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Molecular Characterization of Olfactory Bulb Neural Stem Cells during Proliferation and Differentiation

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ABSTRACT

Objective: This study aimed to demonstrate the fate of human olfactory bulb neural stem cells (hOBNSCs). Reportedly, these cells can be expanded *in vitro* under prolonged mitogen stimulation without propensity to transform. **Material and methods:** We assessed their possible ability to proliferate and differentiate into different neurons, oligodendrocytes and astrocytes through monitoring changes in expression profile of proliferation (NES, NR4A1, SOX2, MSI1) and differentiation (FOXO4, CSPG4, MAP2, GFAP)-related genes. **Results:** *In vitro* induction of hOBNSCs proliferation by addition of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF) to basal serum-free medium (DMEM/F12) resulted in significant up-regulation of proliferation-related genes. Differentiation of hOBNSCs, which was initiated by replacing bFGF and EGF by triiodothyronine (T3), significantly increased expression of differentiation-related genes. Among the differentiated cells, GFAP-expressing astrocytes constituted the highest population of cells followed by CSPGS-expressing immature oligodendrocytes, then MAP2-expressing immature neurons, and finally FOXO4-expressing mature oligodendrocytes. **Conclusion:** These data will enable us to understand the mechanism of proliferation and differentiation of hOBNSCs before and after their engraftment during cell-based therapy for neurodegenerative diseases.

INTRODUCTION

Stem cells (SCs) are defined as cells having the ability to self-renew, and to differentiate into multiple phenotypic lineages (Tuszynski *et al.*, 1996). Recently, there has been an increasing interest for their use in various biochemical applications. However SCs heterogeneity causes a significant obstacle to the clinical implementation of cell-based therapies (Young *et al.*, 2016). In humans, neural SCs (NSCs) have successfully been isolated from the olfactory bulb (OB) (Marei *et al.*, 2012). Thereby, human adult olfactory bulb NSCs (hOBNSCs) provide an attractive tool for transplantation-based therapy of neurodegenerative diseases that avoids the ethical and moral questions associated with the use of human embryonic or heterologous material (Casalbore *et al.*, 2009; Marei *et al.*, 2016).

In vitro, NSCs can be induced to proliferate when they are exposed to growth factors (Vescovi *et al.*, 1993). In the OB, NSCs differentiate into inter-neurons in the granular and glomerular layer. Although none of the markers are completely exclusive for NSC, some of them including nestin, NR4A1, SOX2, and MSI1, are highly enriched in SC populations (Nakano *et al.*, 2005). Nestin is a type VI intermediate filament protein responsible for mechanical integrity of the cell and involved in formation of tissue architecture and in tissue regeneration (Neradil and Veselska, 2015). Upon differentiation, nestin is down-regulated and substituted by other intermediate filament proteins such as glial fibrillary acidic protein (GFAP) in astrocytes or neurofilaments in neuronal cells (Michalczyk and Ziman, 2005). The orphan nuclear hormone receptor NR4A1 is involved in cell survival and apoptosis (Li *et al.*, 2006). SOX2 is a transcription factor essential for maintaining self-renewal of undifferentiated embryonic SCs (Pevny and Nicolis, 2010). However, SOX2 is down-regulated during differentiation when they become post mitotic (Suh *et al.*, 2007). The MSI1

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RNA-binding protein is a specific marker for stem/progenitor cells of neural origin (Uren *et al.*, 2015). Neural progenitors express high levels of MSI1, which is rapidly turned off during terminal differentiation, and its expression is virtually absent in post-mitotic neurons (Glazer *et al.*, 2012).

Mature neurons have distinctive and definite morphological criteria. However, it is usually hard to undoubtedly recognize a cell as being a neuron *in vitro* or *in vivo* (Nakano *et al.*, 2005). Microtubule-associated protein 2 (MAP2) is involved in maintenance of neuronal morphology, development of dendrite, and outgrowth of oligodendrocyte and neurite (Lim and Halpain, 2000; Zamora-Leon *et al.*, 2001). Similar to neurons, mature oligodendrocytes have a distinctive morphology. However, *in vitro*, oligodendrocytes may not be easily distinguishable from other process-bearing cells. Markers used to identify oligodendrocytes for instance are the forkhead box protein O4 (FOXO4) and chondroitin sulfate proteoglycan 4 (CSPG4) (Nakano *et al.*, 2005). FOX proteins contribute in cellular proliferation, transforma-

tion, differentiation and longevity (Tsai *et al.*, 2007). CSPG4 cells have the ability to generate oligodendrocytes *in vitro* as well as *in vivo*, and so they are often called oligodendrocyte precursor cells (Ozerdem *et al.*, 2001; Schiffer *et al.*, 2017). *In vivo*, astrocytes appear stellate and *in vitro* they appear flattened polygonal or have long processes. Glial fibrillary acidic protein (GFAP), an intermediate filament protein present approximately exclusively in astrocytes, is used to identify astrocytes *in vivo* and *in vitro* (Nakano *et al.*, 2005). Together with actin filaments and microtubules, they form an integrated system that regulates many cellular processes, such as cell morphology and proliferation (Moeton *et al.*, 2016).

This study aimed to demonstrate the fate of hOBNSCs through assessment of their ability to proliferate and differentiate into different neurons, oligodendrocytes and astrocytes and to detect gene expression profile that is specific for each cell lineage during proliferation and differentiation processes.

Table 1: Human adult olfactory bulb neural stem cells (hOBNSCs).

Sample index	Age/Sex	Diagnosis	Source
OB1	45/M	Olfactory groove meningioma	Institute of Neurosurgery, Catholic University School of Medicine, Rome, Italy
OB2	39/M	Anterior communicating artery aneurysm	Institute of Neurosurgery, Catholic University School of Medicine, Rome, Italy

MATERIAL AND METHODS

Isolation and culturing of hOBNSCs

The olfactory bulbs (OBs) were collected from two patients undergoing craniotomy at the Institute of Neurosurgery, Catholic University, Rome, Italy (Table 1). Prior signed agreements were obtained according to protocols approved by the Ethical Committee of the Catholic University. The experiments were undertaken with the understanding and written consent of each subject, and the study conforms with World Medical Association Declaration of Helsinki. Obtained OBs were directly broken down at 37 °C in 0.1% Papain (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Resultant cells were then grown in basal serum-free medium (DMEM/F12; Invitrogen, Carlsband, CA, USA) that contain progesterone 0.025 mg/mL, L-glutamine 2 mM, glucose 0.6%, sodium selenite 5.2 ng/mL, insulin 0.025 mg/mL, apo-transferrin sodium salt 0.1 mg/mL, putrescine 9.6 µg/mL, sodium bicarbonate 3 mM, Hepes 5 mM, BSA 4 mg/mL and heparin 4 µg/mL in the presence of 20 ng/mL epidermal growth factor (EGF; Pepro-Tech, Rocky Hill, NJ, USA), 10 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA), and 20 ng/mL leukemia inhibitory factor (LIF; Immunological Sciences, Rome, Italy). For 4 min at 37 °C the primary neurospheres were broken down using accutase (Invitrogen) then diluted and plated (single cell/mini-well) onto 96-well plates. Existence of one single cell in mini-wells was confirmed via microscopic examination. After 7 days these wells were examined for secondary neurosphere generation. Secondary neurospheres were dissociated then cultured (10^3 cells/cm²) in serum-free medium containing EGF and bFGF, and passaged up to P30. Parallel cultures of cells grown as adherent mono-layers were

confirmed between P7 and P10 in medium containing EGF and bFGF with 5% fetal calf serum supplement (Hyclone, Logan, UT). Differentiation investigations were done next to 5 days after culturing 10^5 proliferated cells which were plated in 24-well plate supplied with a poly-L-lysine coated glass cover slips in presence of 1% fetal calf serum (Hyclone) supplemented with cAMP (50 mM), all-trans retinoic acid (5 mM) and T3 (30 nM) (Sigma-Aldrich, St. Louis, MO, USA) and in absence of EGF and bFGF.

Morphological examination

hOBNSCs cells were plated on 12-well plates at 2×10^4/well, and allowed to reach 70%-80% confluence. All cultures were incubated at 37 °C in an atmosphere containing 5% CO_2 for 24 h. After this, cells were washed 3 times with 1X PBS (ice cold), fixed for 30 min with 4% formaldehyde solution in PBS and the morphologic changes of hOBNSCs cells were examined and photographs were taken using an inverted microscope (Nikon, Japan) at 40X magnification. For confirmation the experiment was repeated three times with nearly similar results.

Detection of cell viability by MTT assay

Cell viability of differentiated hOBNSCs was assessed using conventional MTT assay. The cultured cells in 96-well plates (5×10^3/well) were incubated with 20 µL of MTT stock solution (5 mg/mL) for 4 h at 37 °C. Absorbance of extracted resultant MTT formazan with 150 µL dimethyl sulfoxide (DMSO) was recorded at 570 nm using a microplate reader (Bio-Teck, USA). The absorbance of untreated cells was set to 100%, and the results for each treated sample are expressed as the percentage of MTT reduction.

Detection of relative gene expression by real-time PCR

Isolation of hOBNSCs' total RNA was performed using Gene JET RNA Purification Kit (Thermo Scientific, # K0731, USA) and RevertAid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) was used to reverse transcribe isolated total RNA (5 μg/sample) into cDNA. cDNA was then used as a template to determine the relative expression of the candidate genes using StepOnePlus real time PCR system (Applied Biosystem, USA). Primer 5.0 software was used for primers (Table 2) designation. The reference used to calculate the fold change in target gene expression was the housekeeping gene GAPDH. cDNA template (2 μL) was mixed with 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA) (12.5 μL), forward primer (1 μL), reverse primer (1 μL), and nuclease free water (8.5 μL) to form 25 μL of PCR

mix. Thermal cycling conditions included initial denaturation at 95 °C for 10 min, 40-45 cycles of amplification of DNA denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The temperature was increased at the end of the last cycle from 63 to 95 °C for analysis of the melting curve. Target genes and GAPDH gene cycle threshold (Ct) values were calculated then $2^{-\Delta\Delta Ct}$ method was used to determine relative gene expression.

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to evaluate the statistical significance using SPSS software version 18.0. Duncan's multiple range test (DMRT) was used for the individual comparisons. $P<0.05$ was considered statistically significant.

Table 2: Sequences of forward and reverse primers used in real-time PCR.

Gene	Forward primer (′5 ------ ′3)	Reverse primer (′5 ------ ′3)
NES	GGGCCTACAGAGCCAGATCG	CAGGAGGGTCCTGTACGTGG
NR4A1	CCATCCAGAGTGAGATGCCC	TGGGCTTGATGAACTCAGGG
SOX2	GGATAAGTACACGCTGCCCG	CTGTCCATGCGCTGGTTCAC
MSI1	ACGACCCCTGCAAGATGTTC	CCCGAACTGGCCGAAGTATT
FOXO4	CTGGGGGAAAAGGCCATTGA	GTGGCGGATCGAGTTCTTCC
CSPG4	CACAGAGGAACCCTGGATGG	CTTCAGCGAGAGGAGCACTT
MAP2	GGGTGCATCCAGTTTCTGCG	CCCAATCAATGCTTCCTCGGT
GFAP	GCTCAATGTCAAGCTGGCCC	CTTTTGCCCCCTCGAATCTGC
GAPDH	GTT CGA CAG TCA GCC GCA TC	CGA CCA AAT CCG TTG ACT CCG

RESULTS

Morphological characters of the proliferated and differentiated hOBNSCs

The proliferated hOBNSCs formed clusters of cells called neurospheres which contained undifferentiated NSCs, as revealed by the lack of any specific morphology (Figure1A and B). The formed neurospheres were in form of either free floating or loosely attached growing spheres. These cells generated clonally related progeny that differentiated into neurons, oligodendrocytes, and astrocytes. The *in vitro* culture showed that a common progenitor existed for oligodendrocytes, astrocytes, and unipolar single neuron cell in the second differentiation day (Figure 1D) which converted into multipolar neuron by forming synaps with other neurons (Figure 1E).

Effects of differentiation media on cell viability of hOBNSCs

MTT assay was used to evaluate the effect of the different differentiation compounds (3'–5'-cyclic adenosine monophosphate (cAMP) 50 mM, all-trans retinoic acid 5 mM, triiodothyronine (T3) 30 nM, and ascorbic acid) on cell viability of hOBNSCs. Obtained results showed that the percent of cell viability following addition of proliferation media (90 ± 10) did not significantly differ from that of differentiation media (80 ± 7.0) (Figure 2).

Relative expression of proliferation-related genes in hOBN-SCs

Relative expression of the 4 proliferation-related genes (NES, NR4A1, SOX2, and MSI1) was determined in hOBNSCs by real-time PCR (Figure 3). A significant ($P<0.05$) up-regulation in expression of these genes was detected in the proliferated hOBNSCs as compared to control group (MSI1 gene in differentiated cells). SOX2 gene showed the highest significant up-regulation level as compared to other proliferative genes. However, MSI1 gene showed the lowest expression level. No significant difference was detected in the expression of NES and NR4A1 genes ($P>0.05$).

Relative expression of differentiation-related genes in hOBN-SCs

To check whether hOBNSCs had the capability to differentiate into both neuronal and glial cell types, primary neurospheres were dissociated into single cells and induced to differentiate in medium lacking mitogens. Data obtained from qPCR revealed a significant ($P<0.05$) up-regulation in expression level of the 4 differentiation-related genes (FOXO4, CSPGS, MAP2, and GFAP) in hOBNSCs as compared to control group (FOXO4 gene in proliferative cells) (Figure 4). GFAP gene showed the highest significant up-regulation level as compared to other differentiated genes. However, FOXO4 gene showed the lowest expression level. No significant difference was observed between

the expression of MAP2 and CSPGS genes (P>0.05). Thus, it is likely that the OB harbors multipotent progenitor cells that can be differentiated to neurons, astrocytes, and oligodendrocytes.
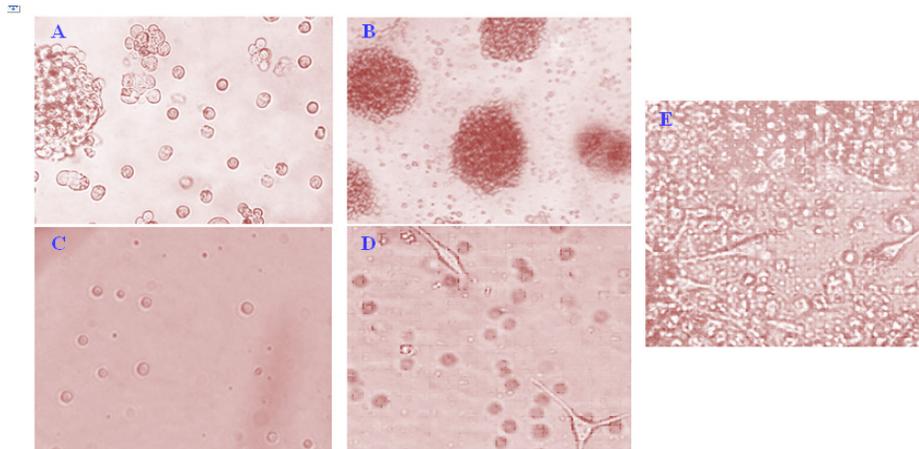

Fig. 1: Morphological characters of proliferated and differentiated hOBNSCs. (A) Neurospheres formed after one week of proliferation. (B) Typical neurospheres indicating the high proliferation ability of cultured cells. (C) First hours in the first day of differentiation showing perfect single cells. (D) Second day differentiation showing unipolar neuron differentiated from single cells. (E) The third day differentiation showing multipolar neuron making synapse to another neuron.
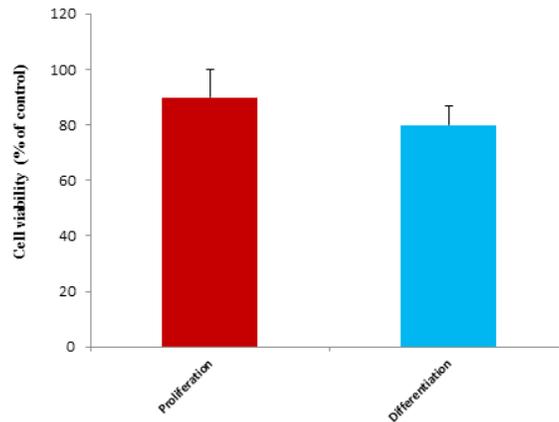

Fig. 2: Effects of differentiation media on human olfactory bulb neural stem cells (hOBNSCs) viability using MTT assay. Data are represented as mean ± SEM (n = 8).
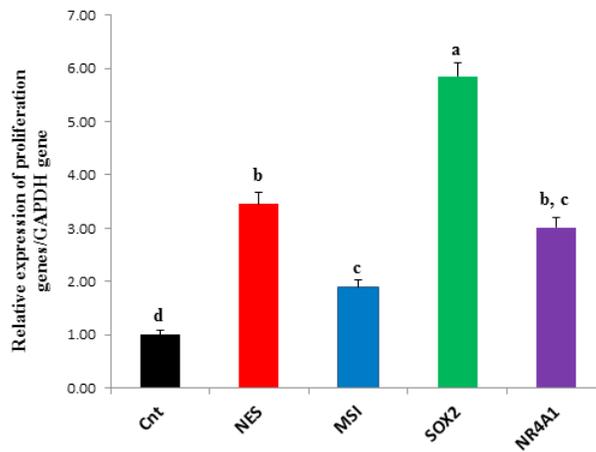

Fig. 3: Real-time quantitative PCR analysis of the expression of proliferation related genes in human olfactory bulb neural stem cells (hOBNSCs) as compared to control (Cnt). Values are expressed as means ± SEM (each group was tested in triplicate). Means carrying different superscript letters are significantly different (P<0.05).
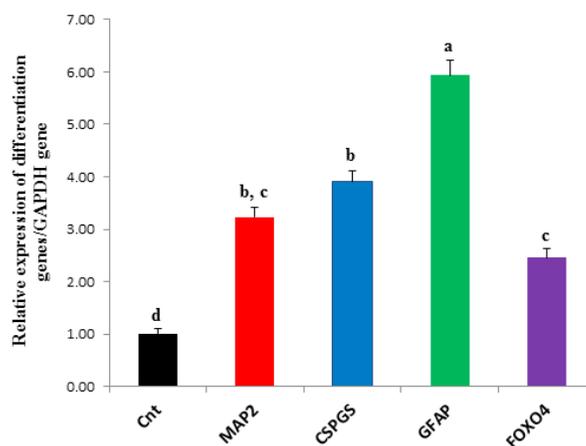
Fig. 4: Real-time quantitative PCR analysis of the expression of differentiation related genes in human olfactory bulb neural stem cells (hOBNSCs) as compared to the control (Cnt). Values are expressed as means ± SEM (each group was tested in triplicate). Means carrying different superscript letters are significantly different (P<0.05).

DISCUSSION

The main aim of the present study was to provide more details about the changes in the expression profile of some pro-liferation- and differentiation-related genes in hOBNSCs follow-ing addition and removal, respectively, of some trophic agents. Isolation of NSCs from the hOB has some advantages if com-pared to other sources of stem cells as induced pluripotent stem cells (iPSCs), and human embryonic stem cells (hESCs). Human OBNSC provides less active mature adult cells which might exclude the rapid proliferation and potential tumorigenic poten-tial of hESCs and iPSCs (Isobe *et al.*, 2014; Suzuki *et al.*, 2014). Besides, hESCs-derived oligodendrocytes precursor cells need more cytokines, demanding techniques, and longer time for dif-ferentiation (Hu *et al.*, 2009; Sundberg *et al.*, 2010). Thus, *in vitro* differentiation of adult hOBNSCs could be a promising tool to overcome some of the practical and ethical concerns of fetal tissue transplantation in neurodegenerative diseases.

Stopping the addition of neurotrophic agents, EGF, bFGF, and LIF inhibits hOBNSCs proliferation, but favors for differentiation. To check whether this withdrawal can affect cell viability, MTT assay was conducted and its results showed no significant effect on cell viability. This means that neither neuro-trophic agents' withdrawal nor addition to differentiation-induced factors has any effect on hOBNSCs cell viability but rather they directed the cells toward differentiation.

During proliferation phase, hOBNSCs have an increased level in the expression of proliferation-related genes (NES, NR4A1, SOX2, and MSI1) compared to control group (MSI1 gene in differentiation cells). Among these 4 genes, SOX2 showed the highest significant up-regulation level. In consistent with our findings, a previous study reported early activation of SOX2 in NSCs derived from adult human OB (Alizadeh *et al.*, 2017) and in the embryonic nervous system, predominantly in the proliferat-ing, undifferentiated precursors (Pevny and Nicolis, 2010). Unlike SOX2, MSI1 gene, which is a marker for neuronal progenitor cells (Uren *et al.*, 2015), showed the lowest expression level. Thus, we would expect a decrease in the proliferation of neuronal progeni-

tors as compared to other progenitors. The other two markers for undifferentiated neural progenitors, NES and NR4A1 showed a moderate up-regulation in the proliferated OBNSCs.

During differentiation phase, there is an up-regulation in expression level of the all 4 differentiation-related genes (FOXO4, CSPGS, MAP2, and GFAP) in hOBNSCs as compared to the control (FOXO4 gene in the proliferative cells). The astrocytes marker GFAP showed the highest significant up-regulation level as compared to other differentiation genes. In agreement with our results, it has been documented that during differentiation Nestin is down-regulated and replaced by GFAP in astrocytes (Michalczyk and Ziman, 2005). Therefore, the astrocytes may constitute the highest percent of the differentiated OBNSCs. The expression of GFAP and its polymerization to gliofilaments are important hall-marks of the process of astroglial morphological differentiation (Messam *et al.*, 2000). On the other hand, FOXO4 gene, which is a marker for mature oligodendrocytes, showed the lowest expres-sion level. This suggests the presence of few numbers of mature oligodendrocytes than astrocytes in the differentiated hOBNSCs. Moderate expression was observed for MAP2 (which is a marker for immature neurons) followed by CSPGS (which is a marker for immature oligodendrocytes). *In vitro* CSPG4 cells represent multipotent cells, since they are capable of generating oligoden-drocytes, neurons and astrocytes in the presence of specific growth factors. However, *in vivo*, CSPG4 cells in the adult intact central nervous system produce only CSPG4 cells and oligodendrocytes (Honsa *et al.*, 2016).

CONCLUSION

Data obtained from the differential expression of dif-ferentiation- related genes revealed that hOBNSCs can be differ-entiated into a large variety of neural cells. Among these cells, GFAP-expressing astrocytes constituted the highest population of cells followed by CSPGS-expressing immature oligodendrocytes, then MAP2-expressing immature neurons, and finally FOXO4-ex-pressing mature oligodendrocytes which constituted the lowest population of cells. These data will help us to control hOBNSCs

proliferation and differentiation *in vitro* via addition/deletion of specific growth factors. Further investigations are also required to study the functional characterization of these *in vitro* formed mature cells.

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