

## Comparing Three Methods of Co-culture of Retinal Pigment Epithelium with Progenitor Cells Derived Human Embryonic Stem Cells

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### ABSTRACT

**Background:** Close interaction between retinal pigment epithelium (RPE) and photoreceptors plays an essential role in visual function. The objective of this study is to determine the effects of RPE cells in the differentiation of progenitor derived human embryonic stem cells (hESC) into retinal cells; we developed *in vitro* co-culture models and compare these models to investigate in which model the expression of photoreceptor markers is superior. It seems the effects of RPE cells on differentiation of retinal progenitor cells (RPCs) through the cell-to-cell contact or with the use of insert and compare of these methods has not been reported yet.

**Methods:** Initially, retinal progenitors (RPs) were differentiated from hESC. After isolation of RPE sheet from rabbit eyes, demonstrated these cells maintains the integrity and feature after 2 weeks. Next, we examined the induction of photoreceptors by the co-culture of RPE through insert in 1 week and 2 weeks (indirect) or without insert by the cell-to-cell contact (direct). The differentiation of retinal cells was verified by protein and gene expression in these three methods.

The adherent cells were morphologically examined using phase contrast microscopy and characterized by immunofluorescent staining and reverse transcription-polymerase chain reaction (RT-PCR)

**Results:** Evaluation of immunostaining showed that hESC, highly (>80%) can be directed to the RPs fate. Upon co-culture of RPCs with RPE sheet using insert for 2 weeks or by the cell-to-cell contact, these cells differentiated to neural retina and expressed photoreceptor-specific markers. However, in direct co-culture, some mature photoreceptor markers like arrestin expressed in compare with indirect co-culture.

**Conclusions:** The expression of late photoreceptor marker could be improved when RPE cells seeded on RPCs in compare with the use of insert.

**Keywords:** Arrestin, co-culture, differentiation, retinal pigment epithelium

## INTRODUCTION

The retinal pigment epithelium (RPE) is a monolayer of hexagonal pigmented cells that fulfills many tasks and plays a crucial role in visual function.<sup>[1,2]</sup> There is a close interaction between the RPE cells and photoreceptors produced by long apical microvilli that surround the outer-segments of photoreceptors.<sup>[3]</sup> Interphotoreceptor matrix (IPM) fills the space between the RPE and photoreceptor cells. Thus, the IPM plays an essential role in this interface.<sup>[4,5]</sup> Isomerization of visual pigments, preservation of blood-retinal barrier and phagocytosis of outer segment of photoreceptor are some of the important roles of RPE cells.<sup>[6]</sup> On the other hand, RPE cells secrete a variety of factors such as: Pigment epithelium-derived factor,<sup>[7]</sup> the vascular endothelial growth factor,<sup>[8]</sup> that are important for survival as some of them protect photoreceptors against light-induced damage.<sup>[9]</sup> Therefore, the interactions between RPE and photoreceptor cells are critical for the development and differentiation of photoreceptors. Gene defects cause disruption of a wide range of proteins involved in the visual cycle reaction cascade. Several retinal disorders such as retinitis pigmentosa or age macular degeneration are caused by this gene defect. In the eye, mutations in genes expressed in photoreceptors lead to degeneration of RPE and visa-versa.<sup>[1,2,10]</sup> The anatomical relationship between RPE and photoreceptors, in addition to the secretion of several growth factors, cause the selection of RPE cells as a good candidate for inducing neural activity. The first co-culture of RPE and neural retina was done by Tamai *et al.*<sup>[11]</sup> Several studies were showed the co-culture of RPE and embryonic neural retina.<sup>[12-14]</sup> On the other hand, spatial arrangement of RPE cells with neural retina may have an essential role for supplying trophic molecules. Impairment in these molecules leads to failure in neurotransmission that is involved in the visual. German *et al.*<sup>[15]</sup> demonstrated that photoreceptor-RPE cell interactions play a key role in the physiological orientation of photoreceptors cells. During the development of eye, heterogeneous populations of progenitor cells give rise to areas of the retina: the optic stalk, retinal pigment epithelium and neural retina. Under the influence of some factors like cell-to-cell interactions or combinations of regulatory genes

these cells converge to different types of the neural retina. So, here, we mimic an *in vivo* system with co-culturing of RPE cells with progenitor cells derived from human embryonic stem cells (hESC) and compare three methods to find, which method of differentiation of retinal progenitor cells (RPCs) to retinal cells is superior.

Induction effects of RPE cells on RPCs through cell-to-cell contact and the comparison of direct and indirect co-culture have not been reported yet.

This is also to understand whether RPE cells probably affect RPCs differentiation via cell-to-cell contacts rather than by using insert. These data may be helpful for improvement of better approaches for culturing and differentiating pathway programming.

## METHODS

### Animals

Pigmented rabbits that weighed between 1.5 kg and 2.0 kg were used in this study (Department of Physiology, Isfahan University, Iran). All proceedings concerning animals used were performed in accordance with the Ethical Committee at Royan Institute. Pigmented rabbits were sacrificed by an overdose of ketamine and xylazine. After enucleating eyes from anesthetized rabbits, extra ocular tissues were cleaned. Intact globes were washed in Ca<sub>2</sub><sup>+</sup> and Mg<sub>2</sub><sup>+</sup>- free phosphate buffered saline supplemented with penicillin/streptomycin.

Then, globes were incubated in 2% dispase (Gibco, 17105-041) for 20 min. Cornea-iris complex cut-off just 3 mm posterior to the limbus. Vitreous and anterior segment were removed. The posterior eye cup was dissected by four incisions. After incubation of posterior segment in Dubecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum for 2 h, the RPE layer was peeled off in sheet and used for co-culture.

### Culture of hESCs

The Royan H5,<sup>[16]</sup> hESC line was obtained from Royan Institute. The cells were cultured on martrigel under feeder-free culture condition in the presence of media previously described.<sup>[17]</sup> The media were changed every other day, for 7 days.

### Tissue culture

After 7 days, the differentiated cells in the center of colony mechanically discarded and the undifferentiated cells of hESCs, which usually located in the peripheral part was induced to neural ectoderm in the presence of media containing noggin (1 ng/mL; R and D, 1976-NG), human recombinant Dkk-1 (1 ng/mL; R and D, 5439-DK/CF), and human recombinant insulin-like growth factor-1 ([IGF]-1, 5 ng/mL; R and D, 291-GI) in DMEM-F12 medium supplemented with 10% knockout serum replacement, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 1% B27 (Gibco, 17504-044), for 2 days. On the 3<sup>rd</sup> day, the cells were cultured in the presence of retinal determination (RD) medium that consisted of DMEM: F12 supplemented with 1% B27, 2% N2 (Gibco, 17502-048), 10 ng/mL noggin, 10 ng/mL Dkk-1, 10 ng/mL IGF-1, and 5 ng/mL bFGF as previously described.<sup>[18]</sup> The medium was renewed every other day up to 2 weeks to form the neural tube (NT)-like structures. On day 16, NTs were manually dissociated and replanted on 1 mg/mL laminin and 15 mg/mL poly-L-ornithine (both from Sigma-Aldrich)-coated 6-well tissue culture plates (TPP, 92406) in the same medium (20-30 NTs per well).

### *In vitro* explant co-culture experiments

To evaluate the differentiation of RPCs, co-cultures of RPE sheet and these cells were obtained by following three procedures. One day after replanting of the dissociated NTs on day 17, by two methods (Indirect), the

isolated RPE sheet was cultured on filter insert 0.4-mm (Millicell-CMPICMO3050) and transferred into six well plates that included the dissociated NTs for either 1 week or 2 weeks in the presence of RD medium. In the third method, RPE sheet was seeded over the RPCs (direct) for 2 weeks in RD medium. The cells from all groups were cultured for an additional period of 10 days in neurobasal medium (Invitrogen, 21-103) supplemented with 2% N2. Half of medium was renewed every other day.

### Immunocytochemistry staining

Cell cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by permeation with Triton X-100 (0.1%) for 15 min. To block non-specific sites, we used 10 mg/ml bovine serum albumin. Retinal cells were identified by the antibodies listed in Table 1. After exposing the cells overnight to primary antibodies, the cells were washed 3-5 times with PBS for 5 min and exposed 1 h with either fluorescein isothiocyanate-or TRITC-conjugated secondary antibodies, and nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma, D8417).

### Reverse transcription-polymerase chain reaction for gene expression analysis

The total RNA was extracted from the differentiated cells and digested with deoxyribonuclease I (Fermentas, EN0521). Standard RT was performed using random hexamer primers, and RevertAid<sup>TM</sup> First strand complementary

**Table 1:** Antibodies used in this study

	Species	Clonality	Source
Primary Ab			
Otx2	Rabbit	Polyclonal	Sigma-Aldrich, HPA000633
Pax6	Mouse	Monoclonal	Chemicon, MAB5554
Zo-1	Mouse	Monoclonal	Invitrogen, 330100
Rx	Rabbit	Polyclonal	Life Span LS-C53650-50
Cytokeratin18	Mouse	Monoclonal	Chemicon, MAB3234
S-Opsin	Rabbit	Polyclonal	Abcam, ab-65681
Rhodopsin	Mouse	Monoclonal	Santacruzsc-56472
Secondary Ab			
FITC	Anti-rabbit	IgG goat polyclonal	Sigma-aldrich, F1262
FITC	Anti-mouse	IgG goat polyclonal	Chemicon, AP124F
TRITC	Anti-mouse	Goat polyclonal	

IgG=Immunoglobulin G, FITC=Fluorescein isothiocyanate

deoxyribonucleic acid (Fermentas, K1622). PCR reaction was followed using SmarTaq polymerase and specific primers shown in supplementary Table 2. Amplification conditions were as follows: initial denaturation at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 45 s and extension for 45 s at 72°C and a final polymerization at 72°C for 10 min. PCRs were performed in triplicate. PCR products were analyzed by gel electrophoresis on 1.7 or 2% agarose and stained with ethidium bromide (10 µg/ml). They were visualized and photographed on an ultraviolet transilluminator.

## RESULTS

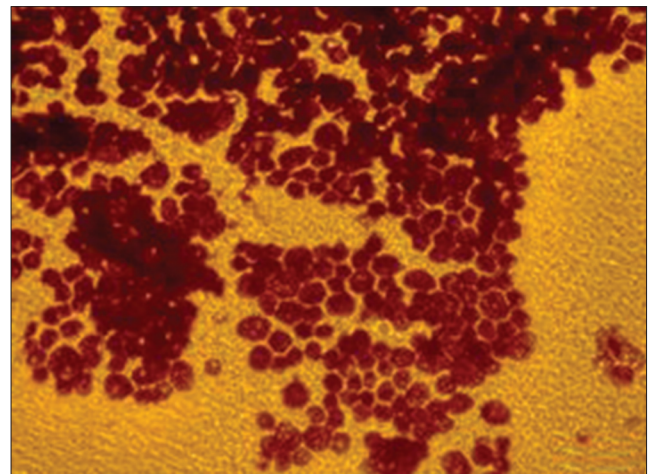
### Characterization of RPE cells

Examination of RPE culture sheets by phase-contrast microscopy showed a monolayer of uniform hexagonal morphology of RPE cells with melanine [Figure 1]. Staining with anti-ZO-1, tight junction protein and cytokeratin 18, an intermediate protein, demonstrated the expression of these proteins in the RPE sheets [Figure 2]. Reverse transcription-polymerase chain reaction (RT-PCR) analysis confirmed the expression of natriuretic peptide receptor-A, a rabbit RPE marker, in these cells, even after 14 days [Figure 3]. In addition, one-way ANOVA analysis of MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) results revealed no significant

different between the viability of RPE cells in first and 14th days (mean OD:  $1.435 \pm 0.32$  and  $1.542 \pm 0.247$ ) respectively.

### Differentiation of hESCs into RPCs

To generate RPCs, the undifferentiated feeder-free hESCs were treated with noggin, Dkk-1 and IGF-1 for 2 days and then switched to RD medium for 2 weeks without embryoid body formation in adherent conditions. Subsequently, the neuro epithelium and NTs emerged from hESCs. These data demonstrate that a large fraction of the hESCs in these cultures are characteristic of retinal progenitors (RPs) [Figure 4]. Quantification



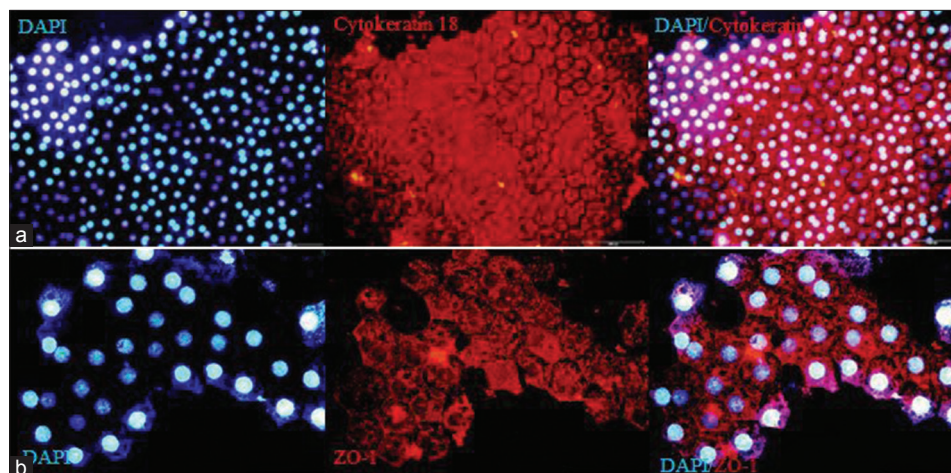
**Figure 1:** Phase contrast photomicrographs of retinal pigment epithelium isolated cells that showed morphology of these cells. Scale bar: 50 µm

**Table 2:** Primer sequences and conditions of RT-PCR

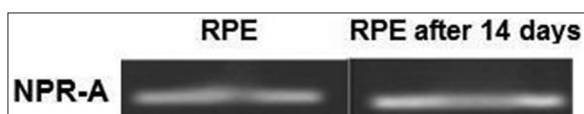
Gene	Primer sequence (5'→3')	AT (°C)	Length (bp)	Cycle	Accession no.
Npr-A	F: AGAGGGAGAACCTGACCAACCG R: ACGATTCTGGAATTCCTGATACTCG	60	215	35	XR_084830
Nrl	F: GAGCCAGAGGAGACAGGA R: TTAGCTCCCGCACAGACAT	58	89	40	NM_006177
Rhodopsin	F: TCATCATGGTCATCGCTTTC R: CATGAAGATGGGACCGAAGT	52	100	40	NM_000539
S-Opsin	F: GATGAATCCGACACATGCAG R: CTGTTGCAAACAGGCCAATA	60	104	40	NM_001708
Recoverin	F: CCAGAGCATCTACGCCAAGT R: CACGTCGTAGAGGGAGAAGG	60	186	35	NM_002903
Arrestin	F: ACCATCAAGGAGGGCATAG R: TCAGATTATGGCGAGCAAAC	61	213	35	NM_000541
β-actin	F: CGTGACATTAAGGAGAAGCTGTGC R: CTCAGGAGGAGCAATGATCTTGAT	55	374	35	NM_001101

RT-PCR= Reverse transcription, Npr-A=Natriuretic peptide receptor-A





**Figure 2:** Isolated retinal pigment epithelium (RPE) *in vitro* and experimental design. (a) isolated RPE expressed intermediate cytokeratin-18 protein and (b) tight junction protein ZO-1. Scale bar: 100  $\mu$ m



**Figure 3:** Reverse transcription chain reaction analysis showing the expression of rabbit retinal pigment epithelium

of immunostaining showed that  $79\% \pm 1.6\%$  of differentiated cells were positive for Rx. In addition,  $78.3 \pm 1.8\%$  of the cells expressed Pax6 and  $86.2 \pm 1.6\%$  of the differentiated cells were positive for Otx2.

### Co-culture of RPCs with retinal pigment epithelium

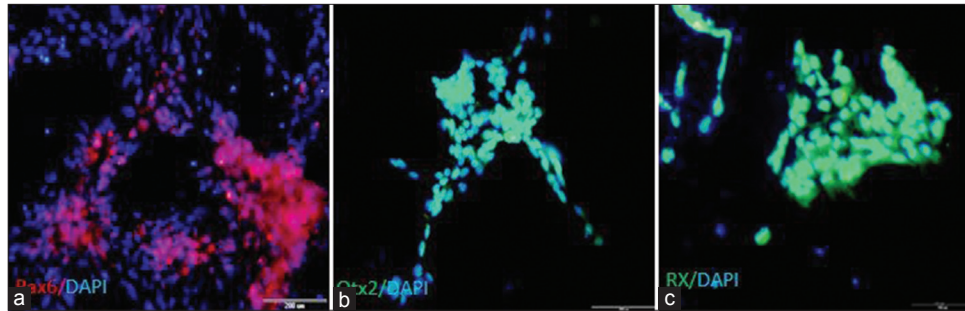
To assess whether the close contact of RPE cells could be effective for the generation of neural photoreceptors, we co-cultured the RPE with hES cell-derived progenitors on 0.4 mm filters for one and 2 weeks or without filters in the presence of RD medium. Before the co-culture, we evaluated the viability and identity of RPE cells after 14 days. The observation of microscopic images suggests that photoreceptor markers are better expressed in use with filter for 2 weeks and direct co-culture as compared to control group [Figures 5 and 6]. In the indirect co-culture, after 1 week, it did not improve the photoreceptor differentiation (data not shown). Direct co-culture expressed some mature photoreceptor markers such as arrestin in compared to the indirect co-culture for 2 weeks and control group. There were no differences between the expressions of other photoreceptor markers [Figure 7].

## DISCUSSION

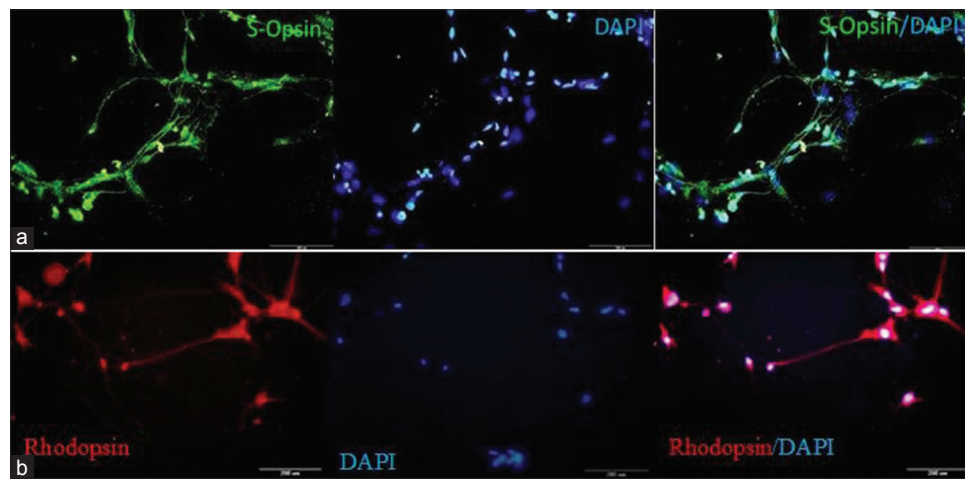
Taken together, our results show that progenitor cells-derived hESC can be directed to a retinal fate by co-culture with RPE sheet. However, in direct co-culture mature photoreceptor markers like arrestin express in compare with indirect co-culture.

The aim of these studies was to improve a procedure for retinal cells differentiation for treatment of some retinal disease such as macular degeneration and retinitis pigmentosa through cell replacement.

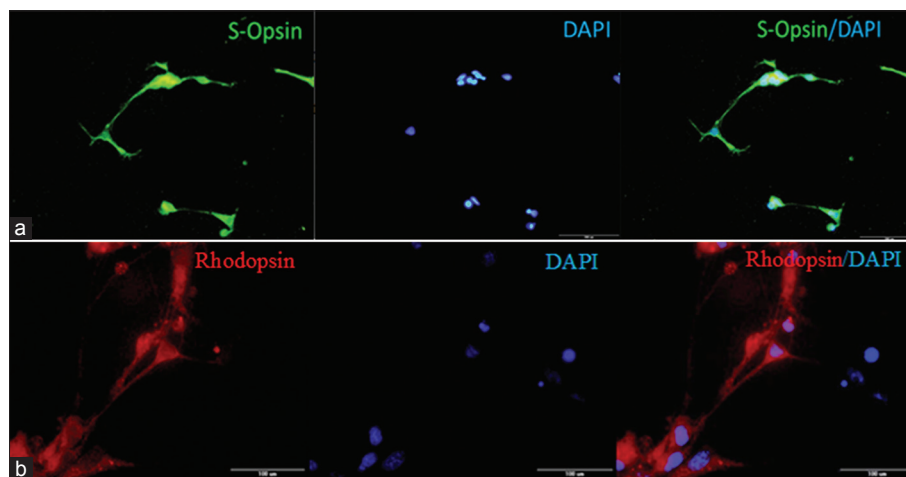
There have been numeral studies on the differentiation of stem cells to retinal cells,<sup>[19,20]</sup> and culture of retinas alone.<sup>[21,22]</sup> However, limited studies have been carried out on the co-culture of retinal cells with underlying RPE or choroid layer<sup>[23-25]</sup> The current study demonstrates that RPE cells as a sheet can reserve their properties such as intact tight junction, intracellular microfilaments and morphologic characteristics, which are important for their functions. On the other hand, hESCs can be differentiated efficiently into RPCs by using defined media as previously described.<sup>[26]</sup> RPE cells promoted the Spatial Orientation of developing photoreceptors when these cells were seeded over RPE cells and rapid reorganization took place.<sup>[16]</sup> Ikeda *et al.* have shown that co-culturing precursors derived from mouse ES with embryonic retinal cells on the culture insert can generate cells with the photoreceptor phenotype.<sup>[27]</sup> Explant of RPCs that were overlaid of RPE cells was previously reported. In a later



**Figure 4:** Quantitative immunofluorescence staining showed high expression of RP markers (a) Pax6 (b) Otx2 (c) RX. Scale bar: 200 μm



**Figure 5:** Immunofluorescence staining showed expression of S-opsin (a) and rhodopsin (b) in retinal progenitors a-derived retinal cells in direct co-culture. Scale bar: 200 μm

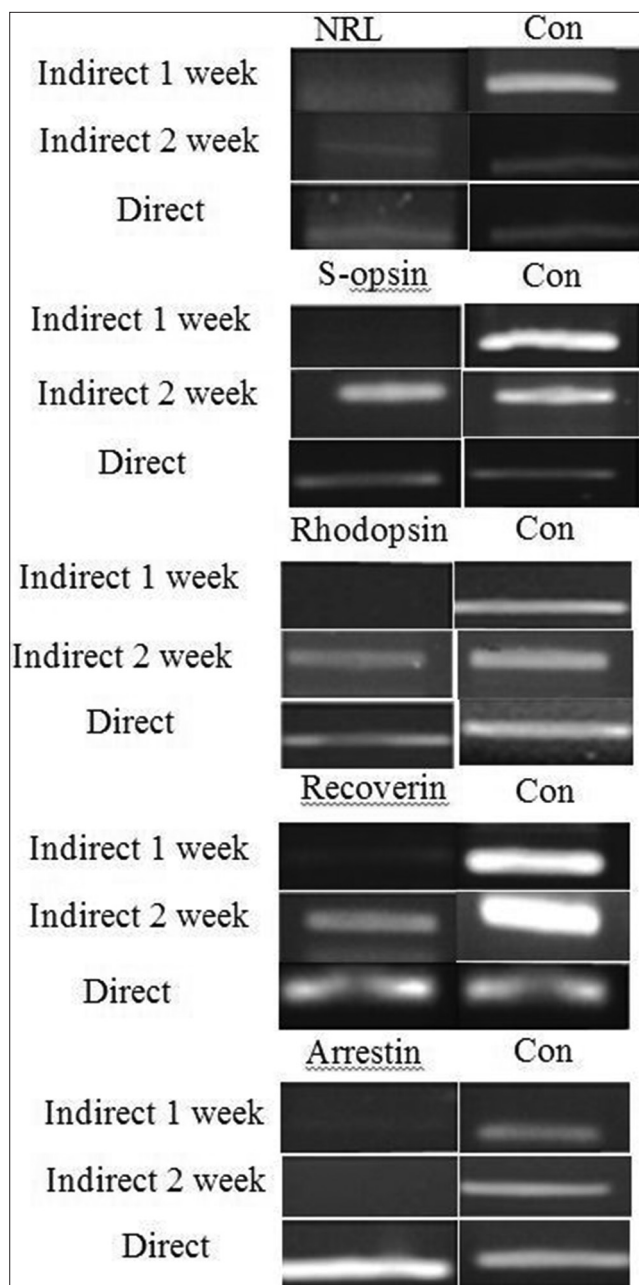


**Figure 6:** Expression of S-opsin (a) and rhodopsin (b) in retinal progenitors-derived retinal cells in indirect co-culture after 2 weeks. Scale bar: Respectively 200 and 100 μm

study, the expression of rhodopsin was increased as compared with control.<sup>[28]</sup> Lamba *et al.* co-cultured the hES cell-derived progenitor cells with retinal explants from adult mice on the culture

insert and showed the expression of photoreceptor markers.<sup>[19]</sup>

To obtain whether RPE cells can direct progenitor cells to neural retina, we mimic the in



**Figure 7:** Reverse transcription chain reaction analysis demonstrated expression of early photoreceptor markers in direct and indirect co-culture and late photoreceptor marker like arrest in in direct co-culture

vivo environment in culture. Hence, we examined three protocols to test the inducing effects of RPE cells in direct and indirect contact with RPCs. The co-cultures were allowed to survive up to 14 days; after 24 days, they were analyzed for evidence of retinal cells differentiation. Our results have shown that a suitable time is the window for co-culture of RPE with RPs to induce mature photoreceptor

differentiation. After this time, in direct and indirect protocol, the expressions of marker of photoreceptor cells were observed. On the other hand, expression of late photoreceptor markers could be improved by seeding RPE overlaid RPs as compared to indirect protocol. Meanwhile, in retina, RPE cells is closely attached to photoreceptor cells; so these expressions in direct protocol may be related to a close interaction between RPs and RPE cells. However, the mechanisms of such intercellular interactions are to be further investigated. In this study, we reported a method that might improve the generation of neural photoreceptors from hESC-derived RPs, and it could be useful for the treatment of retinal diseases by cell replacement. In addition, it is essential to establish a purification system of photoreceptor for future functional analysis and transplantation studies.

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