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

Sara Momenzadeh, M.Sc.1, 2, Fereshteh Karamali, Ph.D.2, Atfeh Atefi, M.Sc.2, Mohammad Hossein Nasr-Esfahani, Ph.D.2*

1. Higher Education Jahad University of Isfahan Province, Isfahan, Iran
2. Department of Cellular Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

*Corresponding Address: P.O.Box: 8159358686, Department of Cellular Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran
Email: mh.nasr-esfahani@royaninstitute.org

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 progenitor 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

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.
This open-access article has been published under the terms of the Creative Commons Attribution Non-Commercial 3.0 (CC BY-NC 3.0).

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 isolated 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 (PDLCs) (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 vitro 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).

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 $5 \times 10^4$ /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).

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), CRX (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-LHX2 (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 △△Ct 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 (V7, 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. |
|---------|------------------------|---------------|
| OCT3/4F: TCTATTGTGGAGATCGTACGAC<BR>R: ATTGTGTGCAGCTCCCTCA | NM_00117531.1 |
| NANOFG: CAGCTACAAACAGGTGAAGAC<BR>R: TGTTGGTAGGAAGAGTAAAGG | NM_024865.2 |
| NESTINF: TTCCTCCCGCATCCCTCAG<BR>R: GCGTCACCCTCCATAGC | NM_006617.1 |
| LHX2F: TAGCACTTACGTCAAGGAAGAC<BR>R: GTATAAACCAAGTCCCGAG | NM_004789.3 |
| PAX6F: TTTGCGGAGGATGTGATGAC<BR>R: CTAATGCTAGTGTTGAGG | NM_000280.3 |
| RAXF: CAACTGGCTACTGTCTGTC<BR>R: GTGATAAACCAAGTCCCGAG | NM_013435.2 |
| SIX3F: TCCCTCCTCCTCCTCCTC<BR>R: GTTTGTGATGTTGCGGTT | NM_005413.3 |
| CRXF: AAAGCCAAGAAGATGACAA<BR>R: GGAAGAGAGAGACAGAAG | NM_000554.4 |
| S-OPSF: GATGAATCCGACACATGCAG<BR>R: CTGGTGCAACAGGCCGATAT | NM_001708.2 |
| RHODOPSINF: TCACATCGTCTACCTTTCT<BR>R: CATGAAATGCGACCGAGGT | NM_000539.3 |
| RECOVERINF: TAACCGGACCACATCGCAAG<BR>R: CCTGGGAGTGTATTCCGG | 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 \textit{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 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), \textit{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).
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 findings 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.

References

1. Barber AC, Hippert C, Duran Y, West EL, Bainbridge JW, Warre-Cornish K, et al. Repair of the degenerate retina by photoreceptor transplantation. Proc Natl Acad Sci USA. 2013; 110(1): 354-359.
2. Luo J, Baranov P, Patel S, Ouyang H, Quach J, Wu F, et al. Human retinal progenitor cell transplantation preserves vision. J Biol Chem. 2014; 289(10): 6362-6371.
3. Pearson RA, Barber AG, Rizzi M, Hippert C, Xue T, West EL., et al. Restoration of vision after transplantation of photoreceptors. Nature. 2012; 485(7396): 99-103.
4. Lamba DA, Gust J, Reh TA. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell. 2009; 4(1): 73-79.
5. Cvekl A, Tamm ER. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. Bioessays. 2004; 26(4): 374-386.
6. Pittenger MF, Discher DE, Péault BM, Phinney DG, Hare JM, Caplan AI. Mesenchymal stem cell perspective: cell biology to clinical progress. NPJ Regen Med. 2019; 4: 22.
7. Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kawanaka Y, Nanakishi S, et al. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron. 2000; 28(1): 31-40.
8. Schwartz CM, Spivak CE, Baker SC, McDaniel TK, Loring JF, Nguyen C, et al. NTeraza: a model system to study dopaminergic...
24. Ikeda H, Osakada F, Watanabe K, Misuseki K, Haraguchi T, Miyoshi H, et al. Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc Natl Acad Sci USA. 2005; 102(32): 11331-11336.

25. Nakano T, Ando S, Takata N, Kawada M, Muguruma K, Sekiguchi K, et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell. 2012; 10(6): 771-785.

26. Osakada F, Jin ZB, Hirami Y, Ikeda H, Danjyo T, Watanabe K, et al. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J Cell Sci. 2009; 122(Pt 17): 3169-3179.

27. Reichman S, Teray A, Slembruck A, Nanteau C, Oriequ G, Habra BA, et al. From confluent human iPS cells to self-forming neural retina and retinal pigmented epithelium. Proc Natl Acad Sci USA. 2014; 111(23): 8518-8523.

28. Liu G, Ma S, Zhou Y, Lu Y, Jin L, Wang Z, et al. Signaling pathways in dental stem cells during their maintenance and differentiation. In: Sahin F, Dogan A, Demirci S, editors. Dental stem cells. Stem cell biology and regenerative medicine. Springer; 2016: 69-92.

29. Nelson BR, Gumucio B, Hartman BH, Reh TA. Notch activity is downregulated just prior to retinal ganglion cell differentiation. Dev Neurosci. 2006; 28(1-2): 128-141.

30. Crawford TQ, Roelink H. The notch response inhibitor DAPT enhances neuronal differentiation in embryonic stem cell-derived embryoid bodies independently of sonic hedgehog signaling. Dev Dyn. 2007; 236(3): 886-892.

31. Völkner M, Zschätzsch M, Rostovskaya M, Overall RW, Buskamp V, Anastassiadis K, et al. Retinal organoids from pluripotent stem cells efficiently recapitulate retinogenesis. Stem Cell Reports. 2016; 6(4): 525-538.

32. Decembrini S, Koch U, Radtke F, Moulin A, Arsenijevic Y. Derivation of traceable and transplantable photoreceptors from mouse embryonic stem cells. Stem Cell Reports. 2014; 2(6): 835-866.

33. Eiraku M, Sasai Y. Mouse embryonic stem cell culture for generation of three-dimensional retinal and cortical tissues. Nat Protoc. 2012; 7(1): 69-79.

34. La Torre A, Lamba DA, Jayabal A, Reh TA. Production and transplantation of retinal cells from human and mouse embryonic stem cells. Methods Mol Biol. 2012; 884: 229-246.

35. Lamba DA, McCusci A, Hiraoka RK, Wang PR, Russell D, Reh TA. Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. PLoS One. 2010; 5(1): e8763.

36. Mellough CB, Semanog E, Moreno-Gimeno I, Steel DH, Lako M. Efficient stage-specific differentiation of human pluripotent stem cells toward retinal photoreceptor cells. Stem Cells. 2012; 30(4): 673-686.

37. Zhou L, Wang W, Liu Y, Fernandez de Castro J, Ezashi T, Telugu BP, et al. Differentiation of induced pluripotent stem cells of swine into rod photoreceptors and their integration into the retina. Stem Cells. 2011; 29(6): 972-980.