·Basic Research·

Differentiation of human bone marrow -derived mesenchymal stem cells into neural-like cells by co-culture with retinal pigmented epithelial cells

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Abstract

• AIM: To detect the differentiation effects of retinal cells or extracts on bone marrow-derived mesenchymal stem cells (BMSC).

• METHODS: Human fetal BMSC were previously labelled by carboxyfluorescein succinimidyl ester (CFSE), and co-cultured with retinal pigment epithelial (RPE) cells which were pre-treated with ultraviolet irradiation at a ratio of 1:1 to induce the differentiation of BMSC for up to 14 days. In some assays, a retinal extract of bovine retinal extract (BRE) was added to detect the potential effects of retinal component on the differentiation of BMSC. In addition, Neuron-specific enolase (NSE), Nestin and Glial fibrillary acidic protein (GFAP) immunostaining were performed to determine the characteristics of BMSC.

• RESULTS: The results indicated that by co-cultured with RPE cells, fetal BMSC were differentiated into neural-like cells expressing special neuronal markers Nestin, GFAP and NSE. And the expression of these markers was obviously increased by BRE.

• CONCLUSION: Retina derived cells and extracts can induce the differentiation of BMSC into neural-like cells.

• KEYWORDS: bone marrow-derived mesenchymal stem cells; retinal pigment epithelial cells; neural-like cells; bovine retinal extract

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INTRODUCTION

According to the origin and potential of differentiation, two types of stem cells can be distinguished: embryonic stem cells (ESC) and somatic stem cells (SSC). SSC are isolated from fetal (after gastrulation) or adult tissues, but classically the differentiation fate of these cells are believed to be limited to the cell types that belong to the tissue from which they originate. However, previously studies have suggested that these tissue-specific stem cells might be able to differentiate into cell types of other lineages[1]. Bone marrow-derived mesenchymal stem cells (BMSC) are one of the major subpopulations of SSC, which is extensively studied with respect to transdifferentiation potential[2]. Recent studies have described that BMSC can be differentiated into neural-like cells *in vitro* under specific induced culture conditions [3,4] or developed spontaneously differentiation under non-induced standard culture conditions [5-7]. Usually the differentiation is induced just by addition of growth factors and/or chemicals in culture medium. The agents include β-mercaptoethanol and dimethylsulfoxide, epidermal growth factor (EGF) and brain-derived neurotrophic factor(BDNF), isobutylmethylxanthine/dibutyryl cAMP, or 5-Aza-C/growth factors. Moreover, a small proportion of BMSC-derived cells differentiated into neuron-like cells expressing NeuN and glial cells expressing Glial fibrillary acidic protein (GFAP) when co-cultured with rat fetal mesencephalic or striatal cells. The retinal pigment epithelium is a hexagonally packed monolayer cell in ocular retina that performs critical functions in the maintenance of the physiology of photoreceptors which is developed from the outer layer of the optic cup.The research of Chiou *et al* [8] shows that co-culture of human BMSC with human retinal pigment epithelium (HRPE) cells facilitates the retina- and photoreceptor-lineage differentiation of adult human BMSC. This system indicates the retinal differentiation potential of BMSC. In this study, we established a co-culture fetal BMSC/RPE system by directly mixing them and showed that under such condition, fetal BMSC was differentiated into neural phenotype-like cells and expressed neuronal markers

such as Nestin, GFAP and neural specific enolase[5]. Besides, our results demonstrated that bovine retinal extract (BRE) could markedly promoted the neural-lineage differentiation of fetal BMSC *in vitro*.

MATERIALS AND METHODS

Materials Human fetal BMSC were cultured in this assay. The use of human tissues in this study was approved by the Shandong Eye Institute Medical Ethics Committee and was in compliance with the Declaration of Helsinki. In order to eliminate unwanted types of cells presented in the marrow aspiration, mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation (lymphoprep, 1073g/L; TBD, China) and BMSC were selected by plastic adhesion. Briefly, a small percentage of cells isolated from the density interface of 1073g/L were seeded in 6 well plates using the medium of L-G DMEM supplemented with 10% FBS (Gibco, USA). After 3 days undisturbed to promote cell attachment, the nonadherent cells were removed by changing the medium. At near-confluence, about two weeks after initial plating, cells were subcultured after trypsin digestion. Fetal BMSC were isolated by Ficoll-Hypaque density gradient centrifugation and the physical property of adherence to plastic culture dishes were confirmed. BMSC cultured in our experiment grew as fibroblastic cells with scant cytoplasm and contain granules around nuclei, which were similar to the cells described in previous reports [8]. Fetal RPE cells were isolated and cultured as described previously[9] and these cells showed typical polygonal shape. Briefly, Sheets of RPE were dissected from the choroids of fetal eye cups and cultured in low-calcium (0.05mmol/L Ca^{2+}) MEM (Sigma, St. Louis, MO; catalog number M-8028) medium containing proposed supplements according to the reports of Hu J and Bok D [9]. Floating RPE cells were collected and passaged with normal-calcium medium (Sigma, St. Louis, MO; catalog number M-2279) containing the same supplements.

BMSC/RPE co –culture Firstly, fetal BMSCs at 3-5 passage were labeled with CFSE (PK-CA705-C375, Probior GmbH) in order to be distinguished from RPE cells. A total of 10^6 to 10^7 cells were washed twice with phosphate buffered saline (PBS) and then incubated with 2.5μmol/L CFSE in PBS for 10 minutes in dark at room temperature. Then cells were washed twice with media containing 100mL/L FBS . Secondly, the second passage of fetal RPE cells were pre-treated under a UV lamp for 15 minutes to abolish its self-proliferation as the method of Chiou *et al* [8] and washed three times with D-Hanks. Then the inactivated cells were digested with 2.5g/L trypsin-0.2g/L EDTA and resuspended in medium containing 100mL/L FBS. Lastly, 2×10^5 of fetal BMSC and RPE cells prepared as above were mix together and plated on gelatin-coated 24-wells plates for up to 14 days. In some assays, 10μg BRE was added in the medium. BRE was prepared by homogenizing 12 fresh bovine retinas per 100mL CMF-BSS and stirring overnight in the dark at 4℃.The mixture was cleared by centrifugation (12 000g for 20 minutes) and the supernatants were collected [9]. The non-induced fetal BMSC were cultured as the control group. In each group four duplicate wells were set up, and the assay was repeated for 3 times independently.

Immunocytochemistry Cells were fixed at -20℃ with cold methanol for 5 minutes. For staining, samples were rehydrated in PBS/2mL/L Triton X-100. Non-specific binding was blocked with 100mL/L normal serum in PBS/2mL/L Triton X-100 for 30 minutes at room temperature. Cells were then incubated overnight at 4℃ with the following primary antibodies diluted in blocking solution, polyclonal rabbit anti-nestin (1:100, Boster, China); polyclonal rabbit anti-NSE (1:100, Boster, China); polyclonal rabbit anti-GFAP (ready to use, Beijing Zhongshan Golden Bridge Biotechnology CO, LTD, China). Rhodamine conjugated Goat anti rabbit IgG (1:100; Beijing Zhongshan Golden Bridge Biotechnology CO, LTD, China) was used as secondary antibody. After washing with PBS for 3 times, samples were counterstained with DAPI (Santa Cruz, USA). Negative controls were performed by omitting primary antibodies.

RESULTS

Differentiation of BMSC In this assay, we detected the differentiation characteristics of BMSC co-cultured with inactivated RPE cells. In order to distinguish BMSC from RPE cells, BMSC were labeled with CFSE before co-culture. After 3 days, we observed that some of cytoplasm of cultured cells retracted toward the nucleus, presenting a more spherical shape and extending processes; the changed cells were CFSE-positive demonstrating the BMSC origin (Figure 1). Furthermore, the co-cultured BMSC aggregated into neurosphere-like body and cells with a spindle-shaped morphology were observed 10 days later (Figure 2). Immunocytochemistry was performed to investigate the expression of neuronal markers, including neural precursor marker (Nestin), neuronal marker (NSE), and astroglial maker (GFAP). These analysis revealed that BMSC expressed the neural makers when co-cultured with RPE. On the contrary, no neural marker was found in untreated fetal BMSC (Figure 3).

Effects of BRE In some assay, 10μg BRE was added to the induction system. The neuron-like morphology of BMSCs treated with BRE in the medium was induced much more markedly at the early induction stage (Figure 1). CFSE is used to fluorescently label live cells and is equally

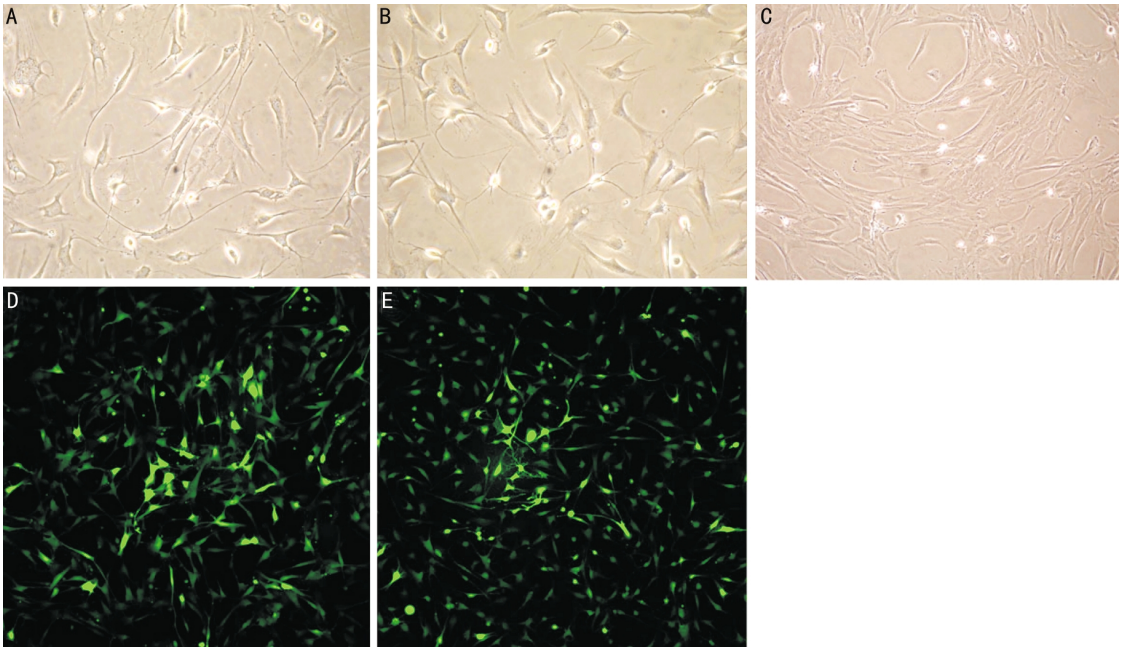

Figure 1 The morphological changes of fetal BMSC after induced for 3 days A and D: BMSC cocultured with RPE cells without addition of BRE; B and E: BMSC co-cultured with RPE cells with addition of BRE;C: untreated BMSC;A, B and C: phase- contrast photomicrograph; D and E: confocal photomicrographs presenting the CFSE-staining cells, ×100

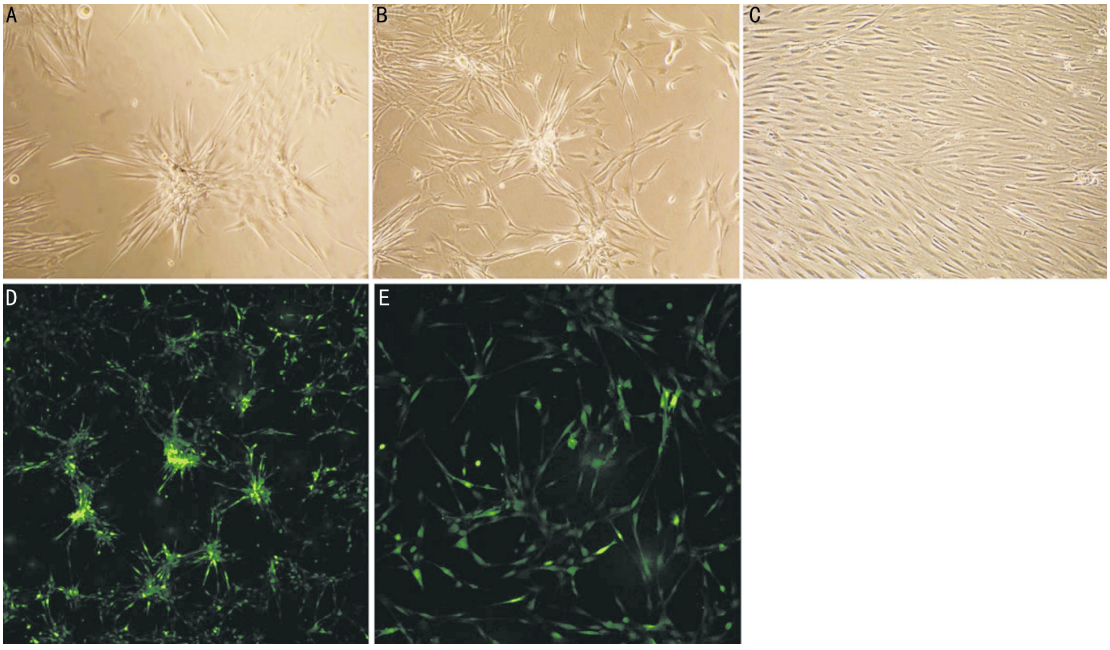

Figure 2 The morphological changes of fetal BMSC after induced for 10 days A and D: BMSC cocultured with RPE cells without addition of BRE; B and E: BMSC co-cultured with RPE cells addition of BRE; C: untreated BMSC; A, B and C: phase- contrast photomicrographs; D and E: confocal photomicrographs presenting the CFSE-staining cells, ×100

partitioned to daughter cells during division and can be used to measure cell proliferation. After 10 days, the CFSE intensity of the cells was markedly decreased by addition of BRE in culture medium(Figure 2), suggesting that BRE may promote the proliferation of the cells. The immunoreactivity for specific neural markers was increased obviously(Figure 3). Especially, the expression of NSE and GFAP was significantly upregulated as compared with the group without BRE.

DISCUSSION

In this study, we detected the effects of RPE cells and BRE on the differentiation of BMSC to determine the potential induction function of retina-derived components on BMSC. Before induction, fetal BMSC had a flat, elongated, spindle-like structure, similar to that of fibroblasts. After induction, the CFSE positive BMSC showed the morphological characteristics of neuronal cells such as long

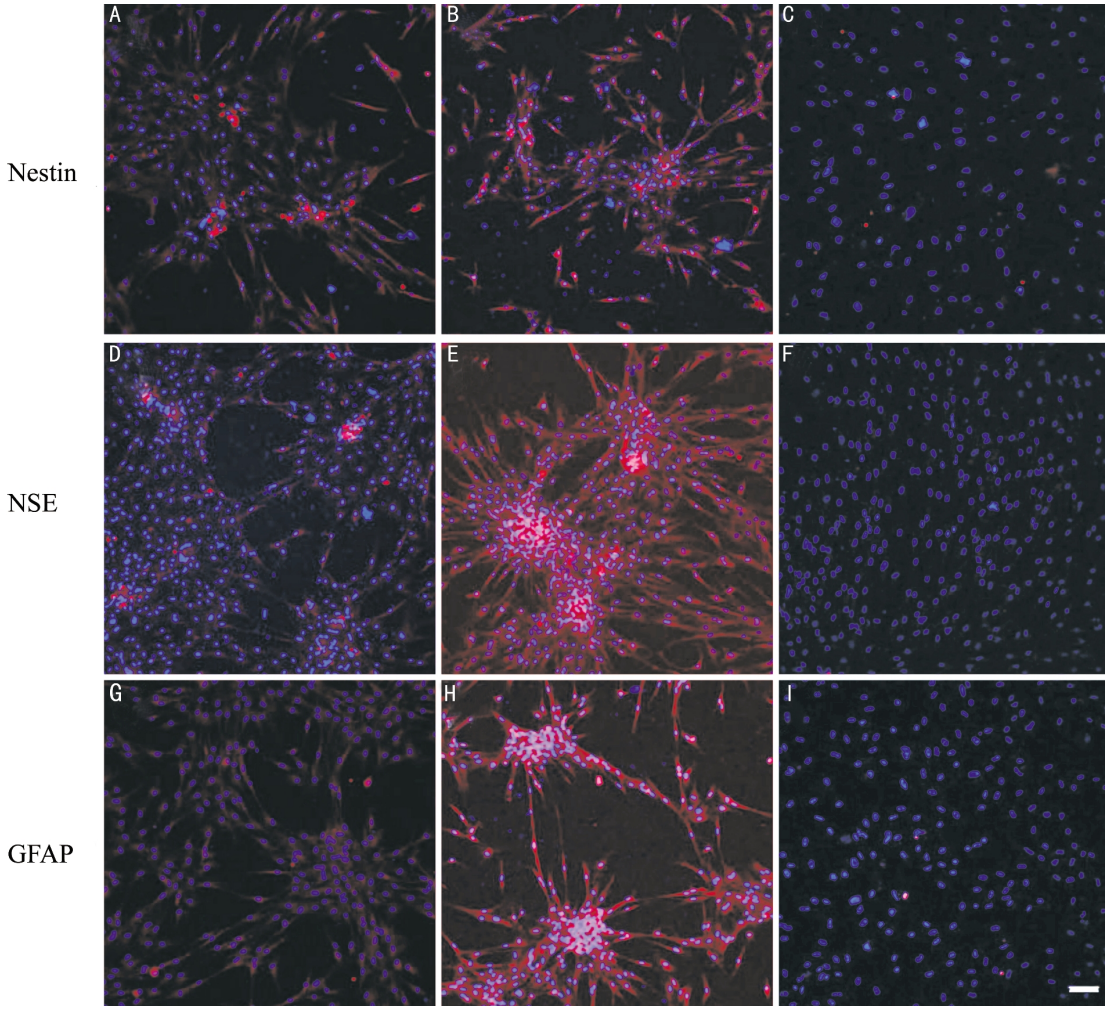

Figure 3 Nestin, NSE and GFAP expression of BMSC upregulated in BRE treated group after induced for 14 days (Bar=100μm)
A, D and G: +RPE cells, -BRE; B, E and H: +RPE cells +BRE; C, F and I: untreated.

multi-polar extensions and branching ends after 3 days; and then aggregated into spheroid 10 days later. And the neural lineage differentiation of BMSC was demonstrated by the expression of some specific neural markers detected by immunocytochemistry assay. In addition, BRE could promote the neural-like cells differentiation of BMSC in this co-culture system. We didn't get the similar results of differentiation BMSC into retinal lineage cells as Chiou *et al* by co-culture of BMSC with RPE cells in this experiment. This may be attributed to the existence of some differences between our induction methods. Chiou *et al* [8] first induced the BMSC to a spheroid body using neurogenic selection medium for 2 weeks, and then followed a further differentiation in the medium containing RPE cells as a feeder layer for another 2-3 weeks. However, we directly combined the undifferentiated BMSC and RPE cells together and induced differentiation for 2 weeks. Considering the neural ectoderm developing origin of RPE, RPE cells may play a role in inducing BMSC into neural-lineage cells.

Previous reports seemed that the protein of BRE has the function of maintenance the proliferation and differentiation of RPE cells [9]. In order to detect whether BRE could have some function in BMSC differentiation, 10μg protein/mL of BRE was used. The results revealed that BRE could promote a higher expression of nestin, an intermediate filament protein that is predominantly expressed during neural development to some degree [10]. Especially, BRE also enhanced the expression of NSE, a unique form of the glycolytic enzyme enolase found in neurons and in virtually all of the neuroendocrine, paraneuronal cell types, and GFAP, a glial protein that is found in glial cells such as astrocytes. These results indicated that BRE could promote the neural differentiation of BMSC. Given that the retina is developed from neural ectoderm and considering that BRE contains prominent portion of intracellular proteins which do not release and contact cell during retinal development; our results suggested that some of the retinal component must have functions in inducing the differentiation of neural lineage cells. Interesting, these neural-like cells differentiated from BMSC in this assay are both NSE and GFAP positive,

we suppose that these neural-like cells are neuron/astrocyte precursor cells which may further differentiated into neuron or astrocyte cells. In conclusion, BMSC from fetal bone marrow were differentiated into neural-like cells expressing the special markers of neural cells by co-culture with RPE cells, and the component from retinal may promote BMSC changing into neural lineage cells.

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