Original Article

Characterization of The Retinal Progenitor Cells Generated Using Co-Culture Systems

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Received: 27/August/2020, Accepted: 03/December/2020

Abstract

Objective: Degeneration of the photoreceptors due to retinal disorders can affect vision, and even lead to blindness. Recently therapeutic progress in retinal degeneration, using human embryonic stem cells (hESCs), has been facing technical challenges, demanding the development of simple and standardized protocols. In addition to the designing of the protocols, characterization of the obtained cells is highly required for confirming the reliability of the applied methods for future medical applications. Previously, we showed that human stem cells from apical papilla (SCAP) have stromal cell-derived inducing activity (SDIA).

Materials and Methods: In this experimental study, we developed an efficient retinal differentiation protocol, based on the co-culture of confluent hESCs and SCAP in the absence of exogenous molecules, such as activators or inhibitors of molecular signaling pathways. This experimental procedure resulted in the generation of self-forming neural retina (NR)-like structures containing retinal progenitor cells (RPCs) within 4 weeks.

Results: We have focused on the characterization of the derived RPCs, as a crucial step towards further verification of the efficiency of our previously suggested protocol. The differentiated cells expressed eye-field markers, PAX6, RAX, LHX2, and SIX3, and also generated neurospheres by a floating culture system for one week.

Conclusion: We have reported that the treatment of hESC-derived RPCs by the Notch pathway-inhibitor induced the generation of photoreceptor precursor cells (PPCs). The presented method demonstrates the fact that a co-culture of hESCs and SCAP without exogenous molecules provides an efficient approach to produce RPCs for the treatment of retinal disease, and act as an *in vitro* model for the development of human retina.

Keywords: Co-Culture, Human Embryonic Stem Cell, Photoreceptors, Progenitor Cell

Cell Journal(Yakhteh), Vol 24, No 3, March 2022, Pages: 127-132

Citation: **Momenzadeh S, Karamali F, Atefi A, Nasr-Esfahani MH. Characterization of the retinal progenitor cells generated using co-culture systems. Cell J. 2022; 24(3): 127-132. doi: 10.22074/cellj.2022.7764.**

Introduction

Rod and Cone photoreceptors convert electrical signals into electrical messages, initiating the visual transduction cascade, which sends visual information to the brain. Recent advances in cell therapy have opened a window of hope for patients who have visual impairments or blindness. To obtain an expandable source of cells for transplantation, *in vitro* differentiation of human pluripotent stem cells (hPSCs) into retinal cells has been studied (1-4). During eye development, mesenchymal cells play a critical role through the secretion of morphogens and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type fates. The released bioactive factors, some of which are packed as extracellular vesicles, have a different role during eye development. They include the factors triggering signaling pathways affecting cell survival, proliferation, differentiation, anti-apoptotic pathways, and immune modulation (6). This phenomenon, which is called stromal cell-derived inducing activity (SDIA), has been well studied in mesenchymal cells such as PA6 and MS5 (7, 8), as well as dental stem cells (DSCs) (9). Human DSCs, which are originated from cranial neural crest cells, are considered as multipotent cells with rapid proliferation rate and mesenchymal characteristics (10, 11). DSCs are isolated from different regions of the tooth and are named accordingly; such stem cells are stem cells from apical papilla (SCAP) (12), dental pulp stem cells (DPSCs) (13), stem cells from human exfoliated deciduous teeth (SHED) (14), and periodontal ligament stem cells periodontal ligament stem cells (PDLSCs) (15). Secreted proteins from DSCs could affect different biological phenomena (16, 17).

To induce differentiation of hESCs, we selected the co-culture system according to previous *in vivo* studies on cells involved in eye field development (5). In a co-culture system, multiple cell types were cultured directly or indirectly with each other and the cell fates were affected by the secreted factors in each culture. Although, during the direct co-culture system, physical contact is also provided (18).

Our previous study showed that SCAP could induce differentiation of hPSCs to retinal fate via secretion of Wnt pathway inhibitors (9). As an indicator for the accuracy of our previous approach for generating

RPCs, in this experimental research, we have mainly focused on the biological methods which were used in characterization of the differentiated cells. Therefore, the suggested approach in this study may have preclinical and therapeutic applications in the future.

Materials and Methods

Cell culture

In this experimental study, the hESC line RH6 and the SCAPs were maintained as previously described by Baharvand et al. (19) and Karamali et al. (9), respectively. Briefly, RH6 was passaged enzymatically and re-plated on matrigel-coated dishes (1:30, Sigma, St. Lois, MO in DMEM/F12, Gibco Life Technologies, UK) in the presence of 20% knockout serum replacement (KSR, Gibco Life Technologies, UK). SCAPs were kept in DMEM medium (Sigma, St. Lois, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, UK). All experimental cell cultures were done according to the research Ethics standards of the Royan Institute Committee (IR.ACECR.ROYAN.REC.1396.100).

Co-culture of hESCs with SCAP

SCAPs were used as inducing stromal cells to design a co-culture system. At first, SCAPs were inactivated with 10 µM Mitomycin C (Sigma, St. Lois, MO, USA) for 2 hours. then, they were cultured at a density of 5×10^4 /cm^2 in DMEM medium supplemented with 10% FBS. Subsequently, the mechanically isolated RH6, as mentioned above, were cultured on top of the SCAP cell layer at a density of 100 colonies/SCAP (Fig.1). The cells were maintained at 37˚C, 5% CO_2 and refreshed the medium twice a week.

Culture and maintenance of hESC-derived RPCs

Four weeks after the start of the co-culture, tube-like neural structures were isolated mechanically using glass pipettes, dissociated by accutase (Millipore, Temecula, California, USA), and re-plated on matrigel-coated dishes (Sigma, St. Lois, MO, USA). The cells were allowed to expand in DMEM/F12: neurobasal (Gibco Life Technologies, UK) supplemented with 5% KSR (Sigma, St. Lois, MO, USA), basic fibroblast growth factor (bFGF, 20 ng/ml, Royan Biotech, Iran), epidermal growth factor (EGF, 20 ng/ml, Royan Biotech, Iran), L-ascorbic acid (200 µM, Sigma, St. Lois, MO, USA) and Y27632 (10 µM, Sigma, St. Lois, MO). The RPCs from the first three passages were used for further analysis.

Differentiation of RPCs to PPCs

To assess the potential of RPCs to differentiate into photoreceptors, the attached RPCs were washed with PBS-, dissociated into single cells using accutase and plated on matrigel-coated dishes at a density of around 10^5/cm^2. The photoreceptor differentiation medium containing DMEM/F12: neurobasal supplemented by N2 (2%, Gibco Life Technologies, UK), B27 (1%, Gibco Life Technologies, UK), and 5% KSR was applied. One day later, notch inhibitor DAPT (Sigma, St. Lois, MO, USA) was added at the final concentration of 10 µM for two additional weeks (20).

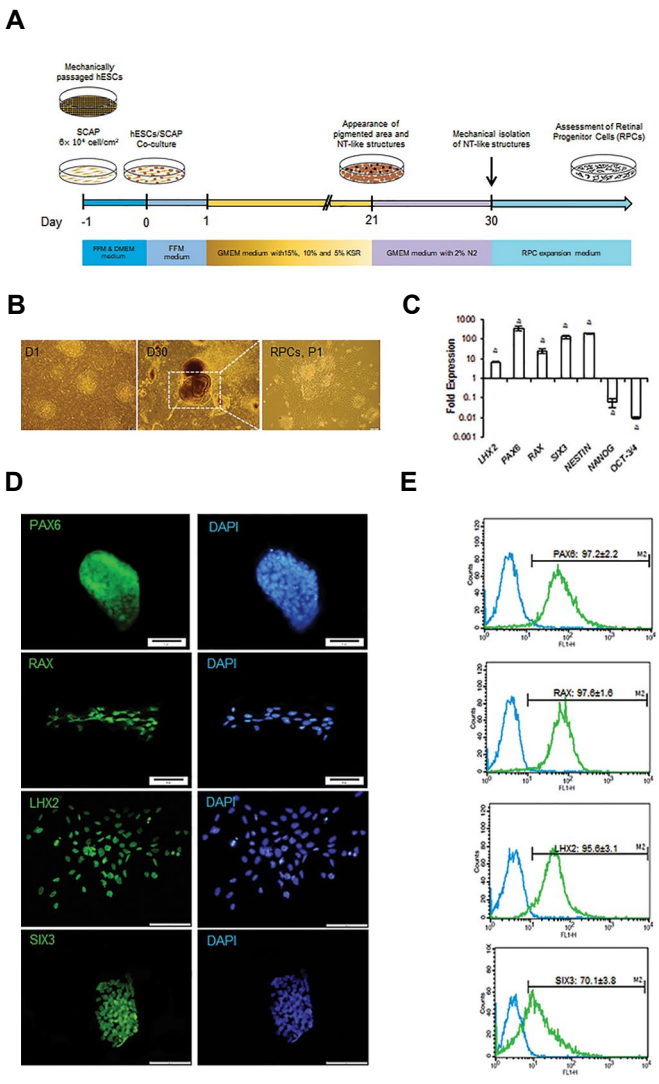

Fig.1: Eye field differentiation of hESCs by SDIA. **A.** Schematic diagram showing stages of the differentiation protocol. **B.** Phase contrast images of differentiated hESCs on SCAP. Left: hESC colonies on SCAP one day after co-culture. Middle: neural-tube like structures on day 21 (white rectangle). Right: isolated and cultured neural tube like structures (passage 1) (scale bars: 100 µm). **C.** RT-qPCR analysis of eye field transcription factors *LHX2, PAX6, RAX, SIX3,* and *NESTIN* as well as pluripotency markers *NANOG* and *OCT4* in RPCs at passage 4 (P4). Data were normalized to hESC at D0, which is present as one-fold and therefore, folds increase or decrease were relative to hESC at D0. Data are presented as mean ± SEM of three independent replicates (one-way ANOVA was used to examine statistical differences, a; P≤0.05. **D.** Immunofluorescence staining of the RPCs at P4 for PAX6, RAX, LHX2, and SIX3 (scale bars: 100 µm). **E.** Flow cytometry analysis of eye field markers LHX2, PAX6, RAX, and SIX3 in RPCs at P4. hESCs; Human embryonic stem cells, SDIA; Stromal cell-derived inducing activity, SCAP; Stem cells from apical papilla, qRT-PCR; Quantitative reverse transcription polymerase chain reaction, and RPCs; Retinal progenitor cells.

Neurosphere generation

To generate neurosphere from hESC-RPC, single

cells were cultured in suspension on 1% agar coated dishes at a density of 10-15 cells/µl using DMEM/F12 containing: neurobasal, N2 (Gibco Life Technologies, UK), B27 (Gibco Life Technologies, UK), bFGF (20 ng/ml), EGF (20 ng/ml) and KSR (5%) was added. One week later, the images of neurospheres provided by inverted microscopy (Olympus, Center Valley, PA, USA) equipped with an Olympus DP70 camera were employed for analyzing their size using the ImageJ software (version 1.6.0, NIH).

Immunofluorescence analysis

For the analysis of the intracellular markers, after fixation of the cells by paraformaldehyde 4%, the cells were permeabilized by 0.4% Triton 100-X for 30 minutes at room temperature. For cytoplasmic markers, 0.2% Triton was used. Next, the fixed and permeabilized cells were incubated with primary antibodies [goat anti-SIX3 (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-PAX6 (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti- RAX (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-LHX2 (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CRX (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-short-wavelength-selective (S)-Opsin (1:50, Abcam, Cambridge, MA, USA), rhodopsin (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), recoverin (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA)]. Subsequently, secondary antibodies [goat anti-mouse IgG-FITC (1:50, Sigma, St. Lois, MO) and goat anti-rabbit IgG-FITC (1:50, Sigma, St. Lois, MO) secondary] were used. The expression of specific markers was then evaluated by a fluorescence microscope (Olympus, Center Valley, PA, USA) equipped with an Olympus DP70 camera. Further characterization of the hESC-RPCs was performed via flow cytometry. The single cells were stained with specific markers mentioned earlier and the results were quantified using a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA) and CellQuest software.

Real-time polymerase chain reaction analysis

To extract total RNA, Trizol reagent was used. Reverse transcription was done using the Takara cDNA synthesis kit (TaKaRa, Japan) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicate. The results were normalized to *GAPDH*, and ΔΔCt method was selected to calculate the relative expression of the experimental genes in comparison to the control groups. The sequences of the primers used are shown in Table 1.

Statistical analysis

All data were collected from three independent experiments and analyzed using GraphPad Prism software (V.7, GraphPad Software, Inc., San Diego, CA) with Student's t test. The data were presented for evaluation as means ± SEM and the statistical significance were achieved when $P<0.05$.

Table 1: Primers used for gene expression analysis by quantitative reverse transcription polymerase chain reaction

Genes	Primer sequence (5´-3´)	Accession no.
OCT3/4	F: TCTATTTGGGAAGGTATTCAGC R: ATTGTTGTCAGCTTCCTCCA	NM_001173531.1
NANOG	F: CAGCTACAAACAGGTGAAGAC R: TGGTGGTAGGAAGAGTAAAGG	NM_024865.2
NESTIN	F: TTCCCTCCGCATCCCGTCAG R: GCCGTCACCTCCATTAGC	NM_006617.1
LHX2	F: TAGCATCTACTGCAAGGAAGAC R: GTGATAAACCAAGTCCCGAG	NM_004789.3
PAX6	F: TTGCTGGAGGATGATGAC R: CTATGCTGATTGGTGATGG	NM_000280.3
RAX	F: CAACTGGCTACTGTCTGTC R: CTTATTCCATCTTTCCCACCT	NM_013435.2
SIX3	F: TCCTCCTCTTCCTTCTCC R: GTTGTTGATAGTTTGCGGTT	NM_005413.3
CRX	F: AAGCCAGGAAGAGTGACAA R: GGAAGAGGAGGACAGATAAGG	NM_000554.4
S-OPSIN	F: GATGAATCCGACACATGCAG R: CTGTTGCAAACAGGCCAATA	NM_001708.2
RHODOPSIN	F: TCATCATGGTCATCGCTTTC R: CATGAAGATGGGACCGAAGT	NM_000539.3
RECOVERIN	F: TAACGGGACCATCAGCAAG R: CCTCGGGAGTGATCATTTTG	NM_002903.2

Results

Generation of RPCs from hESCs and SCAP in a co-culture system

To achieve neural retinal cells from hESCs, we developed an easy and effective co-culture method. At first, hESCs were cultured according to the timeline proposed in Figures 1A and B (left). Three days after co-culture, boundaries of the colonies started to change morphologically and exhibited rosette-like structures between 2 to 3 weeks, and subsequently, neural tubes were appeared (Fig.1A, B).

Expansion and culture of RPCs

To obtain a homogenous population of RPCs, we cultured

the mechanically-isolated tube-like structures on matrigel-coated dishes, providing a suitable condition for RPCs to attach. Previous reports have shown that the presumptive eye field is defined by a group of transcription factors (eye field transcription factors; EFTFs), including *RAX*, *PAX6*, *SIX3,* and *LHX2* (21). After neural tube cell expansion, the expression of EFTFs was assessed at both RNA and protein levels in the attached RPCs (Fig.1C-E). The RT-qPCR our analysis showed a significant reduction in the expression of stemness factors including OCT4 and NANOG and a significant increase in RPC-specific factors (Fig.1C) compared to undifferentiated cells. Immunostaining assessment of eye field markers in hESC-RPC revealed the expression of RPC markers (Fig.1D). Quantitative flow cytometric analysis confirmed that the cells expressed PAX6 (97.2 ± 2.2%), RAX (97.6 ± 1.6%), LHX2 (95.6 ± 3.1%) and SIX3 (70.1 ± 3.8%) (Fig.1E). These data have demonstrated that a large fraction of hESC-derived RPCs were kept in the progenitor state at least for three passages in retinal culture medium. But after the third passage, the morphology of the cells began to change, thus we did not assess these cells after the third passage.

Generation of PPCs

The RPCs were dissociated into single cells, and subsequently, they were cultured on matrigel-coated dishes in the presence of Notch inhibitor DAPT. Three days later, some cells displayed neurite processes. While, these morphological changes did not observe in DMSO group (Fig.2A). CRX, as a cone and rod homeobox gene, has been considered to direct cells for differentiation towards photoreceptors via accelerating chromatin remodeling (22). Therefore, increased expression of CRX as it is shown in Figure 2B and C, committed the RPCs to differentiate into PPCs. Two weeks later, evaluation of differentiation markers showed that DAPT-treated cells expressed S-OPSIN (a mature cone marker) and RHODOPSIN (a rod marker) (Fig.2C). Additionally, we analyzed the expression levels of the genes associated with photoreceptor maturation by qRT-PCR. These results showed a significant increase in the levels of *CRX* (the first PPC marker), *S-OPSIN*, *RHODOPSIN*, and *RECOVERIN* one week after DAPT treatment (Fig.2B).

Generation of neurospheres

Figure 3A illustrates schematic of RPC culture to form neurospheres and its preparation for further analysis. As depicted in Figures 3B and C, RPCs were able to produce neurospheres and increase in size in a time dependent manner during one week. We further showed that these neurospheres express Nestin as a common neural progenitor marker and PCNA as a proliferating cell marker, which confirmed the identity of neurospheres induced by RPCs (Fig.3D).

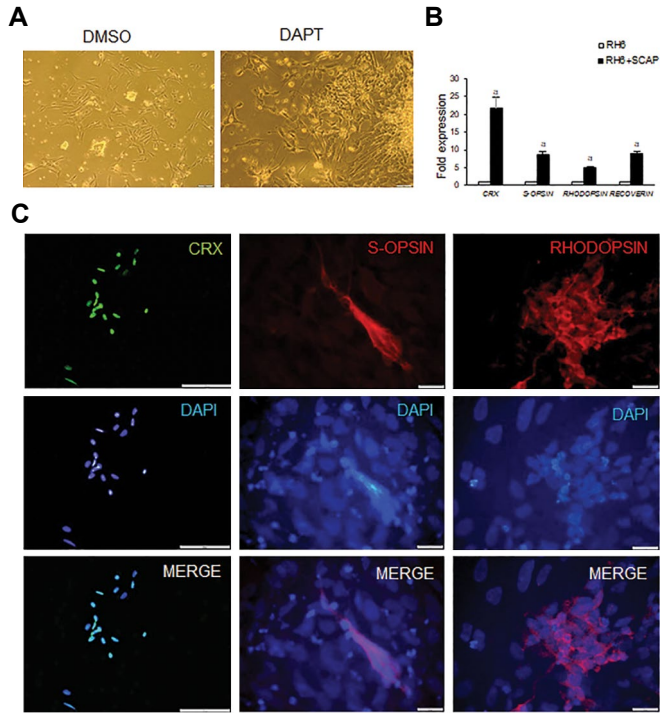

Fig.2: *In vitro* acceleration of mature photoreceptor-like cells generation from human ESC-derived RPCs by Notch inhibition. **A.** Morphological changes of RPCs after treatment of the cells with DAPT and DMSO as the solvent. **B.** qRT-PCR analysis of *CRX*, *S-OPSIN*, *RHODOPSIN* and, *RECOVERIN* markers in RPCs at P4. After calculating the relative expression to *GAPDH*, the data were normalized to cells treated with DMSO at D42 which considered as one-fold change. **C.** Immunofluorescence staining of RPCs at P4 for CRX, S-OPSIN, RHODOPSIN (scale bars: 100 µm). ESC; Embryonic stem cell, RPCs; Retinal progenitor cells, DAPT; (N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl), DMSO; Dimethyl sulfoxide, and qRT-PCR; Quantitative reverse transcription polymerase chain reaction.

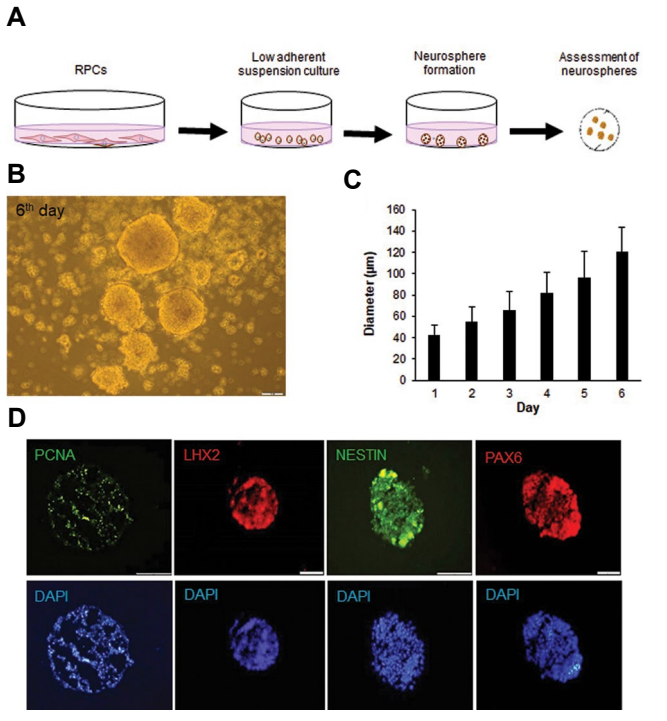

Fig.3: *In vitro* generation of neurospheres from human ESC derived RPCs. **A.** Schematic diagram showing stages of the neurosphere generation protocol. **B.** Sphere formation of RPCs after six days and **C.** Diameter changes over days one to six. **D.** Immunofluorescence analysis of cryo-sectioned RPCs at passage 4 (P4) for LHX2, PAX6, NESTIN, and PCNA (scale bars: 100 µm). ESC; Embryonic stem cell and RPCs; Retinal progenitor cells.

Discussion

In this study, we generated RPCs from hESCs via a co-culture system that induces both differentiation of hESCs into PPCs and formation of neurospheres. Therefore, inconsistent with previous studies (9, 16), it is speculated that SCAP secret various factors that participate in the induction of differentiation of hESCs toward RPCs. These RPCs from our co-culture systems were characterized and the identity of the cells was confirmed using PAX6, RAX, LHX2 and SIX 3 expression at both the RNA and the protein levels. Besides, according to our knowledge, for the first time we have shown that these RPCs, like other neural precursor cells, can produce neurospheres. The proliferative capacity of the cells into neurospheres was proven by the expression of PCNA as a proliferative marker, as well as the increase in the sizes of the neurospheres over time. The differentiated cells also expressed Nestin as a neural progenitor marker in addition to the retinal neural progenitor markers PAX6 and RAX. To our knowledge, there is no report on the derivation of RPC neurospheres from hESCs. It is important to note that the only report on the human retinal neurospheres is by Gamm et al., who obtained neurospheres from prenatal retinal tissue (23).

In order to efficiently differentiate hESCs into RPCS, researchers have introduced different recombinant proteins and/or small molecules to inhibit Wnt and BMP signaling pathways (24-26). In this study, we achieved the same goal by eliminating extrinsic factors. These founding might highlight future clinical applications of the introduced procedure. In this regard, Reichman et al. (27) stated that introducing a simple retinal differentiation method without the formation of embryoid bodies and/or exogenous molecules is widely applicable to future research.

Our results showed a high percentage of cells expressing eye field markers following a decrease in the expression of stem cell markers *OCT4* and *NANOG*. The efficiency of our findings is likely modulated in part by the presence of IGF and DKK (Wnt inhibitors) and Noggin (BMP inhibitor) expressed by SCAP or DPSCs (9, 28), which are commonly added as exogenous factors in most studies of anterior neural differentiation (4, 9, 24-26).

After mechanical isolation of the neural tube like structures, over 90% of the cells expressed specific markers of RPCs including PAX6, RAX, and LHX2, thereby indicating that these isolated neural tubes, in addition to the anterior neural identity, revealed neural retinal specification.

As previous studies have demonstrated, RPCs are committed to form a photoreceptor lineage that due to the increased expression levels of CRX, the cone and rod homeobox transcription factor (20). Nelson et al. (29) were the first researchers who demonstrated that exposure to the secretase inhibitor, DAPT, at an early RPC culture stage, induces differentiation into various retinal cell types. DAPT treatment also increases the number of CRX photoreceptor precursor and ganglion cells. One of the important safety concerns regarding the transplantation of hESC derivatives is their tumorigenicity. In this regard, the use of a notch inhibitor during differentiation of RPCs to PPCs induces RPCs to exit from the cell cycle and thus reduces their ability to form tumors. The hESC-derived RPCs induced by DAPT showed extended cytoplasmic neurite-like processes (30). However, this treatment was sufficient to enhance the expression of the photoreceptor precursor markers such as S-opsin, CRX, recoverin and rhodopsin (31). The RPCs derived in this study are appropriate candidates for disease modeling and photoreceptor cell replacement therapy (5, 27, 32-37).

Conclusion

The simple and efficient protocol described in this study is highly suitable for the production of a high-percentage hESC-derived RPC culture as a potential source for cell replacement studies in preclinical animal models.

Acknowledgments

This work was financial supported by the grants from Royan Institute [no. 95000180] and the Iranian Council of Stem Cell Research and Technology [no. REP141]. This study has been approved by Royan Institute and Jahad University of Isfahan Province. The authors declare that they have no competing financial interests.

Authors' Contributions

S.M., F.K., M.H.N.-E.; Contributed to conception and design. S.M., F.K.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. M.H.N.-E.; Was responsible for overall supervision. S.M.; Drafted the manuscript, which was revised by F.K., and M.H.N.-E. All authors read and approved the final manuscript.

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