Title: Human Olfactory Ectomesenchymal Stem Cells (OE-Mscs) Display Schwann Cell-Like Phenotypes and Promote Neurite Outgrowth in Vitro

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Abstract

Strategies of Schwann cell (SC) transplantation for regeneration of peripheral nerve injury involve many limitations. Stem cells can be used as alternative cell source for differentiation into Schwann cells. Given the high potential of neural crest-derived stem cells for the generation of multiple cell lineages, in this research, we considered whether olfactory ectomesenchymal stem cells (OE-MSCs) derived from neural crest can spontaneously differentiate into SC lineage. OE-MSCs were isolated from human nasal mucosa and characterized by the mesenchymal and neural crest markers. The cells were cultured in glial growth factors-free medium and further investigated in terms of the phenotypic and functional properties. Immunocytochemical staining and real-time PCR analysis indicated that the cultured OE-MSCs expressed SCs markers, SOX10, p75, S100, GFAP and MBP, differentiation indicative. It was found that the cells could secrete neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Furthermore, after co-cultured with PC12, the mean neurite length was enhanced by OE-MSCs. The findings indicated that OE-MSCs could be differentiated spontaneously into SC-like phenotypes, suggesting their applications for transplantation in peripheral nerve injuries.

Keywords: Spontaneous differentiation, Olfactory ectomesenchymal stem cells, Schwann cell, Peripheral nerve regeneration
1. Introduction

The application of cell-based therapies is one of the most promising strategies for treating peripheral nerve injuries (PNIs), whereby is provided trophic factors and cytokines by engrafted cells (1, 2). Schwann cells (SCs) as major myelin-forming cells and a key component in peripheral nerve tissue, play important roles in the process of nerve regeneration (3, 4). Following nerve injury, these cells proliferate rapidly and dedifferentiate while clear the myelin debris (5). SCs promote and direct axonal outgrowth from the proximal to distal neural ends by releasing various neurotrophic factors and extracellular matrix (ECM) and forming the Büngner bands that act as longitudinal guidance channels (5-7). Since during large neural defects, the number of SCs is not adequate, it is critical to supply a sufficient rate of SCs to the repair site (8). However, the supply of cultured autogenic SCs is limited by lack of availability, donor site morbidity and low capacity to proliferate remarkable that delays treatment. Thus, alternative cell sources that can differentiate into functional SCs are desirable (9, 10).

Neural crest stem cells (NCSCs) are known as a kind of multipotent progenitor cells that give rise various phenotypic lineages including SCs. In the craniofacial region, these stem cells are found in some tissues such as dental pulp (11-13), periodontal ligament (11), dermal papilla (14) and nasal mucosa (15).

Olfactory ecto-mesenchymal stem cells (OE-MSCs) are a new population of neural crest-derived stem cells (15) that can be obtained from the olfactory nasal mucosa niche using non-invasive techniques(16, 17). Ecto-mesenchymal stem cells (EMSCs) possess unique properties, including multilineage differentiation and self-renewal capacity, higher mitotic activity in comparison to mesenchymal stem cells isolated from other tissue sources especially bone marrow mesenchymal stem cells (BM-MSCs) (18), immunoregulatory function (19), and lack of apoptotic and tumorigenic activities (20-22). Moreover, it has been demonstrated that EMSCs have a higher potential than the other sources of stem cells to secret neurotrophic factors and differentiate into different neurogenic cells including SCs due to originating from the neural crest (NC) (23, 24). These stem cells are also known as a Schwann progenitor cells and express SC markers S100 and p75NTR (25). Therefore, they can be considered as a good choice for cell therapy in PNIs. Recent researches have shown that EMSCs are capable of committing to spontaneously differentiate into various lineages such as melanocyte (26), smooth muscle and osteoblast lineages (27), whereas
their differentiation into glial cell lineage has not been well understood. EMSCs differentiation into SC-like cells has previously been represented in a specific glial differentiating medium (23, 28). In this research, for the first time, we investigated spontaneous differentiation of EMSCs from human olfactory mucosa into functional SC-like cells by examining SC-specific markers expression, neurotrophic factors secreted by OE-MSCs monolayer at culture media, and neurites outgrowth from PC12 cells on a co-culture assay.

2. Materials and methods

2.1. Isolation and culture of human OE-MSCs

Human nasal mucosa cells were obtained using a modified technique as previously described (29). Nasal mucosa biopsies were collected from individuals age ranges from 20 to 50 years, undergoing nasal surgery, at Iran University Hospital Research Center. In brief, after a local anesthetic, biopsy was performed from nasal superior conchae. The samples were immediately washed with Phosphate-buffered saline (PBS) and placed in DMEM/Ham's F12 (Gibco, USA) supplemented 1% penicillin/streptomycin (Pen/Strep; Gibco, USA). Lamina propria (LP) was isolated under a dissecting microscope and incubated for 40 min at 37°C in Dispase II solution (2.4 U/mL). Then, the suspension of the cells were placed into flasks and cultured in a growth medium, DMEM/F12 supplemented with 10% Fetal Bovine Serum (FBS; Gibco, USA) and 1% Pen/Strep at 37°C with 5% CO₂. The media was changed every 72 hours and when the adherent cells reached 80% confluence, they were passaged. The cells at 4th passage were used for the study.

2.2 Characterization of human OE-MSCs

The cells were confirmed by morphological assessment, flow cytometry analysis for CD105, CD90, CD73 as positive markers, and CD45 and CD34 as negative markers, immunofluorescence staining for examining the neural crest markers, nestin and vimentin. In addition, multilineage differentiation capacity of OE-MSCs were assessed by induction into osteogenic and adipogenic lineages.
2.3 Cell proliferation assay

To determine the growth rate of human OE-MSCs and compare that with human adipose tissue-derived stem cells (ASCs) as an identified and accessible source from human MSCs that can be differentiated into SCs (30, 31), MTT assay was done. Moreover, the viability rate of OE-MSCs was compared with human SCs separately. The cells at passage 4 plated at a density of 2×10^3 cells/well in a 96-well plate for 1, 4 and 7 days. Then, MTT solution was added to the cells and incubated for 4 hours. The insoluble formazan crystals were dissolved using DMSO and the absorbance was measured at 570 nm.

2.4. Evaluation of SCs like phenotypes in human OE-MSCs

To evaluate the potential for spontaneous differentiation of OE-MSCs into SC-like cells, human OE-MSCs cultured in medium, DMEM/F12 supplemented with 10% FBS, 1% Pen/Strep for 7 days (25). Then, the expression of SC-specific markers, genes and secreted proteins were tested through Immunocytochemistry (ICC), quantitative Real-Time PCR (qRT-PCR) and Enzyme-Linked Immunosorbent Assay (ELISA), respectively.

2.5. Immunofluorescence assay

For Immunostaining (30), the cells were fixed in fresh 4% paraformaldehyde (PFA) for 15-20 min. After washing with PBS, the fixed cells were blocked with 10% goat serum for about 1 h. Then, primary antibodies, mouse monoclonal anti-S100 (1:400; Abcam), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:200; Abcam) and mouse anti-myelin basic protein (MBP; 1:20; sigma) were added, and the cells incubated at 4°C overnight. The cells were treated with Texas Red goat anti-mouse, Alexa Fluor 488 goat anti-mouse and FITC goat anti-rabbit (IgG) as secondary antibodies (1:500; abcam), respectively, for 1 h in the dark at room temperature. Finally, cell nuclei were labeled with 4′,6′-diamidino-2-phenylindole dihydro-chloride (DAPI; Sigma) and evaluated by a fluorescence microscope. For quantification of ICC, the number of total cells and positively stained cells were counted.
2.6. qRT-PCR analysis

The total mRNA within human OE-MSCs was extracted using TRIzol. Then isolated RNAs were used for cDNA synthesis by reverse transcription kit (Fermentas, Germany). Real-time PCR was done using the SYBR Master Mix (Takara, Japan). The primer sequences are listed in Table 1:

**Table 1.** Primer sequences in real time-PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>SOX10</td>
<td>R TCGCAAAAGAGTCCAACG</td>
</tr>
<tr>
<td>GFAP</td>
<td>R ACTCCTTAATGACCTCTCCAAC</td>
</tr>
<tr>
<td>S100</td>
<td>R TCACCTCTGGTCTCTTG</td>
</tr>
<tr>
<td>P75</td>
<td>R TGATGACACAGTTCAACCACTC</td>
</tr>
<tr>
<td>MBP</td>
<td>R ACTCCCCCTGAATCCCTTTTG</td>
</tr>
<tr>
<td>β-actin</td>
<td>R GTCTTTCGCGATGTCCAC</td>
</tr>
</tbody>
</table>

The DNA amplification was performed under the following conditions: after predegeneration at 95°C for 2 min, 40 cycles in two steps were performed at 95°C for 5 sec and at 66°C for 30 sec. The reactions were done in triplicate. The relative expression of each mRNA was calculated through ΔΔCT method and normalized to that of β-actin as the housekeeping gene.
2.7. Human OE-MSCs -secreted neurotrophic factors

The released neurotrophins, NGF and BDNF in the OE-MSCs-conditioned medium (OE-MSCs-CM) were analyzed by an ELISA test (32). Conditioned media from the human Schwann cells (hSCs) were purchased from Stem cell Research Institute (Bonyakhteh, Iran) and the base medium used as control groups. To collect conditioned media, $1 \times 10^4$ cells/ml OE-MSCs and SCs were seeded on a 48-well plate and incubated overnight. After that, the media was replaced with DMEM/F12 plus 1% FBS and cultured for 72 hours at 37°C and 5% CO$_2$. CM was collected and frozen at -80°C until using for assessment. The protein levels of NGF and BDNF were measured by ELISA, human β-NGF DuoSet and human BDNF Quantikine ELISA kits (R&D Systems Inc., USA) and measured at 450 nm absorbance according to the manufacturer’s protocol.

2.8. Co-culture of human OE-MSCs with PC12 cells

To investigate the function of human OE-MSCs on neurites outgrowth, we examined the co-culture of the OE-MSCs with rat pheochromocytoma (PC12) cells applied as an in vitro model for investigating neuronal differentiation (33). The PC12 cells seeded at a density of 500 cells/well on the monolayer of hOE-MSCs. After 24 h incubation in DMEM/F12 plus 10% FBS, it was switched to a medium supplemented with 1% FBS and 50 ng/mL nerve growth factor (NGF; sigma) for 5 days. The media was changed every 72 hours. PC12 grown alone on tissue culture plate (TCP) and PC12 seeded with human SCs as control cultures.

2.9. Neurite outgrowth

The behavior of neurites extending from PC12 was evaluated by immunostaining with anti-βIII tubulin antibody (1:200; Millipore) and Alexa Fluor 488 goat anti-mouse IgG (1:500; Abcam) as a secondary antibody and were imaged under a fluorescence microscope. For quantification of neurite outgrowth, images of 10-15 cells from at least 5 randomly selected fields per well were analyzed. Neurites in each of the fields were measured from soma to the end of neurite using ImageJ software and then averaged. Subsequently, maximal neurite length in each field was calculated.
2.9. Statistical analysis

The data were expressed as a mean ± SEM. To analyze the data, GraphPad Prism version 7 was applied. Statistical analysis was done using one-way and two-way analysis of variance (ANOVA) for comparison of differences between groups. *P < 0.05 was considered as significant difference statistically.

3. Result

3.1. Identification of human OE-MSCs originated from NC

Morphologically, olfactory stem cells appeared as fibroblast-like cells and spindle-shaped. Here, these cells proliferated rapidly and after a short time they were harvested at 80% confluency (Fig. 1A). The multipotency capacity of OE-MSCs was confirmed through differentiation toward two lineages, osteogenic and adipogenic by Alizarin Red S and Oil Red-O staining, respectively (Fig. 1B, C). Immunofluorescent staining revealed that almost all of the human OE-MSCs expressed nestin and vimentin (Fig. 2A). Moreover, flow cytometry analysis showed that the isolated human OE-MSCs expressed CD105 (97.18%), CD73 (98.12%), and CD90 (98.98%), while were negative for hematopoietic markers CD45 and CD34 (Fig. 2B).

Fig. 1. Characterization of human olfactory ecto-mesenchymal stem cells (OE-MSCs). (A) OE-MSCs at 4th passage formed a monolayer of fibroblast-like cells under phase-contrast. (B) the differentiated OE-MSCs into osteocytes stained with Alizarin Red S. (C) the differentiated OE-MSCs into adipocytes stained with Oil Red-O; scale bar= 40µm.
Fig. 2. Characterization of hOE-MSCs by immunocytochemistry and flow cytometry analysis. (A) The immunocytochemical staining showed that hOE-MSCs expressed nestin and vimentin; Scale bar=20µm. (B) Flow cytometric analysis indicated that the cells were positive for CD105, CD73 and CD90, and negative for CD45 and CD34.

3.2. Proliferation rate of human OE-MSCs

For MTT assay, OE-MSCs, ASCs and SCs isolated from human tissues at passage 4 were plated at a density of 2×10³ cells/well. As shown in Fig. 3A, the proliferation rate of MSCs taken from nasal mucosa was significantly higher than those from the adipose tissue. Remarkable growth of OE-MSCs started at the early hours, and after 7 days displayed an almost 3-fold increase compared to ASCs (Fig.3A). The same result was also obtained when the growth rate of OE-MSCs was compared to that of human SCs. (Fig.3B). MTT showed that the expansion of OE-MSCs was
more rapidly compared with human SCs in all evaluated days, suggesting the lower proliferation ability of human SCs (****P < 0.0001).

Fig. 3. Proliferation of EMSCs from human olfactory mucosa (hOE-MSC), MSCs from human adipose tissue (hASCs) and human SCs (hSCs). MTT assay determined the growth rate of (A) hOE-MSC (▴) and hASCs (▪), and (B) hOE-MSC and hSCs for 1, 4 and 7 days in absorbance (570 nm). *P < 0.05 and ****P < 0.0001; NS: not significant.

3.3. Evaluation of SC-like phenotypes in cultured hOE-MSCs

One week after OE-MSCs culture, the expression of SC-specific markers was tested. Immunofluorescent staining showed that hOE-MSCs were positive for markers S100 (86.03 ± 9.41%), MBP (39.22± 3.66%) and GFAP (39.8 ± 8.4%) (Fig. 4A). To confirm the results, RT-qPCR was performed on hOE-MSCs (Fig. 4B). The levels of mRNA SOX10, S100, p75, GFAP and MBP were analyzed, and the results showed the expression of whole of genes in hOE-MSCs. However, SOX10 marker showed highest expression in between genes, and the level of S100 marker was significantly higher than that of other mRNAs (***P < 0.001, *P < 0.05) No remarkable differences in levels of p75, MBP and GFAP mRNAs were found in hOE-MSCs.
Fig. 4. The expression of glial cell markers in human OE-MSCs. (A) Immunocytochemical staining showed S100, MBP and GFAP in OE-MSCs cultured in nerve growth factors-free media; Scale bar=20µm. (B) Real-time PCR analysis showed mRNA levels of SCs different states in OE-MSCs differentiated in nerve growth factor-free media. S100 and SOX10 significantly higher than other genes. *P < 0.05 and ***P < 0.001.

3.4. Functional properties of cultured hOE-MSCs

In order to evaluate the similarity of OE-MSCs function with that of SC-like, the levels of secreted neurotrophins BDNF and NGF in OE-MSCs-conditioned medium were detected by ELISA quantification (Fig. 5A, B). The results showed that OE-MSCs secreted detectable levels of BDNF and NGF (**P < 0.001). Also, there was no statistically significant difference between OE-MSCs and SCs in levels of both neurotrophic factors. The ability of OE-MSCs to promote extension of neurites was revealed by assessing their interaction with PC12 cells after 6 days (Fig. 6). Analysis
and quantification of co-culture images showed that neurites of PC12 were more elongated in the presence of OE-MSCs and SCs, and neurite extension increased significantly to 131.9±49.5 µm and 154.2±83.8 µm, respectively (***P < 0.01, **P < 0.001) (Fig. 6B). In contrast, PC12 cultured alone on TCP have shown short neurites (87.9±38.2 µm). Likewise, the longest neurite length significantly increased to 214 ± 83.8 µm, and 262.6 ± 72.6 µm, in groups of co-culture with OE-MSCs and Schwann cells, respectively (*P < 0.05, **P < 0.01), in comparison with PC12 alone (139 ± 26.5 µm) (Fig. 6C). There was also no significant difference between co-cultured groups.

Fig. 5. The neurotropic factor secreted from OE-MSCs. (A, B) Detectable levels of BDNF and NGF (pg/ml) in human OE-MSCs. ***P < 0.001.

Fig. 6. PC12 co-cultured with human OE-MSCs and hSCs. (A) ICC staining for beta III-tubulin showed neurites outgrowth from PC12 on TCP, co-cultured with OE-MSCs and SCs (arrows); Scale bar= 20µm. (B and C) The average neurite length and the longest neurite length were determined in different groups. *p < 0.05, **p < 0.01 and ***P < 0.001.
Discussion

Current cell transplantation strategies for facilitating remyelination and nerve regeneration face limited SCs expansion. The various types of stem cells can be considered as promising alternatives to overcome these limitations (34, 35). Given the high potential of neural crest-derived stem cells as a source of autologous stem cells for generation of glial cell types, recent studies have focused on ectomesenchymal stem cells originated from neural crest and their differentiation into SC-like cells (28). The results have shown that these stem cells differentiate to SC phenotypes under different differentiation conditions (23, 36). In the present study, spontaneous differentiation of EMSCs obtained from human nasal olfactory mucosa into SC-like cell phenotypes was investigated. The isolated human OE-MSCs were characterized in terms of having fibroblastic-like and spindle-shaped morphology, expression of a set of ectomesenchymal stem cells-associated markers, and multipotent differentiation capability. Our experiments confirmed the three characteristics mentioned in human OE-MSCs, in agreement with previous reports on other EMSCs (19, 25). The growth rate of OE-MSCs was determined and further compared with that of cells isolated from human adipose tissue and human SCs separately. The both of results showed that OE-MSCs proliferated significantly faster than ASCs and SCs. These findings were confirmed by Delorme and colleagues in a study that displayed a near 3-fold proliferation rate of OE-MSCs compared to that of bone marrow MSCs (18), and also several researches that demonstrated poor expansion and prolonged in vitro culture of SCs compared to stem cells specially MSCs (10, 32). Given that a large number of cells are usually required in transplantation (33), high proliferation of hOE-MSCs showed that these stem cells can be considered as a reliable source for cell therapy. In this research, we found that OE-MSCs cultured without the presence of glial growth factors expressed typical markers of SCs, they secreted high levels of neurotrophins and enhanced the neurite outgrowth from PC12 cells on co-culture system, displaying trophic effects and phenotypic characteristics similar to those of SCs (27). Consistent with previous studies, our study showed that almost all OE-MSCs expressed neural crest markers nestin and vimentin. Nestin and vimentin are intermediate filament proteins that can be used for the identification of neural stem cells (37). Recently, the expression of nestin has been observed in MSCs from bone marrow and adipose tissue, and its importance has been demonstrated for the committed differentiation along the glial lineage (30, 38). It has been reported that the potential of glial cells formation by adipose and bone
marrow-derived MSCs may be due to the presence of neural crest-derived stem cells (36). During the development, neural crest multipotent stem cells give rise to a variety of lineages, including SCs (39, 40). These cells transform to mature Schwann cells (myelinating and non-myelinating SCs) through the generation of Schwann cell precursors (SCPs) and immature Schwann cells (iSCs) (41). The extensive changes take place in a set of phenotypic features, including expression of genes and proteins at every stage of SC development that used to identify a state of SCs. For instance, there were no expression of GFAP and S100 markers in SCPs, whereas they were up-regulated in immature SCs differentiated from SCPs (42, 43). Also myelinating SCs expressed a range of myelin-associated genes such as MBP. Nevertheless, some markers like p75NTR and SOX10 show no significant change and present in a whole of early developmental stages (43). Although it has been demonstrated that p75, S100, GFAP and MBP as glial markers were expressed strongly in EMSCs differentiated to SCs phenotypes under induction medium (28, 36), the expression of some of them has been observed in EMCSs cultured at growth factor-free medium (20, 23, 25). In this study, Immunofluorescent staining of spontaneously differentiated human OE-MSCs displayed that most cells were immunopositive for S100 and GFAP markers, and a few cells were GFAP and MBP-positive. This result was confirmed by quantitative RT-PCR analysis and showed the co-expression of SC-specific genes, comprising SOX10, S100, p75, MBP, GFAP. However, the mRNA level of p75, MBP and GFAP was significantly lower than that of other genes. In agreement with our researches, a recent study showed that EMSCs from respiratory nasal mucosa could differentiate spontaneously into SC phenotype on a fibrin gel substrate (25). In another research, spontaneous differentiation of EMSCs from dental papilla into mature melanocytes was demonstrated (26), and showed that some EMSCs of the first branchial arch could differentiate spontaneously into osteoblast and smooth muscle lineages, but not glial lineage (27). It has been also suggested that the fate of migrating pluripotent and uncommitted neural crest cells may be dictated by local factors in their environment (44, 45). These cells and their derived-EMSCs after migration to the target tissues could obtain a degree of commitment to be spontaneously promoted without various growth factors (45). It could be the reason that EMSCs isolated from different sites have a tendency to differentiate spontaneously along specific lineages. In our study, this expressed markers showed that hOE-MSCs were similar to immature SCs.

The phenotypic characteristics are not usually enough data to justify whether the function of OE-MSCs is similar to SCs (32). As mentioned before, SCs dedifferentiate and convert into an
immature state in response to peripheral nerve injury (42). These SCs proliferate rapidly and secrete various neurotrophic factors that promote axonal regrowth (46, 47). In this regard, the ability to release neurotrophic factors and extend neurites by OE-MSCs was investigated. The results from ELISA test indicated that human OE-MSCs-conditioned medium produced high levels of neurotrophins NGF and BDNF. These factors as biological regulators could play important roles in the differentiation, myelination and neuronal growth (48). Many studies have used an in vitro model to investigate the function of the derived SCs on the axonal outgrowth and nerve regeneration, and co-cultured them with PC12 cells or primary neurons used as representative of peripheral neuronal cells (32, 33). After co-culturing OE-MSCs and PC12 cells, it was shown the positive efficacy of these stem cells for neurite outgrowth promotion through intercellular interactions and release of growth factors such as NGF and BDNF. These findings are in agreement with a study by Crigler and colleagues who recently showed that human MSCs subpopulations could promote neuritogenesis and neuronal survival by expression of various neuro-regulatory factors such as NGF and BDNF (49). However, some other studies demonstrated that differentiated MSCs are more beneficial rather than untreated ones in stimulating neurite growth and nerve regeneration (32). Although the tendency of hOE-MSCs to spontaneous differentiation into SCs-like cells have been demonstrated in vitro models, further researches are required in animal models of PNI to investigate whether the implantation of these stem cells could improve nerve regeneration.

Conclusion

The findings of the present study provided evidence that human OE-MSCs could show properties similar to immature SCs in terms of phenotypic and functional features. It appears that a degree of commitment along SCs lineage already exist for EMSCs migrated to nasal mucosa. Given the high potential of neural crest stem cells to produce sufficient SCs for applications in clinic, our results suggested that hOE-MSCs as a valuable source of autologous stem cells can be used for the transplantation in peripheral nerve injuries.
Acknowledgments

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Conflict of interest

There is no conflict of interest to declare.
Reference


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