• Basic Research •

Differentiation of human olfactory mucosa mesenchymal stem cells into photoreceptor cells *in vitro*

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Abstract

• AIM: To investigate whether the human olfactory mucosa mesenchymal stem cells (OM-MSCs) can differentiate into photoreceptor cells *in vitro*.

• METHODS: Through the olfactory mucosa adherent method, olfactory mucosa was isolated, cultured and identified *in vitro* among mesenchymal stem cells. The cell surface markers were analyzed by flow cytometry, induced to differentiate into retinal photoreceptor cells *in vitro*, and the expression of rhodopsin was observed and identified by Immunofluorescence and Western blot methods.

• RESULTS: OM-MSCs from human were spindle cell-based, and showing radial colony arrangement. OM-MSCs were negative for CD34, CD45 and CD105, but positive for CD73 and CD90. Following induction, a strong positive reaction was produced by photoreceptor specific marker rhodopsin in the cells.

• CONSLUSION: This novel finding demonstrates that OM-MSCs can be cultured and expanded *in vitro*. They possess biological characteristics of mesenchymal stem cells, and have the ability to be induced into retinal cells.

• KEYWORDS: human olfactory mucosa; mesenchymal stem cells; differentiation; retinal cells

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INTRODUCTION

Mesenchymal stem cells (MSCs) are one type of pluripotent stem cells with the potential of multiple differentiation and capacity of trans-germ layer differentiation. *In vitro*, MSCs have a strong capacity of clonogenicity and proliferation[1-2]. Current research fields on the role of MSCs in human diseases include organ transplantation, auto-immune disorder, vascular diseases, degenerative diseases, injured neuron repair and joint repair[3]. MSCs primarily originate from the human bone marrow. It has been reported that bone mesenchymal stem cells (BMSCs) were capable of being induced to differentiate into retinal neurons[4-5]. However, the number of BMSCs and its capacity were susceptible to multiple variables such as age, gender and history of radiation therapy[6].

Considering the invasiveness of bone marrow aspiration as well, the application of BMSCs in the clinic setting remains limited. Olfactory mucosa mesenchymal stem cells (OM-MSCs) behave similarly to BMSCs in terms of pluripotency, which can be induced to differentiate into osteoblast, adipocyte, neuron and smooth muscle cells *in vitro*[7]. In addition, compared to BMSCs, OM-MSCs bear advantages such as a superior capacity of proliferation, feasibility of autologous transplantation, convenient isolation, zero immune rejection, high safety, no gene mutation after infinite passaging *in vitro* and free ethic issues[8-11]. All these characteristics allow OM-MSCs to be an idea seeding cells for transplant therapy. Our objectives are isolating MSCs from olfactory mucosa, culturing of OM-MSCs *in vitro*, investigating cellular phenotype and cell cycle compared to BMSCs and inducing OM-MSCs differentiation into cells that express photo-receptors. These works aim to exploit OM-MSCs into developing retinal neuron.

SUBJECTS AND METHODS

Ethics Statement Olfactory mucosa was taken from healthy volunteers at the Second Affiliated Hospital of Hunan Normal University. This study was approved by the Institutional Review Board of the Second Affiliated Hospital of Hunan Normal University, and followed the tenets of the Declaration of Helsinki. All volunteers gave their written informed consent. This study was registered by the Chinese Clinical Trial Registry under registration number ChiCTR-OOC-15007627.

Isolation and Culture of Cells Three days before isolation of olfactory mucosa, 20 patients signed their volunteering paperwork, then were examined to clear any olfactory mucosa diseases such as olfactory mucosa injury or atrophic rhinitis, then their nasal hair were trimmed off followed by treatment with Chloramphenicol. After nasal anesthesia, mucosa was taken from the inner side of the bottom of the middle turbinate. Olfactory mucosa was rinsed in DMEM/F12 (v/v=1:1) (GIBCO, USA) mixed medium (penicillin 200 U/mL and streptomycin 200 U/mL) for three times to remove blood and then placed in 10% fetal bovine serum (FBS; HyClone, Australia) in DMEM/F12 medium (containing 100 U/mL penicillin and 100 U/mL streptomycin). Then they were minced with ophthalmic scissors into pieces of 1 mm^3, which were then subjected to centrifugation in order to remove supernatant. Pellets were seeded in a Corning culture flask. When cell confluence reached 100%, the cells were trypsinized and passaged. The fifth passage cells were then centrifuged and seeded in 6-well plate for the analysis of the cell phenotype and induced differentiation studies *in vitro*.

Cell Phenotype Analysis Cells at the 5^{th} passage were digested by 0.125% (w/w) trypsin, then rinsed by phosphate buffer saline (PBS) three times, then split into aliquots at 0.1 mL volume. Negative control tubes were added by anti-human IgG-fluorescein isothiocyanate (FITC) and IgG-PE. Rest tubes were added by mouse anti-human CD34-ECD, CD45-PC7, CD73-FITC, CD90-FITC (Sigma, USA) respectively. After 30min incubation at room temperature, the samples were subjected to flow cytometry to collect and analyze data on the relative abundance of distinct cell surface antigens.

Cell Cycle Examination Cells later than the 4^{th} passage were digested by trypsin for 1-5min, then rinsed by PBS three times, then 1 mL pre-chilled 70% (v/v) ethanol. Single cell suspension solution was made after pipetting up and down and then fixed in 4 degree celsius for 24h. Thereafter, cell pellets were obtained after centrifugation and then resuspended in 1 mL Propidium Iodide (10 lg/mL RNAase A were added; concentration of Propidium Iodide was 50 lg/mL). After 20min incubation in 4 degree celsius, flow cytometry was utilized to analyze cell cycle.

Cell Growth Curve Measurement Cells at the 3^{rd} passage were utilized to make a single cell suspension solution. After centrifugation and removal of the supernatant single cell pellets were collected and re-suspend into fresh culture medium. The cell density was adjusted to 5000/mL and then seeded in a 6-well plate for culturing in an incubator. 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide was employed to measure cell growth for 7 consecutive days. The blank medium control was cell free and each experimental group had 6 wells for calculating the mean, which was then used to plot the growth curve.

Induced Differentiation of Olfactory Mucosa Mesenchymal Stem Cells *in Vitro* OM-MSCs at the 4^{th} passage were seeded at a density of 2.0×10^6 /mL into a 6-well plate where sterile poly-lysine treated coverslips were placed. When cells were attached and grew to 80% confluence, epidermal growth factor (EGF) (0.1 mg/L) (GIBCO, USA), Taurine (50 μmol/L) and Retinoic acid (0.5 μmol/L) were added into the complete medium of the induced group. Medium were changed every 3-4d. Using wells without culture medium as negative control, cell morphology and differentiation status were observed by microscopy in a real time manner. At the 10^{th} day of photoreceptor induction, immunofluorescence and Western blot were utilized to investigate the differentiation outcome.

Immunofluorescence Staining Examination Induced cells growing on the glass slide were used for immunofluorescence staining. The steps were listed below. Cells growing on the glass slide were rinsed three times with PBS solution, then fixed in 95% ethanol for 20min, then rinsed with PBS solution another three times. Mouse monoclonal anti-human rhodopsin antibody (1:50) were added onto the glass slides in a volume of 250 μL. After incubation in 4 degree celsius overnight, the glass slides were rinsed by PBS for three times and then FITC-labeled goat anti-mouse secondary antibody was added onto the slides in a volume of 250 μL. After incubation in the dark for 30min, glass slides were rinsed with PBS solution for three times and then DAPI staining solution was added for nuclei staining. After 15min incubation, the glass slides were rinsed with PBS solution for three times and then fluorescence quenching solution was added to mount the slides. The slides were stored in the dark and then observed under the fluorescence microscope and pictures of the slides were taken as well.

Western Blot Examination In brief, lysates from whole cell extracts or membrane pellets containing 50 μg proteins were subjected to gel electrophoresis. The proteins were then transferred to polyvinylidene fluoride membranes. The blots were blocked in 4% bovine serum albumin in Tris-buffered saline with Tween solution for 30min at room temperature and then incubated at 4℃ overnight with the primary antibody, monoclonal anti-rhodopsin (1:50). After incubation with secondary antibodies at room temperature for 1h, the blot was visualized using Chemi Doc XRS Imaging System.

RESULTS

Observation of Cell Morphology After adherent tissue culture, MSCs isolated from adult olfactory mucosa displayed spindle-like, polygonal shape in primary culture. After pass aging, growth speed was accelerated substantially, which typically can be split every two to three days. Passage number could be as high as 10 times. Purity of cells was enhanced after pass aging, which exhibited homogeneous morphology growing in parallel or spiral form (Figure 1).

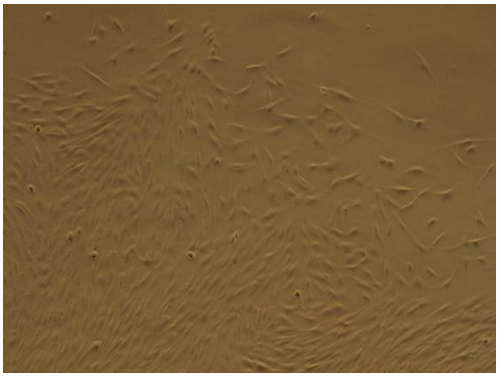

Figure 1 OM-MSCs climbed out of the visible cells *in vitro* for 3d (100×) which displayed spindle-like, polygonal shape and exhibited homogeneous morphology growing in parallel or spiral form.

Examination of Cell Surface Markers After the analysis of flow cytometry, OM-MSCs did express adherent molecule and basal cell surface markers CD73, CD90 and CD105 (Figure 2A, 2B, 2C). They did not express hematopoietic stem cell makers CD34 and CD45 (Figure 2D, 2E).

Measurement of Cell Cycle OM-MSCs cell cycle analysis demonstrated that 82.47% of cells were in G_0/G_1 phase, 6.68% in S phase, 10.85% in G_2 phase (Figure 3). This suggested that majority of OM-MSCs were in quiescence but kept their capacities of self-renewal and proliferation (Figure 4), which are typical characteristics of stem cells.

Measurement of Cell Growth Curve Cells were in quiescence in the first and second day, exhibiting no significant change of cell number. After the third day, the cell number was gradually increased and the cells entered the log phase. Cell proliferation was robust until day 6-7. Thereafter, cell growth reached a plateau state and cell number was barely increased (Figure 4). The growth curve of OM-MSCs showed an "S" shape and was in accordance with normal cell growth. The doubling time of OM-MSCs was 2-3d. The shorter doubling time suggested a highly active growth of OM-MSCs *in vitro* and a strong capability of proliferation.

Induction of Retinal Photoreceptor in Olfactory Mucosa Mesenchymal Stem Cells Induction culturing led to a substantial change of cell morphology of olfactory mucosa-derived cells. Terminus appeared to be analogical to the neuron synapse. Some cells displayed a neuron morphology as aptly illustrated by a spherical shape, extended protrusion, the primary and secondary branches for some cells presented a network structure (Figure 5).

Validation by Western Blot Western blot verified that cellular rhodopsin protein expression was significantly up-regulated upon 2wk induction compared to the control group (Figure 6).

Immunofluorescence Staining Immunofluorescence staining showed the presence of rhodopsin expression as a positive marker for induced photoreceptor-expressing cells whereas the control group showed an absence of such marker (Figure 7).

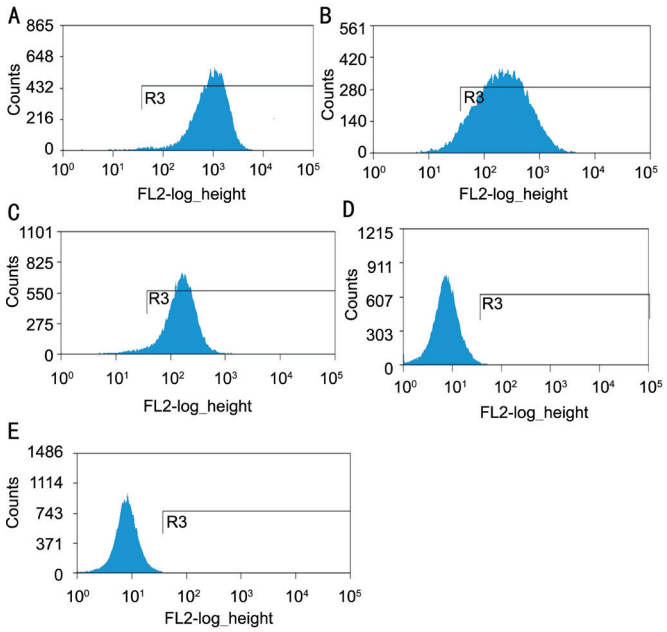

Figure 2 Surface markers expression of OM-MSCs A: CD73; B: CD90; C: CD105; D: CD34; E: CD45.

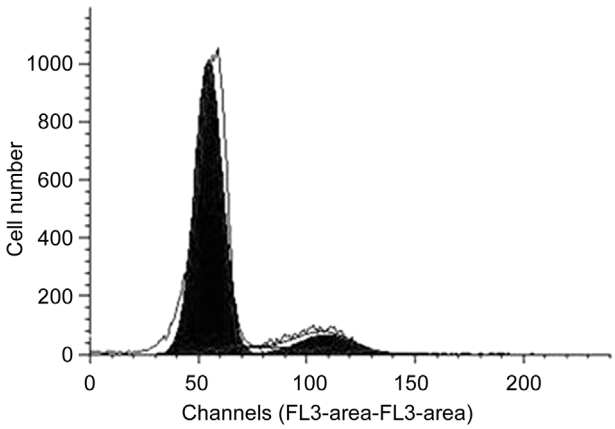

Figure 3 Cell cycle of OM-MSCs.

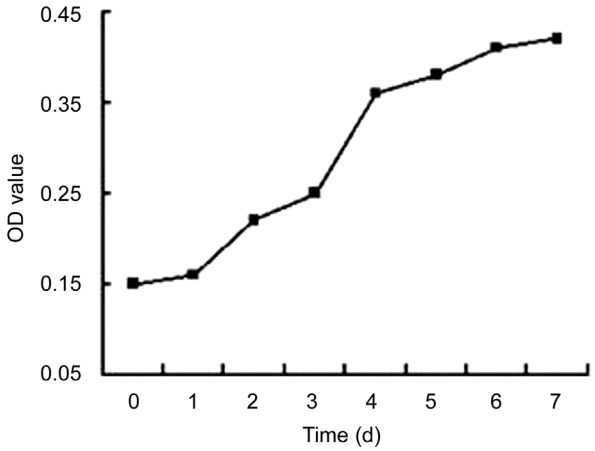

Figure 4 Growth curve of OM-MSCs.

DISCUSSION

Our studies utilized olfactory mucosa adherent tissue culturing to isolate, purify and amplify OM-MSCs. Specifically, DMEM/F-12 medium with 10% FBS was used to culture OM-MSCs from olfactory mucosa. By virtue of replacing the medium and passaging, non-stem cells were eliminated and high pure

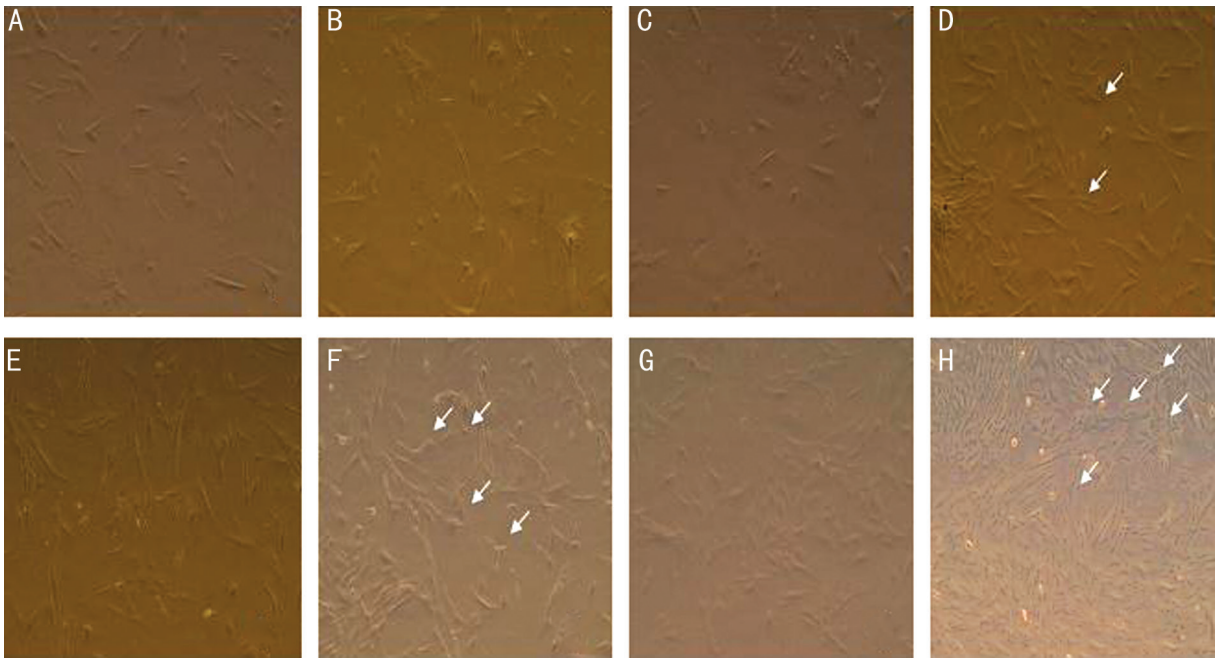

Figure 5 OM-MSCs induced group and the control group at different times after induction of cell morphology change The control group (A) and the induced group (B) had no difference before induction. Induction culturing for 24h led to a substantial change of cell morphology of olfactory mucosa-derived cells. Compared to control group (C), the cell body started to shrink and the cells grew small protrusions (D) (arrows). After 3d, compared to control group (E), cell body shrinkage was evident and protrusion was displayed by triangle, bi-polar and multi-polar cells (F) (arrows). After 6d, compared to control group (G), some cells exhibited an irregular spindle-like shape, and the bi-polar and multi-polar cells were elongated. Some cells displayed neuron morphology as aptly illustrated by a spherical shape, extended protrusion, the primary and secondary branches for some cells presented a network structure (H) (arrows).

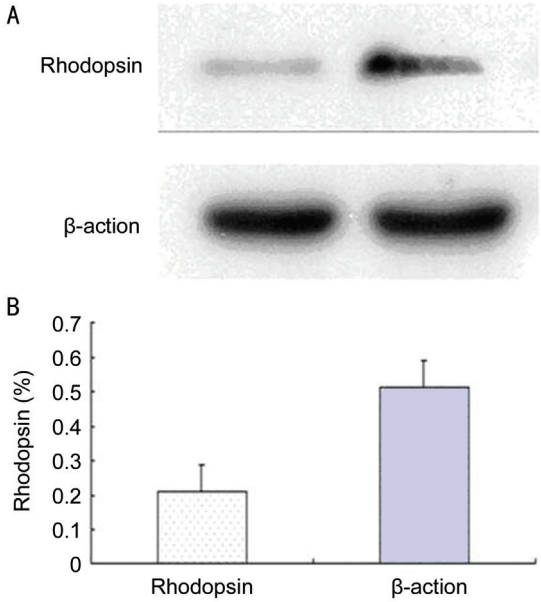

Figure 6 Cellular rhodopsin protein expression A: Western blot; B: Quantitative analysis (cropped gels, run under the same experimental conditions).

OM-MSCs were obtained after the third passage. At this stage, OM-MSCs exhibited a homogeneous spindle-like shape and grew in a parallel or spiral pattern. Flow cytometry analysis showed that these cells expressed high levels of CD73, CD90 and CD105 but did not express CD34 nor CD45, which was consistent with BMSCs phenotype and in accordance with the

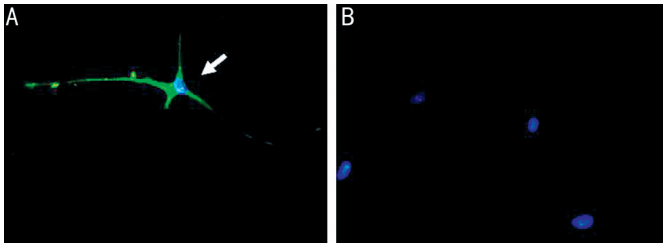

Figure 7 Immunofluorescence of rhodopsin expression A: Immunofluorescence of rhodopsin expression as a positive marker for induced photoreceptor-expressing cells (arrow); B: The control group showed an absence of such marker.

general characteristics of MSCs in the flow cytometry analysis. MSCs are a type of pluripotent stem cells with the potential of multi-differentiation and the capacity of trans-germ layer differentiation, which can be differentiated into adipocytes, osteoblasts, cartilage cells, neuron, glia, insulin-producing cells and liver cells[12]. MSC is an ideal seed cell for cellular substitution therapy and histological engineering. Current research work was focused on BMSCs. Tomita *et al*[13] administrated intravitreal injection of BMSCs in the mouse model with a mechanical-injured retina. Two weeks later, BMSCs were identified to integrate into the injured retina and expressed the photoreceptor marker rhodopsin and astrocyte-specific protein glial fibrillary acidic protein. Inoue *et al*[14] reported that BMSCs were able to mitigate the denaturation

of photoreceptors when mouse BMSCs were implanted beneath the retinal pigment epithelium (RPE)-denatured retina. However, BMSCs stem from the mesodermal layer and are predisposed to naturally differentiate into mesodermal tissue. *In vitro* culturing undermines their potential to differentiate into neuron along proliferation[15]. In addition, bone marrow aspiration is fairly invasive to obtain BMSCs. On the contrary, OM-MSCs are located in the nasal lamina propria and stem from ectodermal layer sharing the similar biological properties and immunological phenotype with BMSCs. Not only can OM-MSCs differentiate into mesodermal derived tissues such as bone and adipocyte, but they can also be induced to differentiate into neurons and glia tissues[16].

In the case of retinal degenerative diseases, RPE cells and photoreceptor cells as main target cells are both originated from the neuronal ectodermal layer. Meanwhile, OM-MSCs bear several advantages such as convenient isolation, feasibility of autologous transplantation, zero immune rejection, and no gene mutation after infinite passaging *in vitro* and free ethic issues. As adult stem cells, both BMSCs and OM-MSCs have potential of pluripotent differentiation and steady ability of proliferation *in vitro*. This suggests that commonality between OM-MSCs and BMSCs provides another solution to treat retinal disease and may have a broader application and better clinical benefits for more patients in the future.

In addition, referring to the method from other studies[17-20] that incubation of rat BMSCs in medium supplemented with EGF, taurine and activin A led to induced differentiation into photoreceptor cells that express marker rhodopsin *in vitro*, we selected EGF, taurine and retinoic acid as inducing reagents during the process of OM-MSCs differentiation into retinal cells. These reagents are able to promote the growth, repair and protect embryonic photoreceptor cells in adults. Our experimental data demonstrated that, in the presence of including reagents, OM-MSCs can be induced to display neuron morphology *in vitro* and express a positive marker for induced photoreceptor-expressing cells.

Currently, application of OM-MSCs in developing neuron progenitor cells has not been reported in the field of ophthalmology, which aims for replacing current therapies against retinal neuron degenerative diseases. Our study lends support to further research on differentiation of OM-MSCs. It also allows the possibility of an alternate choice for exploration of retinal genetics and treatment of difficult regenerative diseases in the field of ophthalmology.

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Conflicts of Interest: Lu W, None; **Duan D,** None; **Ackbarkhan Z,** None; **Lu M,** None; **Huang ML,** None.

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