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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, βIIITUB, MAP2, NGFR). Differentiation of hOBNSCs, which was initiated by of BMP9, significantly increased expression of these genes, with highest expression in NGFR followed by CHAT, CHAT1, MAP2, NF-L, and lowest expression in βIIITUB. These data provide, for the first time, the ability of hOBNSCs to differentiate in vitro into cholinergic neurons expressing CHAT, MAP2, NF-L, βIIITUB, 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 Alzihmer'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 accutase at 37ºC 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 lM 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

Biosystem, USA). The primers were designed by Primer 5.0 software (Table 1). The housekeeping gene GAPDH was used as a reference to calculate fold change in target gene expression. A 25-µL PCR mix was prepared by adding 12.5 µL of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 2 µL of cDNA template, 1 µL forward primer, 1 µL reverse primer, and 8. 5 µL of nuclease free water. The thermal cycling conditions were as follows: 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, extension at 72 °C for 30 s. At the end of the last cycle, the temperature was increased from 63 to 95 °C for melting curve analysis. The cycle threshold (Ct) values were calculated for target genes and the housekeeping gene, and relative gene expression was determined using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All the data were expressed as means ±S.E. The statistical significance was evaluated by Student t-test using SPSS, 18.0 software, 2011. Values were considered statistically significant when $P<0.05$.

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 ($P\leq0.05$) 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 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.

5. Conclusion

Data obtained from the expression of differentiation- related genes revealed that hOBNSCs can be differentiated into cholinergic neural cells. Among these cells, NGFR-expressing cells constituted the highest population of cells followed by *CHAT*, *CHAT*1, *MAP2*, *NF-L* and finally *βIIITUB*. These data will enable us to understand how to control hOBNSCs differentiation *in vitro*.

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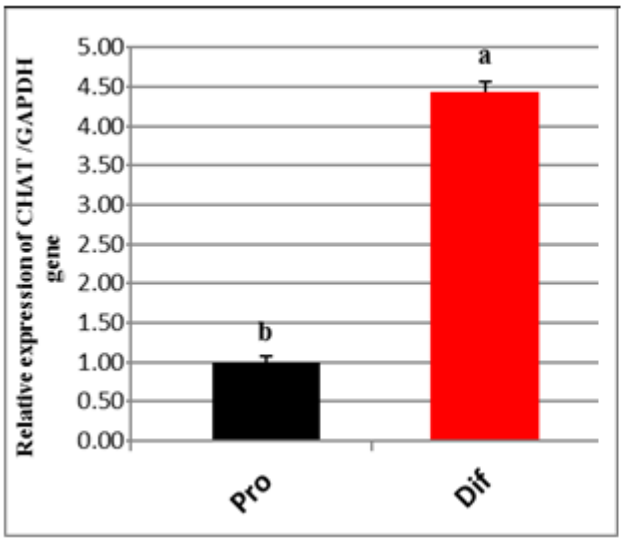
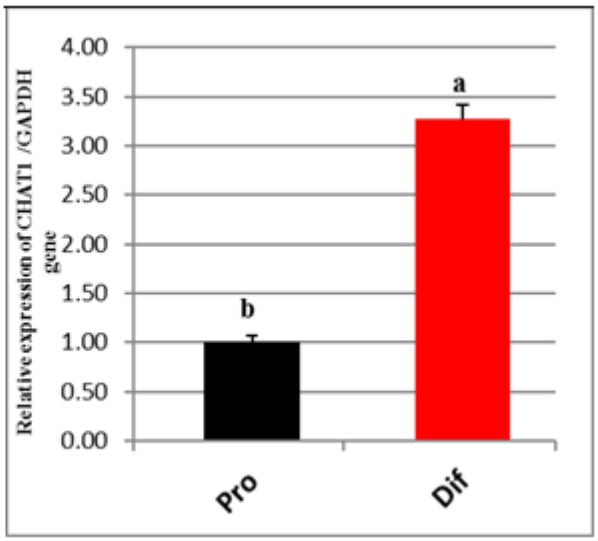
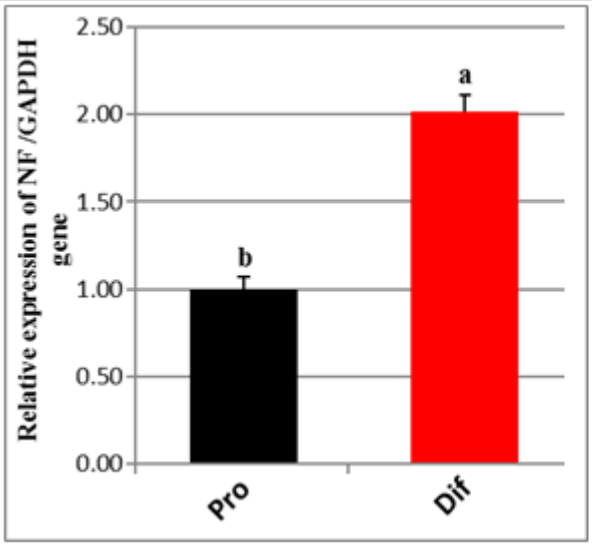
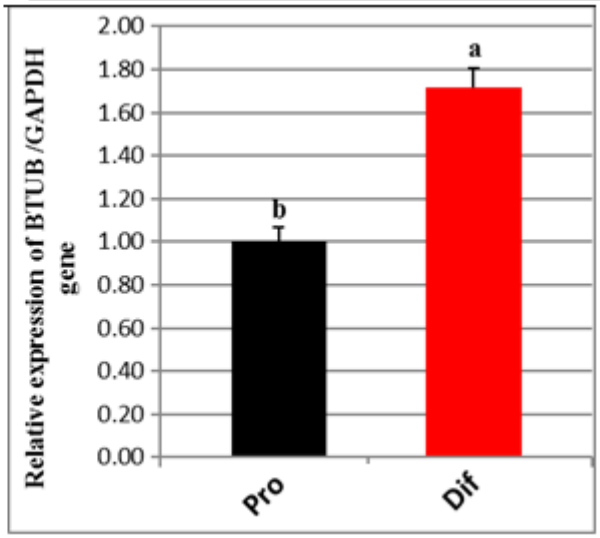
Table 1: Forward and reverse primers sequence used in real time PCR.

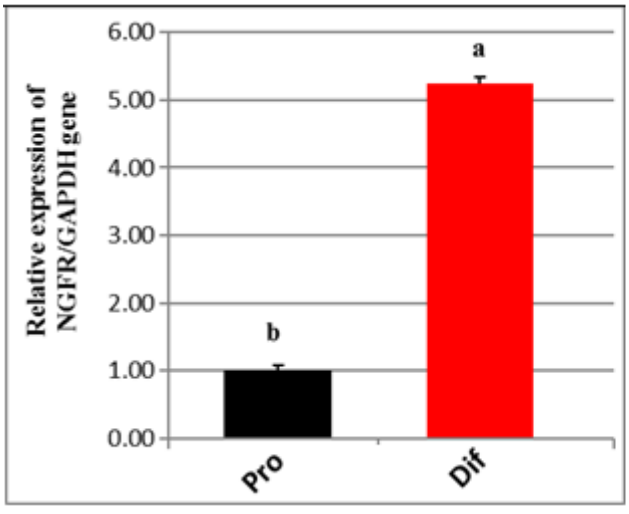
Gene	Forward primer (′5 ------ ′3)	Reverse primer (′5 ------ ′3)
CHAT	CTGTGCCCCCTTCTAGAGC	CAAGGTTGGTGTCCCTGG
CHAT1	ACTGGGTGTCTGAGTACTGG	TTGGAAGCCATTTTGACTAT
NF-L	CTAGGCCTTTGCAACTACACTAC	CCTAAGGTTTAATGGCTGCTG
βIIITUB	CAGATGTTCGATGCCAAGAA	GGGATCCACTCCACGAAGTA
NGFR	CCGAGGCACCACCGACAACC	GGGCGTCTGGTTCACTGGCC
MAP2	GGGTGCATCCAGTTTCTGCG	CCCAATCAATGCTTCCTCGGT
GAPDH	GTT CGA CAG TCA GCC GCA TC	CGA CCA AAT CCG TTG ACT CCG

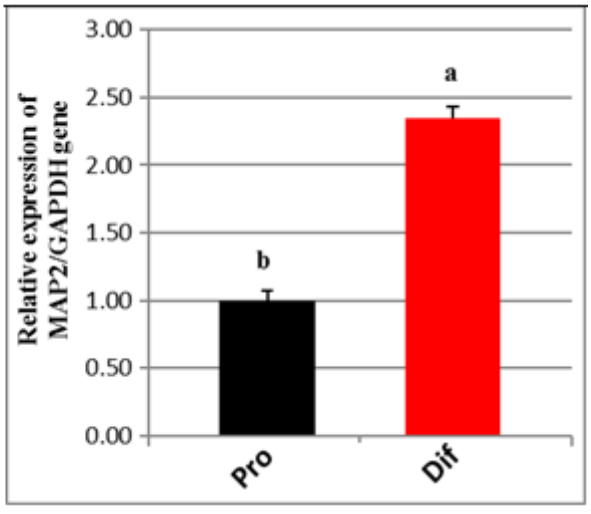












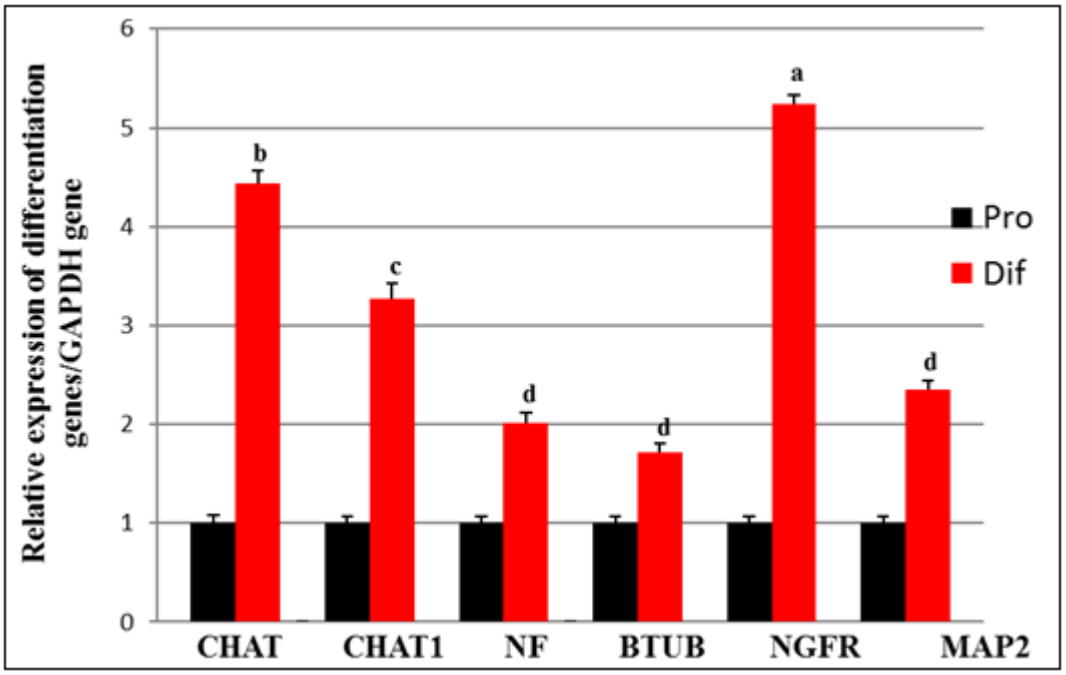


Figure 1: Graphical presentation of qPCR analysis of the expression of the 6 differentiation- related genes (Dif) in hOBNSCs compared to control (Pro). Columns with different letters are significantly different at P <0.05.