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 and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5). This reciprocal interaction results in the determination of both cell type and interaction with epithelial cells (5).
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/cm² 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% CO₂ 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/cm². 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
The 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), 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.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5'-3')</th>
<th>Accession no.</th>
</tr>
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<tbody>
<tr>
<td>OCT3/4</td>
<td>F: TCTATTGTGGAGGAGGTATTCAGC</td>
<td>NM_001173531.1</td>
</tr>
<tr>
<td></td>
<td>R: ATTGTGTTCAGCTTCTCCA</td>
<td></td>
</tr>
<tr>
<td>NANO6</td>
<td>F: CAGCTACAAACAGGGTGAAGAC</td>
<td>NM_024865.2</td>
</tr>
<tr>
<td></td>
<td>R: TGGTGGTGAAGGAAGTAAAGG</td>
<td></td>
</tr>
<tr>
<td>NESTIN</td>
<td>F: TCTCCTCCCAGTCCCCCTCAC</td>
<td>NM_006617.1</td>
</tr>
<tr>
<td></td>
<td>R: GCCGTACACCTCCATTAGC</td>
<td></td>
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<tr>
<td>LHX2</td>
<td>F: TAGCATCTACTGAAAGGAGAC</td>
<td>NM_004789.3</td>
</tr>
<tr>
<td></td>
<td>R: GTGATAAACACAGTCCCGAG</td>
<td></td>
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<tr>
<td>PAX6</td>
<td>F: TTGCTGGAGGATGATGAC</td>
<td>NM_002803.1</td>
</tr>
<tr>
<td></td>
<td>R: CTATGCTTGGTGTTGATGG</td>
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<tr>
<td>RAX</td>
<td>F: CAACTGGCTACTGCTGTC</td>
<td>NM_013435.2</td>
</tr>
<tr>
<td></td>
<td>R: CTATTCCCATCTTCCCCACT</td>
<td></td>
</tr>
<tr>
<td>SIX3</td>
<td>F: TCCCTGCTTCCCTTCCTCC</td>
<td>NM_005413.3</td>
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<td></td>
<td>R: GTGGTGATAGTTGCGGTT</td>
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<tr>
<td>CRX</td>
<td>F: AAGCCAGGAAGAGTGACAA</td>
<td>NM_000554.4</td>
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<td></td>
<td>R: GGAAAGGAGAGAGAGAA</td>
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<tr>
<td>S-OPSin</td>
<td>F: GATGAATCCGGACACATGCAG</td>
<td>NM_001708.2</td>
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<td></td>
<td>R: CTGGTGCAAAACAGGCCAATA</td>
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<tr>
<td>RHODOPSIN</td>
<td>F: TCATCATGTCATCGCTTTTC</td>
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<td></td>
<td>R: CATGAAAGTGGGACCGAAGT</td>
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<tr>
<td>RECOVERIN</td>
<td>F: TAACCGGACACCTCAGCAAG</td>
<td>NM_002903.2</td>
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<td></td>
<td>R: CTCGGGAGGTGATCATTG</td>
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**Results**

**Generation of RPCs from hESCs and SCAP in a coculture 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 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**, 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.](image)

![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.](image)
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

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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

RPCs by Co-Culture System