Generation of Retinal Pigmented Epithelium-Like Cells from Pigmented Spheres Differentiated from Bone Marrow Stromal Cell-Derived Neurospheres

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Abstract

BACKGROUND: Retinal degeneration causes blindness, and cell replacement is a potential therapy. The purpose of this study is to formation of pigmented neurospheres in a simple medium, low-cost, high-performance manner over a short period of time while expressing markers of RPE cells and the activation of specific genes of the pigment cells. Also, these neurospheres have the ability to produce a monolayer of retinal pigment epithelium-like cells (RPELC) with the ability of photoreceptor outer segment phagocytosis.

METHODS: BMSC were isolated from pigmented hooded male rats and were immunoreactive to BMSC markers, then converted into neurospheres, differentiated into pigmented spheres (PS), and characterized using Retinal pigment epithelium-specific 65 kDa protein (RPE65), Retinaldehyde-binding protein 1 (CRALBP) and orthodenticle homeobox 2 (OTX2) markers by immunocytochemistry, RT-PCR and RT-qPCR. The PS were harvested into RPELC. The functionality of RPELC was evaluated by phagocytosis of fluorescein-labeled photoreceptor outer segment.

RESULTS: The BMSC immunophenotype was confirmed by immunostained for fibronectin, CD90, CD166 and CD44. These cells differentiated into osteogenic and lipogenic cells. The generated neurospheres were immunoreactive to nestin and stemness genes. The PS after 7–14 days were positive for RPE65 (92.76–100%), CRALBP (95.21–100%) and OTX2 (94.88–100%), and after 30 days RT-PCR, qPCR revealed increasing in gene expression. The PS formed a single layer of RPELC after cultivation and phagocyte photoreceptor outer segments.

CONCLUSION: Bone marrow stromal stem cells can differentiate into functional retinal pigmented epithelium cells in a simple, low-cost, high-performance manner over a short period of time. These cells due to expressing the RPELC genes and markers can be used in cell replacement therapy for degenerative diseases including age-related macular degeneration as well as retinitis pigmentosa.

Keywords Bone marrow stromal stem cells · Neurosphere · Retinal pigment epithelium · Transdifferentiation

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1 Introduction

Retinal pigmented epithelium (RPE) is a layer of cells located between the sensory retina and the choroid plexus, and any damage to this layer causes degeneration of the sensory retina [1]. Several factors such as age, smoking, genetic background, gender, hypertension, and arteriosclerosis contribute to their injury, and have been shown to be the most important risk factors for the development of age related macular degeneration (AMD). AMD is the most common cause of the central vision deficits in the elderly. RPE have several functions which are vital for physiology of retina [2].

Degeneration of RPE was reported to cause dry type AMD characterized by poor prognosis [3]. The current treatments try to prevent the development of the disease [4]. However, studies revealed that the drug therapy were non-beneficial, because of many side effects and high cost [5]. Cell therapy is a feasible approach for the treatment of this disease [6]. There are several sources of the cells, which can be used either for direct transplantation or transplantation following induction into RPE [7]. Embryonic stem cells (ESCs) were reported to have several disadvantages including tumor formation, immunological rejection, ethical problems and political concerns [8, 9]. Also, the induced pluripotent stem cells (iPSCs) could develop teratoma [9, 10], and studies have been reports of point mutations and copy number variations and shortening the telomere length that is marker for cell nascence and associated with aging DNA [11, 12]. Finally, there is concern that the autologous iPSCs may be immunogenic [13, 14]. The purpose of this study is to generate pigmented spheres (PS) from BMSC (bone marrow stromal cell)-derived neurospheres and evaluate the photoreceptor outer segment (POS) phagocytic activity of the RPE-like cells (RPELC) harvested from the PS.

2 Materials and methods

2.1 BMSC isolation

All the procedures on the pigmented hooded male rats (Razi institute, Iran, Tehran), including euthanasia, were performed in accordance with the Helsinki Declaration of 1975, as revised in 2013. And with the Association for Vision Research and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and with the institutional guidelines for animal research. The rats were housed under standard conditions with a 12-h light/dark cycle.

The BMSC were obtained from the bone marrow of the pigmented rats (6–8 weeks old) according to the method previously described [15]. Briefly, the bone marrow was extracted from the femurs and the tibias with a 5 mL syringe containing Dulbecco's modified Eagle's medium (DMEM: GIBCO-BRL, Eggenstein, Germany). The freshly isolated cells were resuspended in a DMEM supplemented with 10% fetal bovine serum (FBS: GIBCO-BRL, Eggenstein, Germany), 100 U/ml penicillin G and 100 mg/ml streptomycin sulfate (penicillin/streptomycin: GIBCO-BRL, Eggenstein, Germany). Then they were transferred to T25 flasks (Nunc, Wiesbaden, Germany). After 24 h, the non-adherent cells were removed, while the adherent ones were collected and used as the primary culture, and were used in this study after the 3rd passage.

2.2 BMSC characterization

Characterization of the isolated BMSC was done according to a previous study [15]. The lineage of the cultured BMSC was evaluated for lipogenic and osteogenic differentiation. The BMSC at the third passage were cultured in lipogenic medium containing 50 µg/ml indomethacin, 50 µg/ml ascorbic acid and 100 nM dexamethasone (Sigma-Aldrich, Steinheim, Germany). After 3 weeks, the lipid vacuoles were stained with Oil Red O (Sigma-Aldrich, Steinheim, Germany). The osteogenic differentiation was evaluated by incubating the BMSC at the third passage in osteogenic medium containing 10 mM β-glycerophosphate, 60 µM ascorbic acid and 0.1 µM dexamethasone (Sigma-Aldrich, Steinheim, Germany). The calcium deposits and the osteogenic differentiation were identified by Alizarin Red S staining (Sigma-Aldrich, Steinheim, Germany) after 21 days. The BMSC were characterized using the primary antibodies listed in Table 1. This technique was carried out as mentioned in the immunocytochemistry section.

2.3 Neurosphere formation

Generation of neurospheres from the BMSC was performed as previously described by [15]. Briefly, the harvested BMSC at the third passage were seeded (10^4 cells/cm²) into six wells in neurosphere production medium consisting of DMEM/F12 supplemented with 20 ng/ml of EGF (Sigma-Aldrich, Steinheim, Germany), 20 ng/ml of basic fibroblast growth factor (bFGF; Chemicon, Hofheim, Germany) and 2% B27 (Invitrogen, Karlsruhe, Germany). Characterization of the neurospheres was done by using the primary antibodies listed in Table 2. This technique was carried out as mentioned in the immunocytochemistry section.

Table 1 The primary antibodies in immunocytochemistry used for characterizing the BMSC, neurosphere, and pigmented spheres

Primary antibody	Host	Titer	Cell	Supplier
Anti- fibronectin	Mouse	1:500	BMSC	abcam, Cambridge, UK
Anti-CD90	Mouse	1:500	Undifferentiated cell	abcam, Cambridge, UK
Anti-CD166	Rabbit	1:100	Stromal cell	abcam, Cambridge, UK
Anti-CD44	Rabbit	1:100	Stromal cell	abcam, Cambridge, UK
Anti-CD34	Mouse	1:100	Hematopoietic cell	abcam, Cambridge, UK
Anti-nestin	Mouse	1:50	Neural stem cell	abcam, Cambridge, UK
Anti-GFAP	Rabbit	1:400	Neural stem cell	abcam, Cambridge, UK
Anti- Sox2	Rabbit	1:300	Neurosphere	abcam, Cambridge, UK
Anti-Oct4	Mouse	1:300	Neurosphere	abcam, Cambridge, UK
Anti-Nanog	Mouse	1:300	Neurosphere	abcam, Cambridge, UK
Anti-nestin	Mouse	1:50	Neural stem cell	abcam, Cambridge, UK
Anti-Rpe65	Mouse	1:200	Retinal pigment epithelium cell	abcam, Cambridge, UK
Anti-CRALBP	Mouse	1:200	Retinal pigment epithelium cell	abcam, Cambridge, UK
Anti-OTX2	Mouse	1:200	Retinal progenitor cell	abcam, Cambridge, UK

Table 2 Sequences of the primers (F, forward; R, reverse) used for RT-PCR and RT-qPCR experiments

GENE	Primer sequences (5'–3')	Access number	Segment length (bp)
<i>Rlbp1</i>	F: TCTTGAAGAACAAAGCTGCTAC R: CTGTGTTCTCAACTTCAGCC	<i>XM_011250827.1</i>	330
<i>RPE65</i>	F: TCATGTCACAGGCAGGATTC R: GGGGTCTGGAAGACACA	<i>NM_053562.3</i>	260
<i>Otx2</i>	F: GACTACTTTCACGAGGGCAC R: TTCTGTTGCTGCTGCTGTTG	<i>XM_006251839.2</i>	180
<i>b-actin</i>	F: GCTGTGCTGACTGTCCCC R: CCGGTGATTCAAACAACGAC	<i>XM_006247193.2</i>	210

2.4 Differentiation of the neurospheres into pigmented spheres

The BMSC were incubated for 2 days in neurosphere medium. Then replaced with differentiation medium containing (DMEM low glucose with 1% FBS, penicillin/streptomycin and differentiation mix: 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, 2.4×10^{-6} M putrescine and 10^{-11} M triiodothyronine). Then incubated for 15 days [16]. The pigmented spheres were characterized by immunocytochemistry using the primary antibodies in Table 1.

2.5 Immunocytochemistry

Immunocytochemical study was carried out on the cells or neurospheres, which were fixed in 4% paraformaldehyde for 15 min, washed, permeated with triton x-100 (0.3%), blocked in goat serum for 1 h at 37 °C, labeled with

various primary antibodies (Table 1) for 24 h at 4 °C (the negative controls were immunostained simultaneously), incubated with secondary antibodies conjugated with FITC for 1 h at 37 °C, and counterstained with propidium iodide (20 µg/ml, 5 min; for 5 min at room temperature), respectively. For pigmented spheres, after fixation, incubated in blocking buffer containing 2% horse serum, PBS, 1% BSA and 0.1% triton-X100. The spheres incubated with primary antibodies for 24 h at 4 °C and in next day, incubated in secondary antibody at room temperature for 1 h. The cells were visualized using a fluorescent microscope (Olympus 1 × 71, Olympus, Tokyo, Japan). Estimation of the immunoreactive percentages of the induced cells was done according to a previous study [17], where five images were photographed from randomly selected fields and the total number of immunoreactive cells/spheres for a given antibody was divided by the total number of the cells/spheres.

Fig. 1 Isolation and characterization of bone marrow stromal cells (BMSC). Characterization by immunocytochemical methods using immunolabeling with primary antibodies against different markers and immunostained with secondary antibody and counter stained with propidium iodide. **A** Phase contrast image of the isolated BMSC. **B–H** Immunostained BMSC for fibronectin, CD90, CD166, CD44,

CD34, nestin and GFAP, respectively. Induction of the bone marrow stromal cells (BMSCs) into lipogenic and osteogenic phenotypes (**M** and **N**, respectively). Scale bar = 200 μ m for **A–L**, **N**; 100 μ m for **M**. Percentages of immunoreactive cells to fibronectin, CD90, CD166 and CD44 respectively (**O**)

2.6 RT-PCR and RT-qPCR

The total RNA was extracted from the RPE sphere using Trizol reagent (Invitrogen, Karlsruhe, Germany) according

to the manufacturer's instructions. DNA contaminant in the RNA prep was digested with DNase kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized with Strand cDNA Synthesis Kit (Fermentas, Vilnius,

Fig. 2 Phase contrast microscopy of neurospheres derived from bone marrow stromal cells (BMSCs), and their immunostaining with primary antibody followed by secondary antibody and counterstaining with propidium iodide. **A, B** Morphology of the neurospheres at days 1 and 3. Scale bar = 200 µm. **C–**

E Immunohistochemistry of the neurospheres against expression of pluripotency genes including Oct4, Sox2 and Nanog. Scale bar = 100 µm. **F** Immunostaining of the neurospheres with nestin. Scale bar = 200 µm

Lithuania). In brief, the total RNA (10 µl), 1 µl oligo (dT) primer and nuclease free water (1 µl), 12 µl total volume, were mixed gently and incubated, at 65 °C for 5 min, then with 4 µl 5X Reaction Buffer, 1 µl RiboLock RNase inhibitor, 2 µl mM dNTP mix and 1 µl RevertAid M-MuLV reverse transcriptase, 20 µl total volume, for 60 min at 42 °C. Finally, the reaction was terminated by heating at 70 °C for 5 min. The PCR amplification contained 1 µl of cDNA, 12.5 µl Master mix, 2 µl forward and 2 µl reverse primers and 7.5 µl nuclease free water, 25 µl total volume, and then placed in a thermal cycler with a heated lid (see Table 2). The initial denaturation was done at 94 °C for 30 min. Then, denaturation of the cycling process was performed at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 45 s, for 35 times. The electrophoresis instrument was loaded with 5 µl of the RT-PCR product on 1.5% agarose gel. Briefly, 0.75 mg of agarose powder added to 45 ml TAE buffer. Then 2.5 µl of gel red added to it and insert into the instrument. After 30 min in room temperature, the wells was ready for use and loaded with PCR production, loading dye and ladder dye.

For RT-qPCR, 10 µl Maxima SYBER green/ROX qPCR Master Mix (2X), 1 µl forward primer, 1 µl reverse primer, 1 µl template cDNA and 7 µl nuclease free water, 20 µl total volume, were mixed and placed in a Bio-RAD MJ Mini personal thermal cycler sequence detection

system (Weiterstadt, Germany). The initial denaturation was done at 95 °C for 10 min. Then, denaturation of the cycling process was performed at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, for 40 times. The data were normalized using the β -actin as a house keeping gene and the analysis was done as mentioned in the statistical analysis section. The primers used for the RT-qPCR are listed in Table 2.

2.7 Binding and phagocytosis of the photoreceptor outer segment (POS)

For investigation of phagocytosis activity of retinal pigment epithelium like cells (RPELC) *in vitro*, the rat eye enucleated and after removal of neurosensory retina, the photoreceptor outer segment (POS) was isolated and fluorescein-labeled as described by Ref. [16]. The pigmented spheres derived from BMSC cultivated and the RPELC were incubated with 1×10^7 POS/ml for 24 h. The cells washed three times in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min, counterstained with DAPI (in concentration of 1:5000 for 5 min), and examined using a fluorescence microscope (Olympus 1x71, Olympus, Tokyo, Japan). The total number of the cells stained with DAPI was divided by the number of the cells containing cytoplasmic fluorescence.

Fig. 3 Morphologies of the pigmented spheres derived from neurospheres in differentiation medium using phase contrast microscopy. **A–C** Morphologies of the neurospheres incubated in the differentiation medium for 1 week and 2 weeks in low and high magnifications. Scale bar = 200 μ m for **A**; 2.0 mm for **B**; 100 μ m for **C**). **D** Heavily pigmented spheres attached to the surface 2 weeks after induction. Scale bar = 100 μ m. **E, F** Segregated cells from the pigmented spheres following harvesting of the pigmented spheres. Scale bar = 100 and 200 μ m

2.8 Statistical analysis

The cells that are immunoreactive to the BMSC, neurospheres and pigmented spheres markers were counted (200 cells/spheres from 5 randomly selected fields). The total number of cells/spheres was counted in five fields and the number of positive and negative cells/spheres was calculated with image software (1.45 m; National Institutes of Health, Bethesda, MD, USA) and the mean number of positive cells/spheres was enumerated. Kruskal–Wallis test was used for comparison of the results among the groups, ANOVA was carried out followed by post hoc using Dunnet’s test. The RT-qPCR data were analyzed based on $2^{-\Delta\Delta C}$, and the mean fold changes were calculated for each gene [18]. The normality of the data was evaluated by Kolmogorov–Smirnov test, the mean fold changes were calculated and the P values were determined by Student’s t test. All of the quantitative studies were repeated three

times, the data analyzed by SPSS software release 22: www.ibm.com/software/analytics/spss/.

3 Results

The isolated BMSC morphology is presented in Fig. 1A; the cells have typical spindle shape. They were immunoreactive to antibodies raised against fibronectin, CD90, CD166, and CD44 (Fig. 1D, E, G), but they were immune-negative to CD34, nestin and GFAP (Fig. 1H, J, K). Further lineage characterization with lipogenic and osteogenic differentiation is presented in Fig. 1M and N, respectively. The percentages of immunoreactive cells to CD90, CD166 and CD44 are above 95% (Fig. 1O). The morphology of the generated neurospheres at 1 and 3 days is presented in Fig. 2 (A and B, respectively). They were immunoreactive to the stemness genes (Oct4, Sox2 and

Fig. 4 **A** Immunostaining of the pigmented spheres with different markers at 1 and 2 weeks using primary antibodies followed by secondary antibody followed by counterstaining with propidium iodide. **a, b** Immunostaining of the pigmented spheres with anti-RPE65 primary antibody at 1 and 2 weeks, respectively. **c, d** Immunolabeled against CRALBP; **e, f** against OTX2, respectively (scale bar = 200 μ m for **a, c–g** and **i**; 100 μ m for **b** and **h**). **B** Differentiated neurospheres into pigmented spheres have been cultivated and the RPELC migrated from sphere (**a, b**). Also cytoplasmic pigmentation clearly observed in contrast to Native RPE cells isolated (**c**). Differentiated cells expressed RPE65 marker (**e**) as native RPE cells (**f**). (Scale bar = 100 μ m for **a, b** and **d–f**; 200 μ m for **c**)

nanog: Fig. 2C–E respectively) and nestin (a neural stem cell marker) (Fig. 2F). The morphology of the pigmented spheres after 7 days of the differentiation is presented in Fig. 3A, but no pigment was seen in the spheres, while at 14 days pigmentation of the differentiated spheres could be seen at low and higher resolutions (Fig. 3B, C, respectively). Figure 3D shows heavily pigmented spheres following incubation with the differentiation medium for 2 weeks; Fig. 3E, F show low and high resolution of the segregated cells from the pigmented spheres attached to the adherent surface. Immunostaining of the pigmented spheres was done in order to characterize them with RPE65, CRALBP and OTX2 markers at one (Fig. 4A, D, G), and 2 weeks of induction (Fig. 4B, E, H). The percentages of the immunoreactive spheres to the above

markers showed no statistically significant difference between those incubated for one and 2 weeks ($p < 0.05$) (Fig. 5A). The RT-qPCR shows that the markers' expressions increased in folds after 30 days of induction (Rlbp1, RPE65 and Otx2: Fig. 5B). The results of RT-PCR of the pigmented spheres showed the electrophorogram of Rlbp1, RPE65 and Otx2 genes (Fig. 5C). The segregated cells from the pigmented spheres were immunoreactive to RPE65 marker (Fig. 6A) comparing to the positive native RPE cells (Fig. 6C). The functionality of the cells isolated from the pigmented spheres was evaluated by their phagocytic activity, the FITC labeled POS, which was added to the isolated cells culture medium for 24 h, and the cell phagocytized the FITC-labeled POS (Fig. 6E). The

Fig. 5 The expression of pigmented cell markers in pigmented spheres. **A** The percentage of immunoreactive neurospheres at one (1, 3 and 5) and 2 weeks (2, 4 and 6). **B** Histogram of relative expression of OTX2, RPE65 and CRALBP genes using RT-qPCR of RNA extracted from the pigmented spheres subjected to the differentiation protocol for 30 days. **C** Electrophorogram of RT-PCR of RNA extracted from the pigmented spheres subjected to the differentiation protocol for 30 days to detect OTX2, RPE65 and CRALBP genes, non-template control (negative control) and housekeeping gene (β -actin)

phase contrast image of the same cells in E is shown in Fig. 6F.

4 Discussion

The bone marrow stromal cells were transdifferentiated into neurospheres, which were differentiated into pigmented spheres immunostained with RPE65, CRALBP and OTX2. The RPELC derived from the harvested pigmented spheres showed phagocytic activity of retinal sensory epithelium.

Differentiation of embryonic stem cells into RPE cells was achieved in primates [19] and other laboratory animals [20]. Despite the current progress in stem cell biology and its potential therapeutic use, methods for deriving RPE are still inefficient [21]. One of the promising sources for generating RPE cells is embryonic stem cells; generating them from human ESCs and transplanting them in Stargardt's macular degeneration patients improved the vision of patients. Some scientists were concerned with the validity of the results [22], because of the number of the patients included in the study [23]. Also, transplantation of human ESCs into dry age related macular degeneration and Stargardt's macular degeneration patients gave promising results, except that some of the patients showed adverse reaction to immunosuppression, because of the allogenic

source of the human ESCs [24]. Again, other scientists were concerned with the reported results [25], whereas long-term outcome of human ESC transplantation was reported to elicit immunological reaction [26, 27]. On the other hand, human induced pluripotent cells are considered as another potential source for cell replacement therapy, Leach and Clegg considered this cell type as a reasonable source for generating RPE [21], where RPE could be produced directly from fibroblast [28].

Adult stem cells were considered as a potential source for ophthalmic regenerative medicine [29], for example, retinal epithelial cells were generated from adult retinal stem cells [16]. Also, bone marrow stromal cells cocultured with RPE resulted in their differentiation into retinal cells and photoreceptors [30], likewise, coculturing of BMSC with RPE resulted in the expression of the RPE markers, whereas the BMSC showed a tendency to migrate into the lesion retinal site [31]. The novelty of this study is that we induced BMSC-derived neurosphere differentiation into pigmented sphere immunoreactive to RPE markers, consistently, Aruta et al. reported that adult ciliary body-derived retinal stem cells differentiated into a functional RPE cells [16].

In this study, the transdifferentiation of BMSC into neurospheres is consistent with the other studies; the BMSC-derived neurospheres were immunoreactive to

Fig. 6 Phagocytosis of photoreceptor outer segments by retinal pigmented epithelium-like cells (RPELC) harvested from the pigmented spheres. **A** RPELC immunostained with RPE65 marker. **B** Phase contrast image of **A** with intracellular melanin granules. **C** isolated native RPE cells expressing RPE65 (as positive control for **A**). **D** DAPI counterstaining isolated native RPE cells. **E** RPELC phagocytized FITC-labeled photoreceptor outer segments (POS). **F** Phase contrast image of **C**

pluripotency genes confirming that the cells had neural stem cell property [15]. The differentiation of these aggregated cells, forming the neurosphere, into pigmented spheres immunoreactive to RPE65, CRALBP, OTX2 and nestin indicates that the cells in the pigmented spheres had features consistent with RPE-like cells [32], and it was confirmed that the cells isolated from pigmented spheres were immunoreactive to RPE65. The differentiation of neurosphere into pigmented spheres was achieved using a cocktail of inducers. This process occurred in two steps, the initial is transdifferentiation of BMSC into neurospheres, which were pluripotent in nature and immunoreactive to nestin, and the second step is conversion of the neurospheres into pigmented spheres. Moreover, a two-step generation of RPE cells from iPSC was documented, where the first step led to a neuroectodermal fate and the second

to RPE differentiation [33]. Other investigators successfully transdifferentiated BMSC into RPE cells using a coculturing system [34]. Mesenchymal stem cells were also transdifferentiated into RPE cells by incubation in RPE cell conditioned medium with or without VIP [35]. Genetically manipulated MSC by PAX6 overexpression was reported to produce RPE cells [36], in addition, RP progenitor cells were generated by co-incubation either with peripheral mononuclears or with CD3⁺ CD25[−] lymphocyte [37]. In this study, the induction was based on incubation of the neurospheres with a hormone mix and other small compounds such as putrescine, selenious acid and linoleic acid [16]. Small chemicals were used as inducers for human iPSCs, which were induced into RPE by dimeric epidithiodiketopiperazine combined with nicotinamide [38]. Also, small molecules including nicotinamide with either Activin A or IDE-1 were used for direct induction of human pluripotent stem cells into RPE cells [39].

The expression of OTX2 was observed in pigmented spheres, which remained for 30 days after the commencement of induction. This was reported to play an important role in RPE specification and to persist until the late optic vesicle stage [40]. It is a transcription factor essential for establishing the identity of the RPE [41]. Human pluripotent cells were also reported to express OTX2 during conversion into RPE cells [42], their expression was detected at a low level in the initiation of induction and continued to increase with time, while the expression of RPE65 and CRALBP started at the second week of induction [43]; however, a low level of RPE65 and CRALBP expressions then a subsequent increase in the level of the expression was noticed.

One of the main functions of RPE is phagocytosis of the photoreceptors in order to make their length constant [44], and it was considered the most active phagocytic cell in the body [45]. *In vitro* phagocytic activity of RPE is an important feature in evaluation of their functionality [45, 46]. This show that the RPE-like cells harvested from the pigment spheres are able to phagocytize the sensory retinal membrane, which is consistent with other investigations [47–49].

In this study, we produce the pigmented spheres from bone marrow stromal stem cells-derived neurospheres in a simple medium and method, low cost and high yield. These pigmented neurospheres had express adult retinal pigment epithelium cells markers during the 7–14 days after induction. qPCR and RT-PCR of pigmented spheres showed that these cells activate RPE cells genes. Also, the pigmented spheres after cultivation, spread out the cells and formed RPE like cells monolayer. These cells expressed RPE65 marker and have photoreceptor outer segment phagocytosis activity. Therefore, these cells can have hope for cell therapy in retinal degenerative diseases,

including age related macular degeneration and retinitis pigmentosa.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee (IACUC) in Tarbiat Modares University (IRB No. 1392-5-14-25-254). This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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