Research Paper: Neurosphere-Free Transdifferentiation of Rat Bone Marrow Stromal Stem Cells Into Retinal Cells and Retinal Pigment Epithelium

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ABSTRACT

Introduction: Neurosphere-free transdifferentiation of bone marrow stem cells into Retinal Pigment Epithelium (RPE) and Retinal Cells (RCs) in vitro could offer an exceptional opportunity to study cell replacement in degenerative eye diseases. Thus, a simple and efficient protocol for retinal cells production from transdifferentiation of rat BMSCs in the neurosphere-free state is reported.

Methods: Extracted BMSCs from hooded pigment rats were exposed to a single-step protocol, including neurosphere-free, containing a cocktail medium that induced transdifferentiation into Retinal Pigment Epithelium (RPE) and retinal cells.

Results: The results showed morphological differentiation changes in vitro. Also, the expressed retinal pigment epithelium and retinal cell markers, such as retinal orthodenticle homeobox 2 (23.45%), retinal pigment epithelium protein 65 (91.54%), cellular retinaldehyde-binding protein (91.21%), vascular endothelial growth factor (94.79%), rhodopsin (57.19%), glial fibrillary acidic protein (28.33%), and neurofilament 200 (24.55%).

Conclusion: Overall, these findings showed that a protocol without using basic fibroblast growth factor, epidermal growth factor, and B-27 supplements could generate RPE and retinal cells in vitro.

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Highlights

- We showed simple-step method for generation of RPE and retinal cells.
- Generation of in vitro both RPE and retinal cells have been shown.
- The study have been shown use of serum-free medium to induce neurosphere cells into RPE and retinal cells.

Plain Language Summary

Age-related Macular Degeneration (AMD) is associated with Retinal Pigment Epithelium (RPE) cells impairment. The replacement of these cells is the main goal of cell replacement therapy. Many stem cells have been used for AMD treatment. Bone marrow stromal stem cells are used in AMD therapy because of their safeties, accessibility, and differentiation potential. Generating RPE cells in a reliable and straightforward method is interesting. It seems that producing RPE and retinal cells together and based on developmental order will be the future goal of stem cell differentiation and tissue engineering.

1. Introduction

The functional layer of the eye, or retina, comprises three main layers: Outer Nuclear Layer (ONL), Inner Nuclear Layer (INL), and Ganglionic Layer (GL) (Cayouette, Poggi, & Harris, 2006). The photoreceptors in ONL are primary cells that transform light into electrical stimuli (Ramsden et al., 2013). The retinal Pigment Epithelium (RPE) is a layer in the back of the eye and is essential for the healthy photoreceptor. In many disorders such as Age-related Macular Degeneration (AMD) (Ramsden et al., 2013), Retinitis Pigmentosa (RP), and Stargardt Disease (De Jong, 2005; Kicic et al., 2003; Zhong et al., 2014), RPE and photoreceptor dysfunction cause visual impairment. Today, there is no available therapy for these diseases (Li et al., 2013; Ramsden et al., 2013; Tzameret et al., 2015; Zhong et al., 2014), but cell-replacement therapy is a promising approach in treating those conditions (Li et al., 2013).

There are three main cell sources to generate retinal and RPE cells in vitro:

Embryonic Stem Cells (ESCs) (Kicic et al., 2003), induced pluripotent stem cells (iPSCs) (Zhong et al., 2014), and Adult Stem Cells (ASCs) (Mead et al., 2015; Nicoară et al., 2016). In vivo studies have shown that transplantation of stem cells or retinal cells is also helpful (Jeon & Oh, 2015; Ramsden et al., 2013). Meanwhile, the clinical application of ESCs and iPSCs require more investigations (Kicic et al., 2003; Mead et al., 2015; Nicoară et al., 2016). On the other hand, ASCs are primary sources of cell differentiation and transplantation, because of their characteristics, including plasticity (Redi, 2011), neuroprotection, immune-modulating properties, no ethical issues, and autologous fashion (Barry & Murphy, 2004; Becker, Jayaram, & Limb, 2012; Caplan, 1991; Catacchio et al., 2013; Duan, Xu, Zeng, Wang, & Yin, 2013; Kicic et al., 2003; Levkovitch-Verbin et al., 2010; Mead et al., 2015; Nicoară et al., 2016; Tzameret et al., 2015). Studies in the rat and mice models have revealed that signaling factors such as Fibroblast Growth Factor (FGF), Neurotrophic Factor 3 (NT3), Ciliary Neurotrophic Factor (CNTF), basic Fibroblast Growth Factor (bFGF), and heparin can change the fate of fetal stem cells into photoreceptors, glial cells, and the cells express rhodopsin, calbindin, and calretinin.

In the same way, bFGF, Transforming Growth Factor (TGF), KOM, nicotinamide, Retinoic Acid (RA), and taurine have differentiated human and mouse ESCs/iPSCs into photoreceptors, RPE, retinal progenitors, eye-like structure, and retinal cells. The significant limitations of ESCs are the risk of causing tumorigenesis, chromosomal aberrations, and ethical issues (Becker et al., 2012). ASCs, in contrast, originate from different sources (like mouse and rat's RPE, iris, ciliary body, hematopoietic stem cells, bone marrow stem cells [BMSCs], and umbilical cord stem cells [UCBSCs]) and can generate diverse cells (rod photoreceptors, bipolar, Muller glia, RPE, retinal neurons, retinal ganglionic cells) by adding signaling factors like FGF, heparin, N2-supplement, Stromal Cell-Derived Factor (SDF-1), activin A, taurine, Epidermal Growth Factor (EGF) (Kicic et al., 2003), CNTF, BDNF and NT3 (Wong, Poon, Pang, Lian, & Wong, 2011). Iris pigment epithelium and ciliary body are other cell sources for producing cells in vitro. In our previous work, we transdifferentiated the BMSCs into the RPE sphere and eventually to RPE cells while some RPE spheres expressed retinal progenitors cells (Otx2) (Kadkhodaeian et al., 2019). Here, we hypothesized that whether missing the neurosphere and signaling factors in their formation can generate RPE and retinal cells from BMSCs or not. Therefore, Neurosphere-Free Transdifferentiation of Bone Marrow Stem Cells (NFT-BMSCs) into retinal cells and RPE in a short time may be a new strategy for cell-replacement therapy.

2. Methods

Cell culture and characterization

All experimental procedures were performed according to ARVO guidelines for the use of animals in ophthalmic and vision research, and the study was approved by the Ethics Committee at Semnan University of Medical Sciences, Semnan, Iran, with ethical code: IR.SEMUMS.REC.1396.224 and conformed to the ethical guidelines of the 1975 Helsinki Declaration.

BMSCs were obtained from the bone marrow of pigmented rats (6-8 weeks) according to the outlined method (Arnhold et al., 2006). Briefly, the bone marrow was isolated from femurs and tibias with a 5-mL syringe containing Dulbecco's modified Eagles medium (DMEM). Freshly isolated cells were re-suspended in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich, Germany), 100 U/mL penicillin G, and 100 mg/mL streptomycin sulfate (Sigma-Aldrich, Germany) and then was transferred to 25-cm^2 cell culture flasks. After 24 h, non-adherent cells were taken away, and adherent cells were used as the primary culture. The harvested cells at the third or fourth passages were fixed with 4% paraformaldehyde for 15 minutes, rinsed, and blocked in goat or rabbit serum for one hour at 37 °C. Different primary antibodies (Table 1 and 2) were used for 24 hours at 4°C (negative and positive controls were set up simultaneously). Mouse or rabbit secondary antibodies (conjugated with fluorescein isothiocyanate [FITC]) were performed for one hour at 37°C. Finally, the cells were counterstained with propidium iodide (1:1000) for 5 min at room temperature. The cells were visualized using a fluorescent microscope (Olympus 1x71, Olympus, Tokyo, Japan).

Generation of retinal and RPE like cells

Retinal and RPE-like cells were generated corresponding to the method explained with some modifications (Aruta et al., 2011). Briefly, the harvested BMSCs were collected, centrifuged, and seeded (104 cells/cm^2) into a 6-well plate. After reaching 30% confluences, the medium was changed to a cocktail of synthetic components: DMEM low glucose with 1% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin and 1.4 × 10-8 M selenious acid, 2.8 × 10-8 M hydrocortisone, 3 × 10-7 M linoleic acid, 8.3 × 10-7 M insulin, 6.3 × 10-8 M transferrin, and 2.4 × 10-6 M triiodothyronine. The cells were incubated for 60 days. For tracking the transdifferentiation procedure, the sites of cells were tagged with a pen and observed daily under the microscope. At the end of the procedure, immunocytochemistry was performed.

Isolation of native RPE and retinal Cells

We isolated adult RPE cells from control healthy male pigment rats as previously described (Li et al., 2007). The connective tissue of each globe was removed, rinsed with PBS, and incubated in 2% neutral protease (Cat No. 4,942,078,001, Sigma-Aldrich) in DMEM for 5 min at room temperature. The anterior segment and neurosensory retina were removed, and RPE cells were peeled out mechanically with two forceps. The RPE cells were centrifuged at 1200 rpm for 5 min and triturated 10 times with DMEM containing FBS 10%. The cells were resuspended with DMEM containing FBS 10% and gentamicin (Cat No. G1397, Sigma-Aldrich). The culture was maintained for 3 days, and the medium was changed every 3 days until confluence. For retinal cell culture, the retina was removed thoroughly, without RPE contamination. The cells were dissociated by trituration with a fire-polished pasture pipette. The suspension was diluted in growth medium MEM supplemented with 0.3% glucose, 2 mM taurine, 5% rat serum, and gentamicin. The cell suspension contained single cells with a few small cell clumps. The medium was changed then every 2-3 days.

Immunocytochemistry

We used the following primary antibodies: anti-Otx2 (1:200; Abcam), anti-rhodopsin (1:200; Abcam), anti-neurofilament 200 (NF200) (1:100, Abcam), anti-Glial Fibrillary Acidic Protein (GFAP) (1:100; Abcam), and anti- Retinal Pigment Epithelium Protein 65 (RPE65) (1:200; Abcam), anti-cellular retinaldehyde-binding protein (CRALBP) (1:200; Abcam), anti-Vascular Endothelial Growth Factor (VEGF) (1:200; Abcam). Mouse and rabbit secondary antibodies were 1:1000 Fluorescein Isothiocyanate (FITC) conjugated. For quantification of induction, five captures were randomly taken from different sites of each well for any antibody. Then, the total number of immunoreactive cells for an antibody was divided by the total number of the cells counted with ImageJ (IJ 1.46r, Wayne Rasband, National Institutes of Health, USA, Http://imagej.nih.gov/ij) software. The cells were visualized using a fluorescent microscope (Olympus 1x71, Olympus, Tokyo, Japan).

Statistical analysis

The positive cells to the BMSCs markers were counted (200 cells from 5 randomly selected fields). The immunoreactive cells were divided by the total number of the cells, and the mean number of the differentiated cells was counted in five fields (with a minimum of 100 total spheres). The normality of the data was evaluated with the Kolmogorov-Smirnov test. All the quantitative studies were repeated three times, and the data were analyzed by SPSS ver 22: www.ibm.com/software/analytics/spss/.

3. Results

BMSCs characterization

We used BMSCs cells isolated from the normal hooded rats for transdifferentiation to retinal and RPE cells. After the third passage, immunocytochemistry showed the ratio of BMSCs markers in vitro (Figure 1 A-D). Surface antigens CD44 (91.9%, A), CD90 (95%, B), CD166 (98.5%, C), CD106 (99.31%, D), and extracellular fibronectin antibody (96.9%, A) were carried out, respectively. Negative expression of hematopoietic stem cells (Figure 1-F) and cytoplasmic glial fibrillary acidic protein (Figure 1-G) were observed, too. Quantification of BMSCs markers showed the percentage of positive antibody expression was greater than 95% (Figure 1-H). In addition, the percentage of negative antibodies was zero.

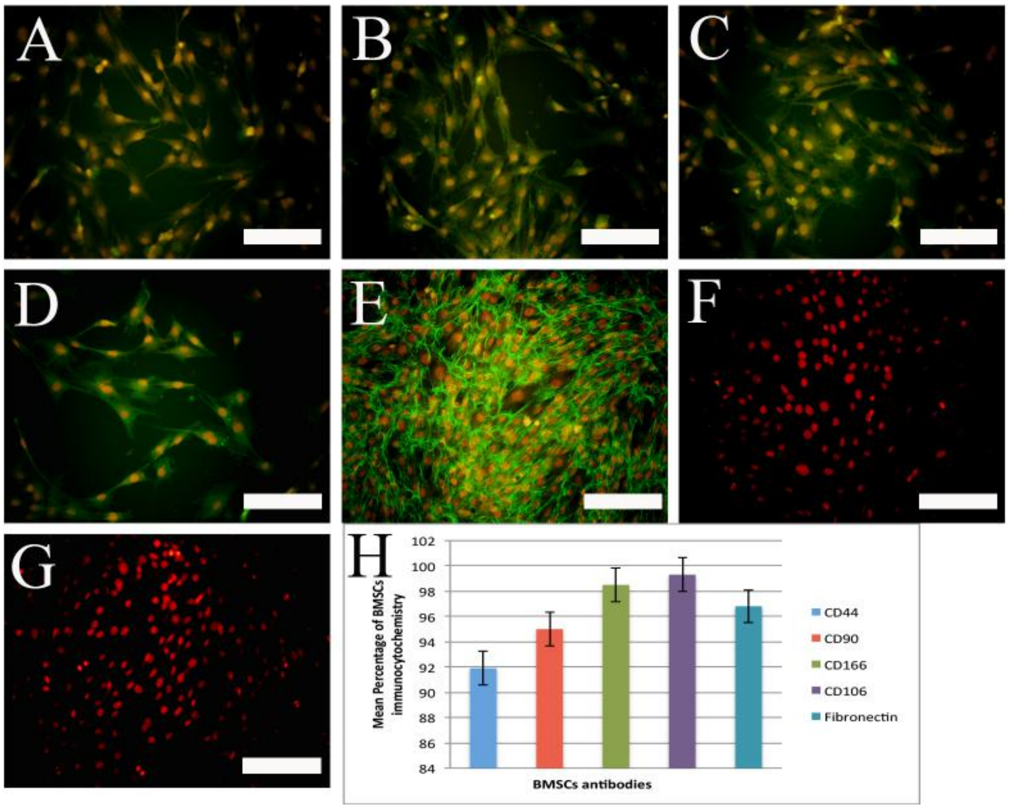

Figure 1. Confirmation of Bone Marrow Stem Cells (BMSCs), expression of mesenchymal markers

A: CD44; B: CD90; C: CD166; D: CD106; E: Fibronectin After the Third Passage; F, G: Showing a Negative Expression of CD34 and Glial Markers.

Scale bar: 200 µm A-G; (H) Histogram Showing the Quantification of Markers.

Data are expressed as Mean±SEM; P<0.05 (Students unpaired t test).

Transdifferentiation of BMSCs into retinal-like Cells

In our study, BMSCs were transdifferentiated into retinal-like cells (Figure 2 A-P). The BMSCs (Figure 2-A) were seeded (104 cells/cm^2) on a 6-well plate and treated with a medium-containing cocktail for 60 days. A pool of cells with various morphologies (round to appended) was observed (Figure 2 B-D). Similar to native adult rat photoreceptors (Figure 2-E), some round cells that had different neuritis all over the cell membrane (Figure 2-D) may be seen in the initial step of differentiation. Following a long time of culturing, thick cell membrane (Figure 2 F-G) was disrupted from one side, and the newest sprout started to grow (Figure 2-G arrowheads), and from the opposite site, the new process has emerged which was smaller than the first one (Figure 2-H).

Some differentiating cells had a morphology like Muller glial cells (Figure 2 I-L) these cells have two long and short appendages, one longer and the other shorter. The terminal end of these cells differed from other cells and are similar to native rat Muller glial cells. Another cell in the culture had a morphology like bipolar cells (Figure 2-N) compared to native bipolar cells (Figure 2-M), and some of them had a morphology like native ganglionic cells (Figure 2 O-P).

We did immunocytochemistry for the expression of various retinal cells markers. Retinal Orthodenticle homeobox 2 (Otx2) is a retinal progenitor protein, likewise the pan-photoreceptor marker. At the endpoint of transdifferentiation, over 23.45% of cells express Otx2 while 57.19% for rhodopsin and 24.55% for neurofilament 200, and 28.33% for Glial Fibrillary Acidic Protein (GFAP) (Figure 3-C). As shown in Figure 3 left panel, several cells expressed the cytoplasmic Otx2 marker. Most of them are round to spindle. Other cells in the culture expressed markers of inner retinal cell types: neurofilament 200 (Figure 3, left panel [D-F], right panel [G, H]), and GFAP (Figure 3 left panel, A-C, right panel, E, F). Also, there were positive cells for photoreceptor markers at the end of induction (Figure 3 left panel [J-L], right panel [C, D]). The BMSCs could be induced into retinal cells and express retinal progenitor markers and differentiated cells, such as ganglionic cells, Muller glial cells, photoreceptor cells, and retinal pigment epithelial cells.

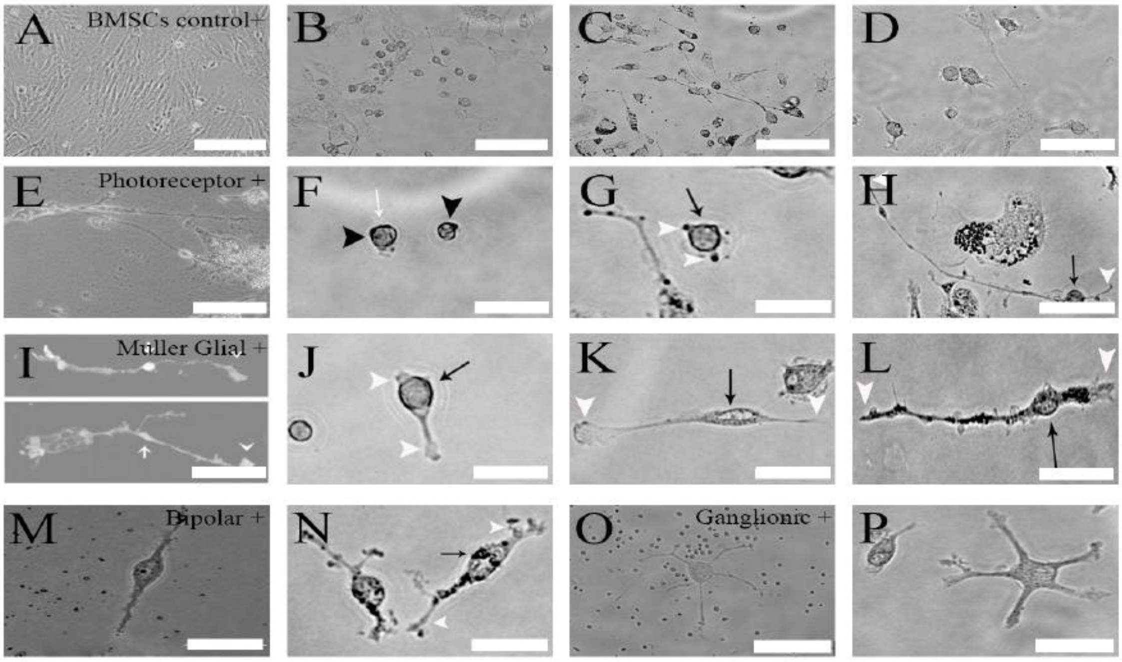

Figure 2. Morphological tracking of the cells in the culture

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A-D: Cells Colony With Different Morphologies Compared to Undifferentiated Bone Marrow Stem Cells (BMSCs); F-H: Cells Showing Differentiation Steps to Final Photoreceptors-Like Morphology; J-L: Muller Glial-Like Cell Differentiation With Two Nerve Sprouts (arrowheads); N: Two Bipolar-Like Cells With A Large Nucleus and Small Processes (arrowheads); P: Ganglionic-Like Cells With Many Processes Similar to Ganglion Cells; E, I, M, O: Showing the Native Rat Photoreceptor, Muller, Bipolar, and Ganglionic Cells

Scale bar: 200 µm for A-E; 100 µm for F-P.

Table 1. Primary antibodies used in immunocytochemistry to characterize bone marrow stem cells

Primary Antibodies	Host	Titer	Cell	Dilution
Anti-fibronectin	Mouse	1:500	Stromal cell	100 µL
Anti-CD90	Mouse	1:500	Undifferentiated cell	100 µL
Anti-CD44	Goat	1:100	Stromal cell	100 µL
Anti-CD166	Mouse	1:200	Stromal cell	100 µL
Anti-CD106	Mouse	1:500	Stromal cell	100 µL
Anti-GFAP	Rabbit	1:400	Neuroglial cell	100 µL
Anti-CD34	Mouse	1:100	Hematopoietic cell	100 µL

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Transdifferentiation of BMSCs into retinal pigment epithelium-like cells

We investigated the ability of BMSCs to transdifferentiate into Retinal Pigment Epithelium (RPE) cells. In a previous paper (Kadkhodaeian et al., 2019), we differentiated the BMSCs into RPE cells in two steps like Aruta et al. (2011), i.e., pre-induction in 2 days and induction in 7-14 days, but this time, we used a one-step modified protocol (2D culture) without formation of neurosphere. Native RPE cells (Figure 4 A-F) were isolated from rats and utilized as a control. Native RPE cells lost their cytoplasmic pigment granules (Figure 4 A-B). However, after a long time, tiny pigment granules were observed

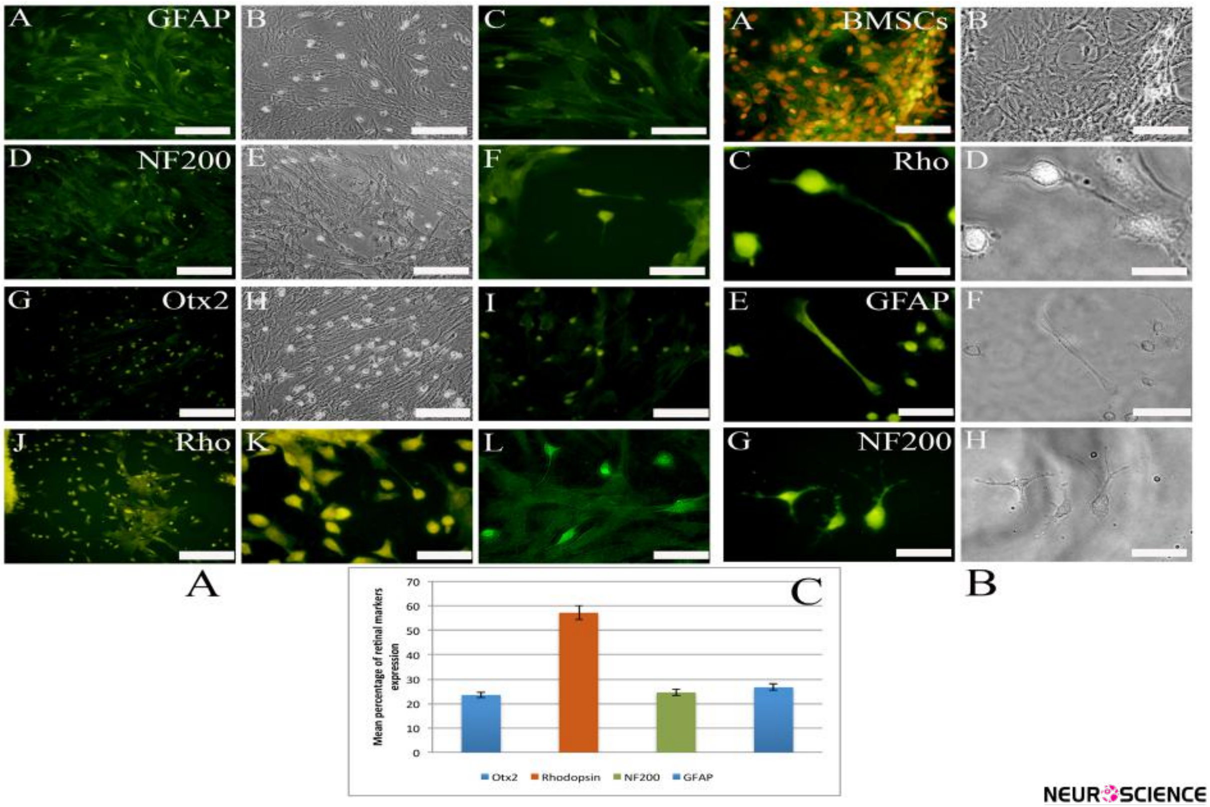

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Figure 3. Immunofluorescence positive retinal cell types derived from neurosphere-free transdifferentiation of bone marrow Stem Cells (NFT-BMSCs); Immunostaining of Orthodenticle Homeobox 2 (Otx2) (Left Panel [G-H]), Rhodopsin (Left Panel [J-L], Right Panel [C, D]), Neurofilament200 (NF200) (Left Panel [D-F], Right Panel [G, H]), Glial Fibrillary Acidic Protein (GFAP) (Left Panel [A-C], Right Panel [E, F]) After 60 Days In Vitro; Right Panel (A, B) Immunostaining of Bone Marrow Stem Cells (BMSCs) as a Control in the Third Passage.

Scale bar: 200 µm for left panel (A, B, D, E, G, H, J) and right panel (A, B); 100 µm for left panel (C, F, I, L) and right panel (C-H); C, Quantification of markers. Data are expressed as Mean±S.E.M; P<0.05 (Students unpaired t-test).

Table 2. Different cell sources and growth factors/signaling molecules used for retinal cell differentiation

Variables	Cell/Tissue Type	Growth Factor/Chemical Modulator	Primary Differentiation
Fetal stem cells	Retinal progenitors (r)	FGF, FGF2, heparin	Photoreceptors
	Neural retinal progenitor cells (r)	FGF2 and NT3 (removal of medium)	Glial cells, neurons expressing rhodopsin, calbindin, calretinin
	Progenitor cells, neural retina (porcine)	CNTF and no bFGF	Photoreceptors
	Human retinal progenitor cells	NT3, FGF2	Retinal cells (cell culture)
	Retinal progenitor cells (m)	EGF	Mature neurons, rhodopsin, or cone opsin
	Photoreceptor precursors (m)	Transplantation of cells into the immature retina	Rod photoreceptors, synaptic connections
	Retinal progenitor cells (h)	Transplantation of cells into 16-18-weeks G.A. B6 mice	Photoreceptors
ESC and iPSC	ESCs (h)	Stepwise treatment with defined factors	Photoreceptors and RPE
	ESC and iPSC (h)	Casein kinase I inhibitor, ALK4 inhibitor, the rho-kinas inhibitor	Retinal progenitors, retinal pigmented epithelial cells, and photoreceptors
	iPSCs (h)	No bFGF	RPE (cell culture)
	ESCs (h)	KOM, nicotinamide, TGF	RPE (cell culture)
	ESCs (m)	bFGF, Dex, cholera toxin	A structure of the lens, neural retina, and pigmented retina (tissue culture) (cell culture)
	ESCs (m)	NMDA-treated eyes	Eye-like structure
	ESCs (h)	bFGF, Xeno-free	RPE (tissue culture)
	ESCs (m)	No LIF, retinoic acid	Neural progenitors, retinal cells
	iPSCs (h)	KOS, zfbFGF, taurine, triiodo	RPE
Adult stem cells	Dissociated cells from the RPE and NR (m)	EGF, FGF2	Rod photoreceptors, bipolar neurons, and Muller glia
	Adult iris, pars plana, and ciliary body progenitor cells	FGF2	Neurons and glia
	Pars plicata and pars plana of the retinal ciliary margin progenitor cells (h)	FGF2, heparin, EGF	Photoreceptors
	Multipotent cells within the IPE of postnatal and adult (r)	bFGF	Neural retinal cells, RPE, photoreceptors (cell culture)
	Adult hippocampus-derived neural progenitor cells (r)	N2, bFGF	Retinal neurons
	Hematopoietic progenitor cells (m)	SDF-1 alpha	RPE
	Hippocampus-derived neural stem cells (r)	N2, bFGF	Neurons and glia
	Adult CD90+MSC (r)	Activin A, taurine, and EGF	Rhodopsin, opsin, recoverin
	UCB-MSCs (h)	TGF-B, CNTF, NT3, BDNF	RGCs (superior colliculus)
	The ciliary body (m)	bFGF, GDNF	Photoreceptor, bipolar cell
	Iris (r)	FGF2	Rod photoreceptor

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H: human; M: Mouse; ESC: Embryonic stem cell; iPSC: Induced Pluripotent Stem Cell; NR: Neural Retina; UCB: Umbilical Cord Blood; MSC: Mesenchymal Stem Cell; RPE: Retinal Pigment Epithelium; RGC: Retinal Ganglionic Cell (Wong et al., 2011); FGF: Fibroblast Growth Factor; FGF2: Fibroblast Growth Factor 2; NT3: Neurotrophic Factor 3; CNTF: Ciliary Neurotrophic Factor; EGF: Epidermal Growth Factor; ALK4 inhibitor: Activin receptor like kinases 4; Dex: Dexametasone; zfbFGF: Xeno-free basic fibroblast growth factor; KOS: SDF1-alpha: Stromal cell-derived factor 1; TGF-B: Transcription growth factor beta; CNTF: Ciliary neurotrophic factor; BDNF: Brain derived neurotrophic factor; GDNF: Glial cell line-derived neurotrophic factor; RGCs: Retinal ganglionic cells.

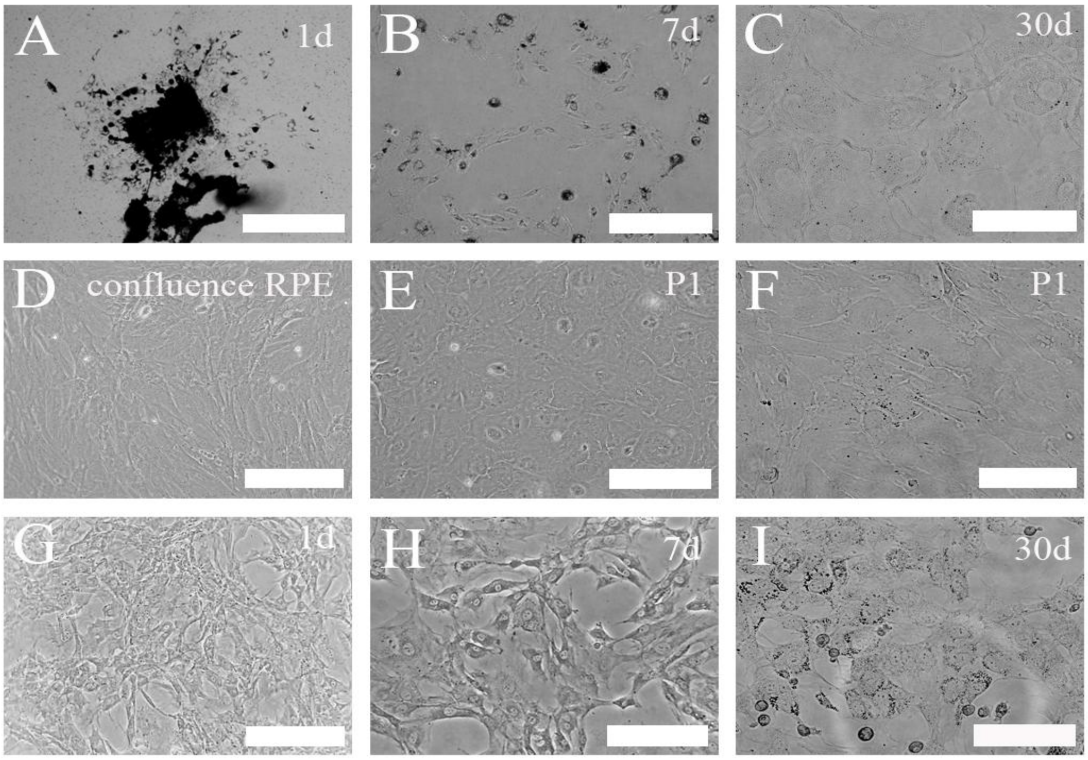

Figure 4. Morphology of rat Bone Marrow Stem Cells (BMSCs) induced into Retinal Pigment Epithelium (RPE)-like cells

A-C: Showing Cultivation and Losing Granules During 30 Days; D-F: Showing Re-Proliferation of Native RPE Cells Containing Small Granules; G-I: Showing Neurosphere-Free Transdifferentiation of Bone Marrow Stem Cells (NFT-BMSCs) Into RPE Without Formation of Neurosphere After 3 Days in a Medium, Having a Cocktail of Chemical Inducers Without Basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF).

Scale bar: 500 µm for A; 200 um for B, D, G, H; 100 µm for C, E, F, I.

in many cells (Figure 4 C-F). Two types of native RPE were seen in the culture: spindle (Figure 4 B-D) and hexagonal (Figure 4 C-E-F). Although NFT-BMSCs did not show a common hexagonal RPE morphology (Figure 4 H-I), some pigment granules were observed after 60 days (Figure 4-I). Immunocytochemistry of these cells showed a firm expression of RPE65 (91.54%, F, G), CRALBP (91.21%, J), and VEGF (94.79%, K).

4. Discussion

In the present study, we showed an in vitro one-step modified NFT-BMSCs into retinal and RPE-like cells. After 60 days, we observed the heavy expression of RPE-specific proteins and diverse cell types, including ganglionic-like, bipolar-like, rod photoreceptor-like, and Muller glial-like cells based on morphology and immunocytochemistry. Using a medium that lacks transcription factors can generate RPE and retinal-like cells without going through the neurosphere step that we did in the previous work (Kadkhodaeian et al., 2019).

RPE originates from the optic vesicle neuroepithelium in the embryonic period, and several eye field transcription factors and signaling molecules like Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), Wingless-Int (Wnt), and Sonic Hedgehog (Shh) are involved in the differentiation of neuroepithelium into RPE and retinal cells (Kadkhodaeian, 2010). Also, we know that BMSCs are related to mesodermal lineage, and their differentiation to the neural and epithelial cells seems difficult. But studies show that mesenchymal stem cells in the adult mammalian will produce both retinal and RPE cells in vitro and are relevant to our results (Catacchio et al., 2013; Chotima, 2007; Hatzistergos et al., 2010; Stern & Temple, 2011; Wong, Poon, Pang, Lian, & Wong, 2011; Xu & Xu, 2011). Studies show that different adult mesenchymal stem cells could produce retinal and RPE cells using signaling factors like FGF, EGF, and bFGF (see supplementary Table for details) (Wong et al., 2011). But we reported here that BMSCs could transdifferentiate into RPE and retinal-like cells without such factors.

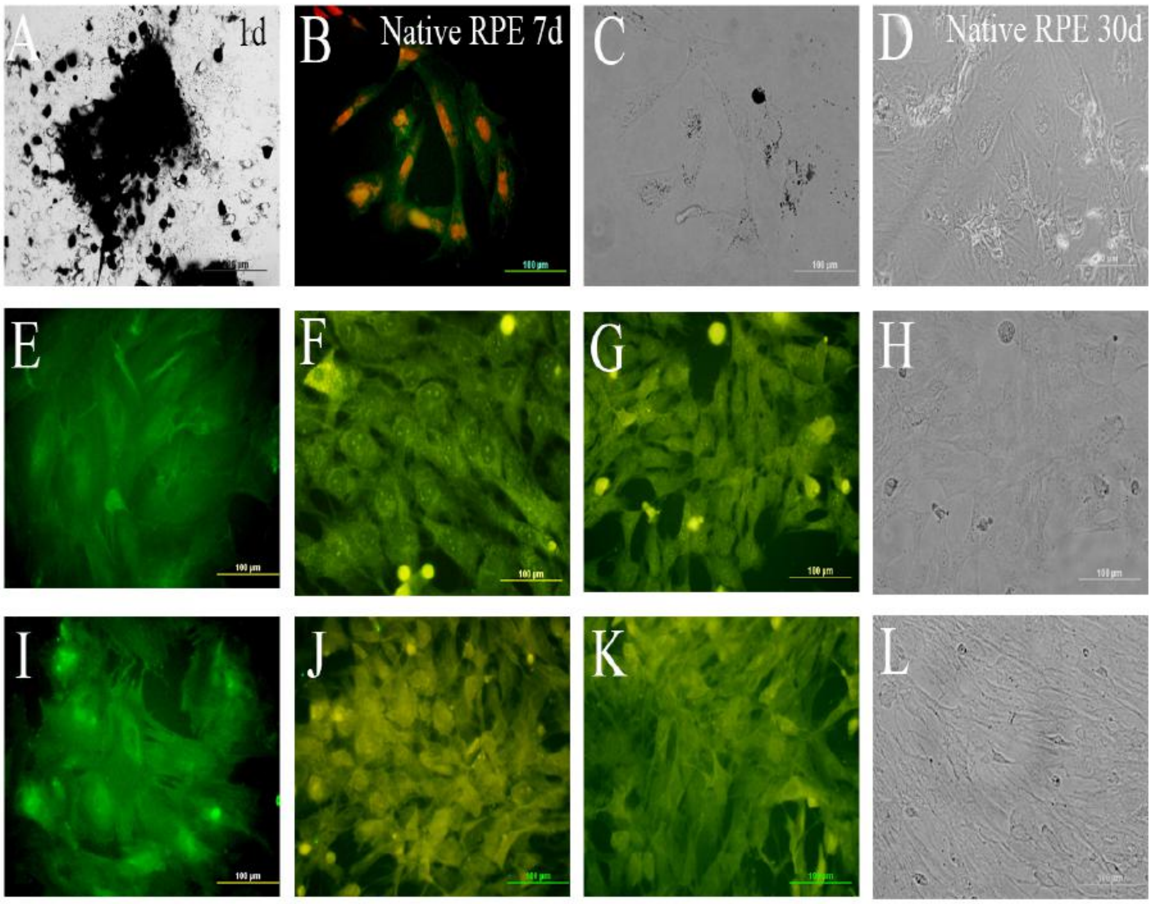

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Figure 5. Immunofluorescence of Neurosphere-Free Transdifferentiation of Bone Marrow Stem Cells (NFT-BMSCs)-Derived Retinal Pigment Epithelium (RPE)-like cells

A-D: The native RPE Isolated and Cultured for 60 days; B: While Immunocytochemistry for Retinal Pigment Epithelium Protein 65 (RPE65) Antibody Carried Out in 7 Days; C: Some Cytoplasmic Granules; and C-D: Epithelial Morphology Observed After 60 Days; Heavy Expression of RPE65, Cellular Retinaldehyde-Binding Protein (CRALBP), and Vascular Endothelial Growth Factor (VEGF) Antibodies After 30 Days (F, G, J, K) Compared to the Control (E, RPE65 and I, CRALBP).

Scale bar: 200 µm for A; 100 µm for B-L. Data are expressed as Mean±SEM; $P<0.05$ (Students unpaired t-test).

The RPE is the first cell that is derived from the neuroepithelium. Following the influence of extraocular mesenchyme and surface ectoderm, retinal cells could be differentiated further. This result corresponds to our study where RPE cells are transdifferentiated firstly, and then retinal-like cells are derived from BMSCs after a long time.

Some studies show that RPE cells could transdifferentiate to retinal cells in vitro (Galy, Neron, Planque, Saule, & Eychene, 2002; Guillemot & Cepko, 1992; Hyer, Mima, & Mikawa, 1998; Mochii, Mazaki, Mizuno, Hayashi, & Eguchi, 1998; Park & Hollenberg, 1989; Pittack, Grunwald, & Reh, 1997) and in our study, because retinal cells appeared after a long time, the results are in line with these studies. However, more investigation in our research is needed to confirm this hypothesis. Similar data were seen in other studies that reported Iris Pigment Epithelium (IPE) isolated from mice or rats has neural stem/progenitor cell properties with expressing transcription factor Pax6 in vitro and in vivo (Asami, Sun, Yamaguchi, & Kosaka, 2007).

Our experimental results revealed that bone marrow stromal stem cells could transdifferentiate into retinal and RPE-like cells in vitro without neurosphere formation. These data confirm our initial hypothesis and agree with other studies (Asami, Sun, Yamaguchi, & Kosaka, 2007; Kicic et al., 2003). In addition, BMSCs differentiated into retinal cells and RPE without cell division. These findings are in contrast to other investigators (Ahmad, Tang, & Pham, 2000; Asami et al., 2007; Tropepe et al., 2000), which noted that intermediate neurosphere forming steps are required for differentiation.

In our study, we proposed that neurosphere formation may be unnecessary to make retinal cells and RPE while others used ciliary body or iris pigmented epithelium that formed neurosphere (Ahmad et al., 2000; Aruta et al., 2011; Asami et al., 2007; Yang, Seiler, Aramant, & Whittemore, 2002) in two steps. In the first step, neurospheres are cultured in the N2 supplement/bFGF, and in the next step, the medium is replaced with a hormone mix. Some studies used a single step with N2 supplement, FGF2, heparin (Ahmad et al., 2000), and EGF/FGF2 (Tropepe et al., 2000). These results are in agreement with our one-step differentiation method. Unlike researchers that used two steps (Aruta et al., 2011) and generated RPE cells after 15 days, we produced both RPE and retinal-like cells. By morphological and immunofluorescence analyses, we showed that this protocol produced RPE and retinal-like cells. Recently, other investigators (Canto-Soler, Flores-Bellver, & Vergara, 2016) reported that adult stem cells from non-neural lineages had been used in various retinal diseases, including AMD, RP, and Stargardt disease as a potential trophic paracrine effect. However, others (Tao, Xu, Yin, & Gibbon, 2010) generated neural and photoreceptor cells by transduction of human BMSCs with a noggin adenoviral vector that expresses rhodopsin, homeobox protein (Chx10), Nestin, and Nrl in vitro. Their results indicated that this technique generated more photoreceptor cells than EGF-induced cells (Tao et al., 2010). The study showed that bFGF leads to the generation of neurospheres (Das et al., 2005) and retinal progenitor-like cells in vitro (Asami et al., 2007). In contrast, our findings indicated that bFGF and neurosphere formation are not required for the generation of RPE and retinal-like cells. Most studies on adult stem cells used FGF or bFGF to generate retinal cells and RPE. However, similar to our study, retinal cells are derived from mouse hematopoietic progenitor cells, adult rat CD90+ MSC, and human umbilical cord blood-MSCs without FGF or bFGF growth factors (Wong et al., 2011).

Expression of Otx2 up to 60 days in our study indicated that differentiated cells might be maintained their capability over a long time. Studies demonstrated that Otx2 is required for the gradual evolution of RPE and the retina. It is a pan-vesicular factor in optic vesicles and is down-regulated in the possible neural retina of the late optic vesicle stage. This factor activates RPE-specific gene expression. In addition, it is essential for optic cup morphogenesis and photoreceptor development (Pébay, 2014). This factor has different roles in the various organisms. The overexpression in mice or rats leads to the excess of photoreceptors, while in frogs causes bipolar cells (Pébay, 2014). The generated RPE-like cells representing RPE-specific markers in our work indicated that Otx2-expressing cells might be transdifferentiated into RPE-like cells.

Noticeably, several neurofilament-positive ganglionic cells were seen in our study. It implies that these cells may be generated in the early phase of retinogenesis. Generation of ganglionic cells firstly mimics the in vivo ordering cells in the retina. In addition, the ganglionic cell is the default phenotype of retinal progenitors (Sernagor, Eglen, Harris, & Wong, 2012).

5. Conclusions

We have derived different retinal and RPE-like cells from BMSCs using a neurosphere-free state. This protocol could enable us to trace the RPE and retinal cells in vitro and transdifferentiation mechanisms. We had limitations in our findings because of the lack of more specific markers for each cell type. Also, failing to examine the functional activity and lacking a marker for cell division and lineage tracing were other limitations. The results of our study may be helpful for other investigators working on BMSCs and RPE/retinal cell biology both in vitro and in vivo.

Ethical Considerations

Compliance with ethical guidelines

The study was approved by the ethical Committee of the Semnan University of Medical Sciences.

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Authors' contributions

Conceptualization, method investigation, preparation, writing-review: Hamid Aboutaleb; Supporting: Hamidreza Sameni and Ali Shahbazi Equal.

Conflict of interest

The authors declared no conflict of interest.

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