate film gelatinized with 10 \times NCCM ($P = 0.0004$), 2% alginate film gelatinized with 1 \times NCCM ($P = 0.00003$) and 2% alginate film gelatinized with 10 \times NCCM ($P = 0.0038$) as compared with polystyrene. The number of hRPE cells on 1% alginate film, gelatinized with 1 \times and 10 \times NCCM and 2% alginate film gelatinized with 10 \times NCCM was 1.3 times that of polystyrene controls. The total number of hRPE cells cultured on 2% alginate film gelatinized

with 1 \times NCCM was 1.8 times that of polystyrene controls [Figure 7]. Human RPE cells showed the highest level of growth, and they built macroscopically giant colonies on 2% (v/w) alginate film gelatinized with 1 \times NCCM [Figure 8].

The Effect of Alginate Film on the Growth of hRPE Cells

MTT cell assay showed that hRPE cells revealed better growth on 1% alginate film gelatinized with 1 \times NCCM ($P = 0.02$), 1% alginate film gelatinized with 10 \times NCCM ($P = 0.02$), 2% alginate film gelatinized with 1 \times NCCM ($P = 0.00008$) and 2% alginate film gelatinized with 10 \times NCCM ($P = 0.02$) as compared to polystyrene.

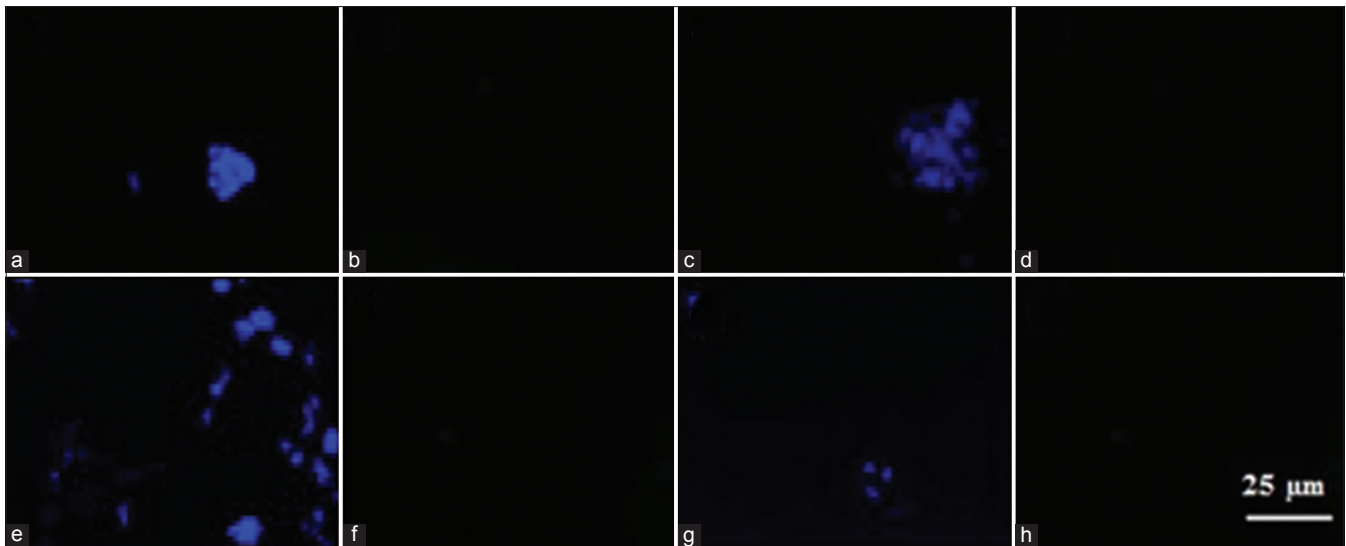


Figure 4. Negative controls in which the primary antibodies for Ki67 and Pax6 (b), Chx10 (d) Oct4 (f) were omitted, showed no immune-reactivity. (a, c and e) Nuclei of the cells were stained blue using DAPI; pictures are related to fields b, d and f respectively. (h) Shows a control without both primary and secondary antibodies. Nuclei of the cells were stained blue using DAPI (g) (magnification: $\times 400$). DAPI, diamidino-2-phenylindole.

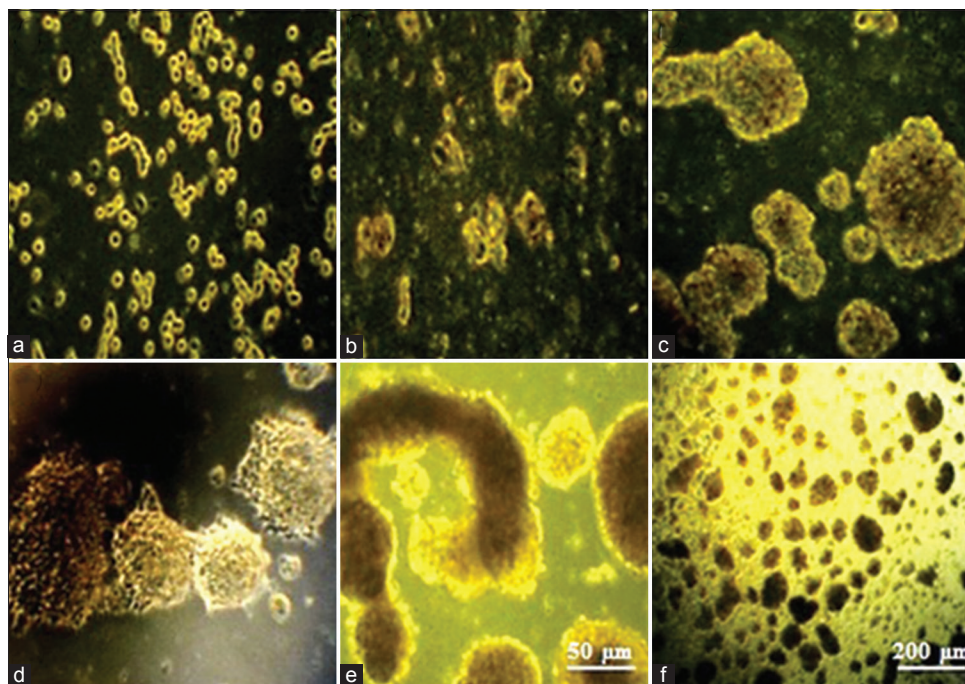


Figure 5. Morphology of hRPE cell line on 2% alginate film gelatinized with 1% NCCM. (a) Single cells on the first day of cultures. (b-d) represent hRPE cell line 3, 5 and 6 days after the beginning of the culture respectively. (e) Giant macroscopic colonies of hRPE cell line were disengaged from alginate film 10 days later. (f) All colonies were dissociated from the alginate film 13 days later. Magnifications (a-e: $\times 200$ and f: $\times 50$). hRPE, human retinal pigment epithelial; DAPI, diamidino-2-phenylindole.

The best condition for culture of hRPE cells was on 2% alginate film, gelatinized with $1\times$ NCCM [Figure 9].

DISCUSSION

This study pursued hRPE cell line development and maintenance on alginate film; its characteristics

were studied and compared with native hRPE cells. Immunocytochemical analysis demonstrated expression of Ki67+ proliferative marker and specific markers of stem cells and progenitor cells including Oct4, Pax6, and Chx10 in the established cell line. This indicated that isolated cells retained proliferative and stem cell potential *in vitro*. While native hRPE cells, as controls, did

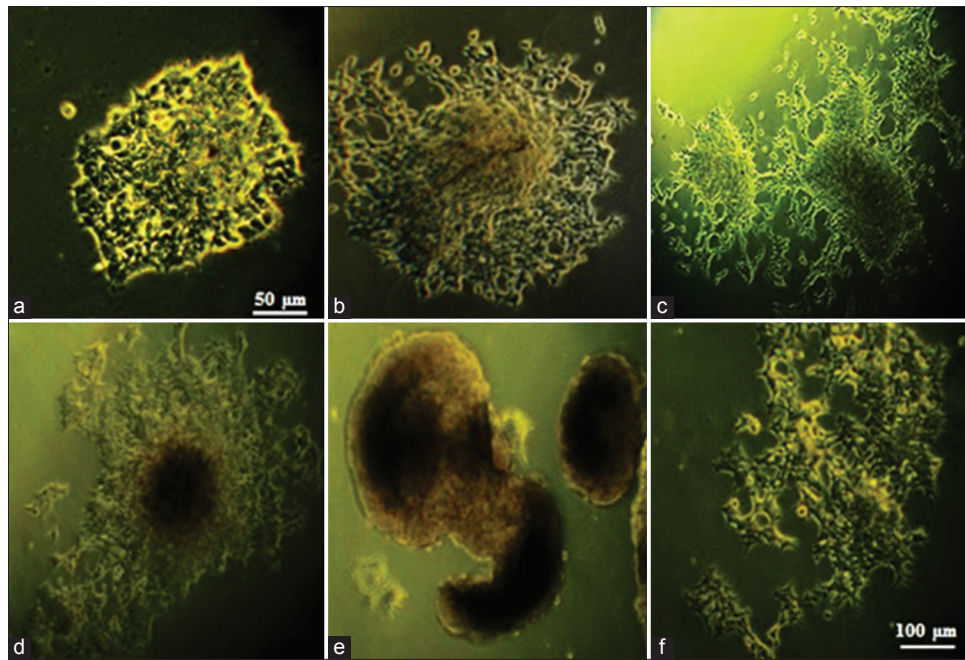


Figure 6. Re-plating hRPE colonies. (a) 1 day after re-plating, evidence of colony adherence to the polystyrene were manifested. (b and c) 2 to 6 days after re-plating, hRPE cells disengaged from colony structures and started to re-adhere to culture surface. (d-f) Represent a colony of hRPE cells that partially dissociated from the culture vessel surface, a suspended colony of hRPE cells and a colony of hRPE cells that completely disengaged from the culture vessel, 9 days post re-plating, respectively. Magnifications (a: $\times 200$ and b-f: $\times 100$). hRPE, human retinal pigment epithelial.

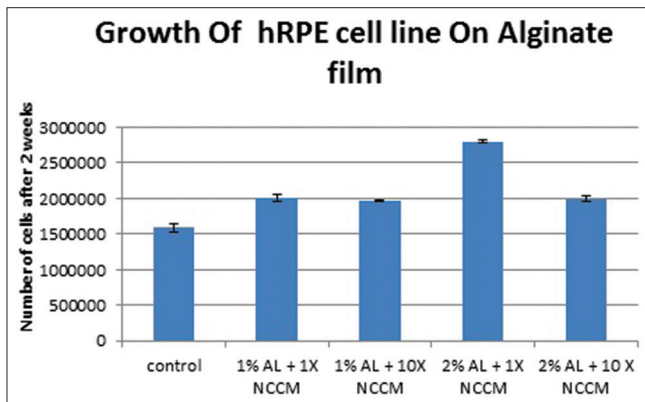


Figure 7. hRPE cell growth on alginate film. Cells showed a faster growth rate on 1% alginate film gelatinized with $\times 1$ NCCM ($*P$ value = 0.0003), 1% alginate film gelatinized with $\times 10$ NCCM ($**P$ value = 0.0004), 2% alginate film gelatinized with $\times 1$ NCCM ($***P$ value = 0.00003) and 2% alginate film gelatinized with $\times 10$ NCCM ($****P$ value = 0.0038) as compared to cultures on polystyrene (control). hRPE, human retinal pigment epithelial; NCCM, neurobasal cell culture medium.

not express the aforementioned markers in a reasonable amount.

In previous experiments, collagen,^[21] gelatin,^[22] fibrinogen^[23] and poly-L-lactic acid (PLLA)^[24] have been reported as promising substrates for development of RPE cells. Machida-Sano et al investigated the effect of five different types of ions cross-linked on alginate

films (i.e., Fe^{3+} , Al^{3+} , Ca^{2+} , Ba^{2+} , and Sr^{2+}) on cellular growth and showed that Fe^{3+} -cross-linked alginate films supported cell growth superiorly.^[25] In another experiment, the effect of Fe^{3+} and Ca^{2+} ions, as reagents inducing polymerization of alginate, was assessed on human dermal fibroblast growth and revealed a faster growth rate for cells cultured on Fe^{3+} -alginate films.^[26]

In this study, a thin layer of alginate film was used as a 3D *in vitro* model for culturing hRPE cells. Different concentrations of NCCM were used for polymerization of alginate and also for evaluation of its growth inducing effect on hRPE cells. Our data showed that the best condition for growth of hRPE cells was culturing the cells on 2% alginate film gelatinized with $1\times$ NCCM.

Previous studies have investigated that human hepatocyte cell line formed adhesive colonies on alginate substrate.^[27] Studies have also indicated that alginate hydrogels were developed as a niche for corneal epithelial cells which formed colony structures on this substrate.^[28] The viability of primary epithelial cells increased when they were cultured on thin alginate discs.^[29] By encapsulating hRPE cells (ARPE-19) within alginate beads their viability and growth got improved.^[30]

Our previous research demonstrated that growth of hRPE cells was increased on alginate or alginate/gelatin substrate (as compared to polystyrene), we indicated that they made giant colonies on these substrates.^[31] Re-plating harvested colonies on polystyrene and examining the

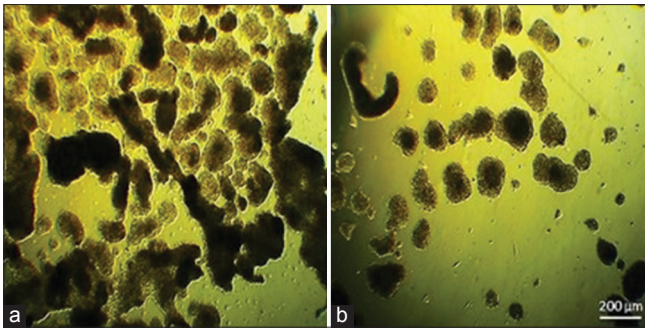


Figure 8. Growth of hRPE cell line on 2% alginate film 10 DIV. (a) hRPE cells on 2% alginate film gelatinized with 1% NCCM. (b) hRPE cells on 2% alginate film, gelatinized with 10% NCCM. hRPE cell line showed a faster growth rate and made larger colonies on 2% (v/w) alginate film, gelatinized with $\times 1$ (NCCM). Magnifications $\times 50$. hRPE, human retinal pigment epithelial; NCCM, neurobasal cell culture medium.

cultures on the next consecutive days demonstrated that alginate film was biologically compatible with growth and expansion of hRPE cells *in vitro*. Cells on 1 mm thick 2% alginate prepared in water showed the highest growth rate.

Soft hydrogels of alginate gelatinized with $1\times$ NCCM, promoted neuritis outgrowth of neural stem cells and increased differentiation of them to neural cells.^[19] In our study, $1\times$ and $10\times$ NCCM was used and we observed that hRPE cells displayed higher growth rates when cultured on 2% alginate film gelatinized with $1\times$ NCCM. Mouse ESCs on alginate substrate did not spread and predominantly established “tissue-like” clumps.^[32] Our study revealed that by passing of time hRPE cells formed tissue-like clumps on alginate film; they joined each other and built macroscopically visible clumps. This study indicated that alginate film could be an ideal substrate for studies aimed at replacing healthy cells instead of diseased ones in injured tissues. Yamamoto et al also used alginate substrate for developing functional and transplantable vascular tissue grafts which increased the efficacy of cell-based therapeutic strategies in tissue engineering.^[33]

Our findings demonstrated that NCCM cross-linked alginate film supports the maintenance, proliferation, cell attachment properties, viability and growth of hRPE cells. Future *in vivo* studies as well as clinical trials are needed to clarify the potential application of alginate film and NCCM, as a biological material, for fabrication of tissue engineering scaffolds and cell transplantation purposes in retinal degenerative diseases.

In summary, 2% (v/w) alginate gelatinized with $1\times$ NCCM made a thin film that supported the survival and growth of spontaneously established hRPE cells. It induced the cells to re-organize in giant macroscopically visible structures like spheroids, and offered a suitable substrate for culturing and expanding potential progenitor cells such as hRPE cells *in vitro* and for further

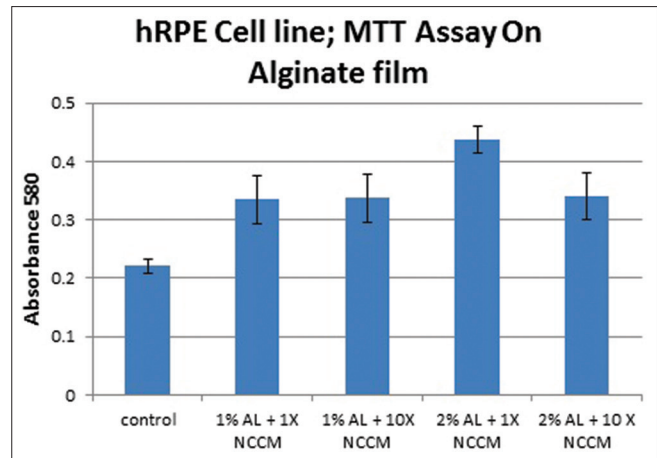


Figure 9. MTT assay. hRPE cell line exhibited a significant increase in total number on alginate film as compared to polystyrene. hRPE cells represented preferred growth on 1% alginate film gelatinized with $\times 1$ NCCM ($*P$ value = 0.02), 1% alginate film gelatinized with $\times 10$ NCCM ($**P$ value = 0.02), 2% alginate film gelatinized with $\times 1$ NCCM ($***P$ value = 0.00008) or 2% alginate film gelatinized with $\times 10$ NCCM ($****P$ value = 0.02) as compared with polystyrene (control). hRPE cell line showed the best growth rate when cultured on 2% (v/w) alginate film, gelatinized with $\times 1$ (NCCM). hRPE, human retinal pigment epithelial; NCCM, neurobasal cell culture medium; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

in vivo or clinical applications in retinal degenerative diseases.

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Nil.

Conflicts of Interest

There are no conflicts of interest.

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