Generation of Cholinergic Neurons from Human Olfactory Bulb Neural Stem Cells Using BMP9 Treatment

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Abstract: The aim of the present study was to obtain a predominantly pure population of cholinergic neurons from human olfactory bulb neural stem cells (hOBNSCs) using diffusible ligands and to study the gene expression profile for the key genes involved in this transformation (CHAT, CHAT1, NF-L, βIIIITUB, MAP2, NGFR). Differentiation of hOBNSCs, which was initiated by BMP9, significantly increased expression of these genes, with highest expression in NGFR followed by CHAT, CHAT1, MAP2, NF-L, and lowest expression in βIIIITUB. These data provide, for the first time, the ability of hOBNSCs to differentiate in vitro into cholinergic neurons expressing CHAT, MAP2, NF-L, βIIIITUB, CHAT1, NGFR genes and this can help us to know the potential mechanism of cholinergic differentiation of hOBNSCs which will be helpful when these cells used in treatment of neurodegenerative diseases.

Keywords: human olfactory bulb, neural stem cells, cholinergic neurons, gene expression

1. Introduction

The Alzheimer’s disease (AD) is the most common age-related neurodegenerative disease estimated to affect approximately 30 million people worldwide (Holtzman et al., 2011). Most prevalent symptoms are confusion and memory loss caused by synaptic dysfunction and neuronal death. One of the primary neuronal populations affected are the cholinergic neurons, which are partly responsible for these cognitive deficits (Holtzman et al., 2011; Everitt and Robbins, 1997). The progress of AD research has been inhibited by lack of accurate models that recapitulate the complex facets of AD (Han et al., 2011). Recent advances in human pluripotent stem cell (hPSC) technology have made it possible to produce regionally specified neuronal populations affected by various neurodegenerative conditions, providing a novel source of human neurons for in vitro disease modeling (Nat and Dechant, 2011; Liu, 2011). It is essential that these neurons are phenotypically accurate and functional, and that they can also be used to model the embryonic differentiation of these populations, which is applicable to their regenerative potential (Liu, 2011).

Previous studies have demonstrated the generation of cholinergic neurons, with a potential basal forebrain phenotype (Nilbratt et al., 2010; Bissonnette et al., 2011). However, high levels of specific extrinsic factors were used to direct differentiation, of which their exact role remains unclear. The overall aim of the present study was to generate a predominantly pure population of cholinergic neurons (CNs) from hOBNSC using diffusible ligands and to study the gene expression profile for the key genes involved in this transformation.

2. Materials and Methods

Isolation and culturing of hOBNSCs

The olfactory bulbs (OBs) were harvested from adult patients undergoing craniotomy at the Institute of Neurosurgery, Catholic University, Rome, Italy. Informed consent was obtained according to protocols approved by the Ethical Committee of the Catholic University. Immediately after removal, the OBs were dissociated in Papain 0.1% (Sigma-Aldrich, St. Louis, MO) for 30 min at 37 °C. Dissociated cells were cultured as previously described by (Marei et al., 2016) to obtain neurospheres.

Generation and programming of neural progenitors with BMP9 treatment

Neurospheres were moved to media supplemented with 100 ng ml⁻¹ FGF8 and 200 ng ml⁻¹ SHH for 72 hours. Neurospheres were dissociated in 500 μl acutase at 37°C for 10 minutes, and were then plated on poly-D-lysine (PDL)-laminin in neuron media 1 for 5 days. For the first 24 hours, media was supplemented with 100 ng ml⁻¹ SHH, 100 ng ml⁻¹ FGF8, and 10 ng ml⁻¹ BMP9. For the next 48 hours, media was supplemented with only BMP9. Cells were moved to neuron media 2, which has been shown optimal for the growth of CN, from D5 to D16–19. From D5 to D10, media was supplemented with 2.66 μM arabinosylcytosine (AraC) to eliminate the growth of bFGF-responsive cells arising from fragments of undissociated neurospheres.

Detection of relative gene expression by Real time PCR

Total RNA was isolated from hOBNSCs using Gene JET RNA Purification Kit (Thermo Scientific, # K0731, USA) according to the manufacturer’s protocol. Total RNA (5μg) was reverse transcribed using RevertAid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) to produce cDNA. The latter was used as a template to determine the relative expression of the candidate genes using StepOnePlus real time PCR system (Applied
approach provides a superior and more accurate approach, where the cells establish their own requirement for a developmental model; a reductionist generating high numbers of CNs in keeping with a main aim of the present study was to provide a method of associated with the use of human embryonic or heterologous diseases that avoids the ethical and moral questions for transplantation the neuronal phenotype. Due to their ability to self-renew and to differentiate towards the neuronal phenotype, 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). The main aim of the present study was to provide a method of generating high numbers of CNs in keeping with a requirement for a developmental model; a reductionist approach, where the cells establish their own developmental cues, in parallel to the developing embryo. Our reasoning for the advantages of this are two-fold; firstly we believe this approach provides a superior and more accurate developmental model to study the innate acquisition of basal forebrain cholinergic fate; furthermore by using intrinsic cues from development we would suggest that the resulting neuronal progeny would be more similar to those in the developing brain. Therefore our work provides a model of human CN population, which meets the criteria required for a multipurpose model of the basal forebrain cholinergic system, both developmentally and also in a mature functional context.

During differentiation, there is an up-regulation in expression level of the all 6 differentiation-related genes (CHAT, CHAT1, NF-L, βIIITUB, MAP2, NGFR) in hOBNSCs as compared to the control (the same genes but in the proliferative cells). The cholinergic marker NGFR showed the highest significant up-regulation level as compared to other differentiation genes. This finding was in agreement with (Michalczyk & Ziman, 2005). Therefore, the cholinergic neurons may constitute the highest percent of the differentiated hOBNSCs. In addition, CHAT and CHAT1 expression is important hallmarks of the process of cholinergic neurons (Messam et al., 2000). We also found a significant up-regulation of these two genes in a level lower than NGFR but higher than the other three genes (NF-L, βIIITUB, MAP2). On the other hand, βIIITUB gene, which is also a marker for cholinergic neurons, showed the lowest expression level among all differentiation genes.

**3. Results**

To check whether hOBNSCs had the capability to differentiate into cholinergic neurons, neurospheres were dissociated into single cells and induced to differentiate in medium containing SHH and BMP9. Data obtained from qPCR revealed a significant (P<0.05) up-regulation in expression level of the 6 differentiation genes (CHAT, MAP2, NF-L, βIIITUB, CHAT1, NGFR) in hOBNSCs as compared to control group (the 6 differentiation genes in proliferative cells) (Fig. 1). NGFR gene showed the highest significant up-regulation level as compared to other differentiated genes. However, βIIITUB gene showed the lowest expression level. No significant difference was observed between the expression of MAP2, NF-L, and βIIITUB genes (P>0.05). On the other hand, CHAT expression was significantly upregulated as compared to CHAT1 expression. Thus, it is likely that the OB harbors progenitor cells that can be differentiated to cholinergic neurons.

**4. Discussion**

Due to their ability to self-renew and to differentiate towards the neuronal phenotype, 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). The main aim of the present study was to provide a method of generating high numbers of CNs in keeping with a requirement for a developmental model; a reductionist approach, where the cells establish their own developmental cues, in parallel to the developing embryo. Our reasoning for the advantages of this are two-fold; firstly we believe this approach provides a superior and more accurate
### Table 1: Forward and reverse primers sequence used in real time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>CHAT</td>
<td>CTGTGCCCCCTTCTAGAGC</td>
<td>CAAGGGTGGTGTCGCTG</td>
</tr>
<tr>
<td>CHAT1</td>
<td>ACTGGGTGTCGATGCTA</td>
<td>TTGGAACCATTTTGACTAT</td>
</tr>
<tr>
<td>NF-L</td>
<td>CTAGGCCCTTTGCAACTACACT</td>
<td>CCTAAAGTCTAATGGCGTCTG</td>
</tr>
<tr>
<td>βIII Tub</td>
<td>CAGATGTGATGCGACGAAGA</td>
<td>GGGATCAAATCCACGAAAGTA</td>
</tr>
<tr>
<td>NGFR</td>
<td>CCGAGGCAACCCCGACAACC</td>
<td>GGGGTGCATCCAGTTCCTCGT</td>
</tr>
<tr>
<td>MAP2</td>
<td>GGGTCATCTCCGCTTCTCGG</td>
<td>CCCAATCAATTCTTCTCGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTT CAG TCA GCC GCA TC</td>
<td>CGA CCA AAT CCG TG ACT CCG</td>
</tr>
</tbody>
</table>

![Graphs showing relative expression of various genes](image-url)
Figure 1: Graphical presentation of qPCR analysis of the expression of the 6 differentiation-related genes (Dif) in hOBNSCs compared to control (Pro). Columns with different letters are significantly different at P < 0.05.