Cellular & Molecular Biology
Cell. Mol. Biol. 2015; 61 (2): 56-59
Published online May 16, 2015 (http://www.cellmolbiol.com)
Received on April 7, 2015, Accepted on May 8, 2015.
doi : 10.14715/cmb/2015.61.2.10

Oligodendrocyte progenitor cells differentiation of nuclear transferred mouse embryonic stem cells

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Abstract

Central nervous system (CNS) injuries cause to variable disorders in people around the world without any decisive treatment. Use of embryonic stem cells (ESCs) would be helpful in repairing of neural system damages. Somatic cell nuclear transfer (SCNT) is a way for implanting ESCs with lowest possible rejection. In the present study, mouse nuclear transfers ESCs (ntESCs) ability in differentiation to oligodendrocyte progenitor cells (OPCs) was investigated by morphological study, RT-PCR and flow cytometry analysis. Bi-polar and tri-polar, OPCs were observed in stem cells cultured in differentiation medium after four weeks. Result of gene expression analysis demonstrated that differentiated stem cells were expressed most important OPCs related gene markers after differentiation period. Moreover, flow cytometry analysis carried out to confirm other results that showed differentiated stem cells significantly expressed NG2 and O4 as critical OPC surface markers. Taken together, it seems that mouse ntESCs showed highly potential for OPC differentiation and could be an appropriate candidate for stem cell therapies.

Key words: Somatic cell nuclear transfer, embryonic stem cells, Oligodendrocyte progenitor cells.

Introduction

The central nervous system (CNS) is composed of two parts including the brain and spinal cord, which are responsible for processing all the information that arrives from our senses. As well as keeping our organs, reflex functions, directing our thoughts and feelings and movements upon it.

CNS injuries are described as a common set of damages to the brain or spinal cord (1). Currently, it has been estimated that approximately 2.5 million people around the world are suffering from Spinal Cord injury (SCI) (2). Less than 1% of SCI patient could be recovered by regularly available methods such as physiotherapy and some medications that sometimes are abortive (3). Demyelination of healthy axons is the core problem in SCI and other traumatic injuries to CNS (1).Significantly, axonal growth regeneration inhibitors and glial scars are the corresponding suppressive causes for regeneration of adult's nervous system (2, 4-6).

Oligodendrocytes are the most important factors in the survival of axons and facilitate the rapid transfer of the action potential in neurons by producing myelin in the CNS. These cells are generated from oligodendroglial progenitor cells (OPCs), which originate from the neuroepithelium during embryonic development.

Stem cells are the main component of tissue engineering and regenerative medicine. Several type of stem cells are classified based on the developmental stage (adult and embryonic) or them differentiation potential (toti-, multi- or uni-potent). Stem cells are undifferentiated cells with self renewal indefinitely and differentiation potential into different cell type.

Several cell-based therapies are reported for SCI by researchers in two past decades. OPCs are one of the available choices for SCI treatment (7). OPCs can be isolated from the CNS directly (8, 9), or differentiation of neural precursor cells (NPCs) and embryonic stem cells (ESCs) in vitro (10). OPC derived from ESCs is one of the best candidates for transplantation therapies to enhance CNS remyelination.

ESCs are pluripotent stem cells that are genetically normal following proliferation in vitro. They have capability to differentiate to all lineage (11). However, transplantation of differentiated cells from ESCs elicited immune responses in the host body. The practical method that can be used to solve this problem is nuclear transfer embryonic stem cell (ntESC) and induced pluripotent stem cells (iPSCs).To obtain ntESCs somatic cell nucleus of a donor is inserted into a removed-nucleus oocyte of the host (12). The host's cell begins to reprogram inserted nucleus and developed until the blastocyst stage. ESCs obtained from these blastocysts are now ready to differentiate to variable cell lines such as oligodendrocytes. In the present study, we evaluated the potential of ntESC differentiation to OPCs.

Materials and methods

Culture of embryonic stem cells

OPCs generated by previously published method using mouse (ntESC) (a Gift from Stem cells Technology Research Center, Tehran, Iran) (13). Mouse ntESCs were cultured in ES medium containing knockout-DMEM medium (Gibco, USA) supplemented with 1% non-essential amino acids (Gibco, USA), 0.1 mM 2-mercaptoethanol (Sigma, USA), 2 mM L-Glutamine (Gibco, USA), 1% Penicillin/Streptomycin (Gibco,

USA), 10% Fetal Bovine Serum (FBS) embryonic stem cell-qualified (Gibco, USA) and 1000 IU/ml mouse leukemia inhibitory factor (mLIF) (ESGRO, Chemicon, Germany). Every 4–5 days, mouse ntESC colonies were detached with 0.25 % Trypsin/EDTA and replated onto inactivated MEF for expansion.

Embryoid body formation and OPC differentiation

NtESC colonies were dissociated and then transferred into non-treated 6-well plates (Jet Biofil, China) for 4 days in embryoid body (EB) medium consisting of ES medium without mLIF. Medium was replaced by ES medium supplemented with 1 µM retinoic acid (RA) (Sigma, USA) and 50 nM purmorphamine (Santacruz ,USA) and EBs were cultured for another 4 days. At day 8, EBs were disaggregated using trypsin/EDTA (5min, 37°C) and plated on 0.2% gelatin-coated 6-well plates in OPC medium (DMEM/F12 supplemented with 1% N2 medium, fibroblast growth factor-2 (FGF-2, 20 ng/ml), 1mM sodium pyruvate, 1% non-essential amino acids and 2mM L-glutamine). The medium was replaced every two days. The cells were trypsinized and replated approximately every week if they had become confluent. At day 28 after induction, cells were used for flow cytometry and gene expression analysis.

RNA extraction and RT-PCR analysis of gene expression

After period of study, the expression of TATA box binding protein (TBP) as internal control, Nestin, Olig2, myelin basic protein (MBP), and Neural/Glial Antigen 2 (NG2) genes were evaluated in differentiated cells and compared with controls.

Total RNA was extracted by using Qiazol reagent (Qiagen) according to manufacturer's protocol. Standard RT was performed using the Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. The primer sequences are illustrated in Table 1.

Flow cytometry

Flow cytometry analysis was performed as previously described. Differentiated cells were detached from the bottom of plates by using 0.25% trypsin/EDTA and the singled cells were washed with phosphate buffer saline (PBS). After washing and preparation stages, Anti-NG2 (1:200,Abcam,USA), and Anti-Oligodendrocyte marker O4 (2.5µg/10^6 cells, Sigma, USA) were added to cells and incubated at 4 ºC for overnight. After incubation cells were centrifuged for 5 minutes at 1200 RPM. Secondary donkey anti-rabbit (1:200 phycoerythrin (PE) conjugated, Santacruz, USA), and goat anti-mouse (1:200 phycoerythrin (PE) conjugated, Santacruz, USA) for NG2 and O4 were added to cells respectively and placed at room temperature in darkness for 45 minutes. Finally cells were centrifuged again after incubation and analyzed with Attune Accoustic Focusing Cytometer (Applied Biosystems, USA) and FlowJo software (Tree Star Inc., USA).

Table 1. Primers used in RT-PCR.

Gene	Primer sequence(F,R, 5´-> 3´)	Product length (bp)
TBP	CCACAACTCTTCCATTCTC CCAAGATTCACGGTAGATAC	170
Nestin	AGCAGGGTCTACAGAGTCAG GTCCTGTATGTAGCCACTTCC	99
MBP	TCAAGAACATTGTGACACCTC GCCTCCGTAGCCAAATC	125
Olig2	ATTCACATTCGGAAGGTTG GACGATGGGCGACTAGAC	98
HPRT1	CCTGGCGTCGTGATTAGTG TCAGTCCTGTCCATAATTAGTCC	125

Results

Stem cell characterization

Mouse ntESCs were seeded on inactivated MEF and after 3 days ntES colonies showed in Fig.1A.The ntES colonies were asymmetrical, and indented. To initiate OPC differentiation, EB formation was allowed to occur after 4 days and then RA and puromorphamine were added to their medium until 8 days (Fig 1B). Trypsinized EBs were transfer to gelatinized six-well culture plates under differentiation medium. So EBs were adhered to the surface of culture plates and started to proliferating and expanded in a radial direction (Fig.2A).Morphological study at day 13 showed that stem cells have growth in colony form still (Fig 2B), but in day 17 it was observed that growth in colony form was ruptured and the cells are individually separating from oligospheres and then dispersed at the surface of culture plates. After 28 days of induction, a grid of OPC cells was then

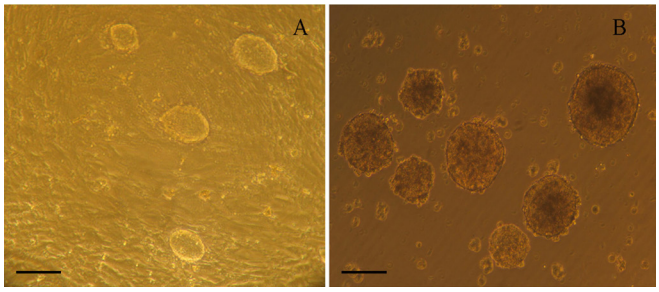

Figure 1. Photograph of ntES colonies after cultured on MEF feeder layer (A) and formed ntES embryonic bodies after 8 days (B), all scale Bars 100µm.

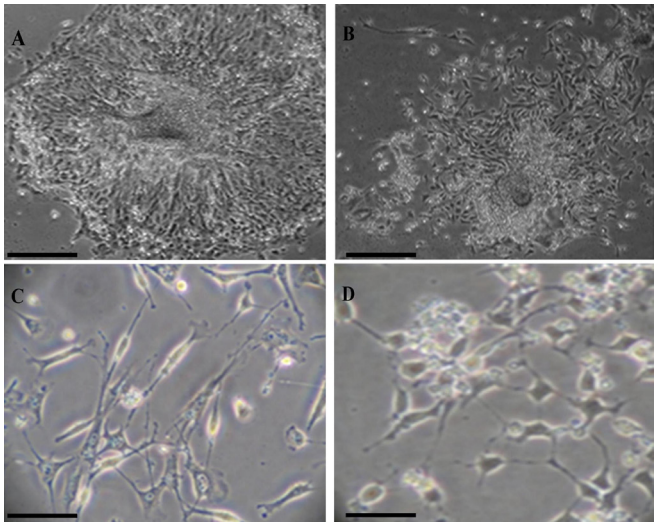

Figure 2. Morphological study of EBs after adhered to the surface of culture plates and expanded in a radial direction during 13 days (A and B) and OPCs differentiated ntESCs were appeared to spherical shape with bipolar or tripolar angles after 28 days of induction times (E and F), all scale Bars 100µm.

appeared after separation of spherical cells as showed in Fig 2E and F. OPCs were spherical with bipolar or tripolar angles.

Gene expression analysis

For more evaluation of OPC differentiated from mouse ntESCs, RT-PCR analysis was used to investigate the expression of four important OPC related genes including Nestin, Olig2, MBP, and NG2. The Olig2 and NG2 as OPC index markers showed high level of expression. In contrast, MBP which is the marker of mature oligodendrocyte cells had low expression (Figure 3).

Flow cytometry

O4 and NG2 were examined as the superficial markers of OPC. At the end of the differentiation process, the cells were investigated using flow cytometry and according to the results NG2 and O4 indicated 68.2% and 21.1% expression respectively. It should be pointed out that O4 is a pre-oligo cell marker (Figure 4).

Discussion

In comparison with other types of stem cells, the application of ntESCs will efficiently decrease the possibility of rejection after transplantation. This property is highly interested and important in the field of cell therapy and regenerative medicine. In this study, the ntESC were differentiated into OPCs under inductive medium in vitro. Wakayama and colleagues demonstrated that the ESCs lines derived from SCNT technique had the capacity of differentiation into all chimeric mice embryonic layers in in-vitro (12). This was the first investigation that showed ntESCs have the same capabilities as

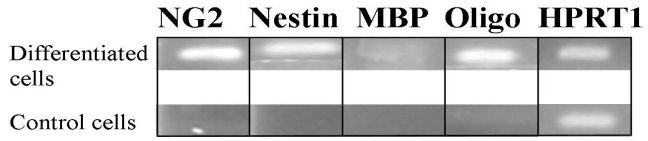

Figure 3. RT-PCR analysis. Expression of important OPC-related gene markers included Olig2, NG2, Nestin and MBP after 28 days cultured in differentiation medium.

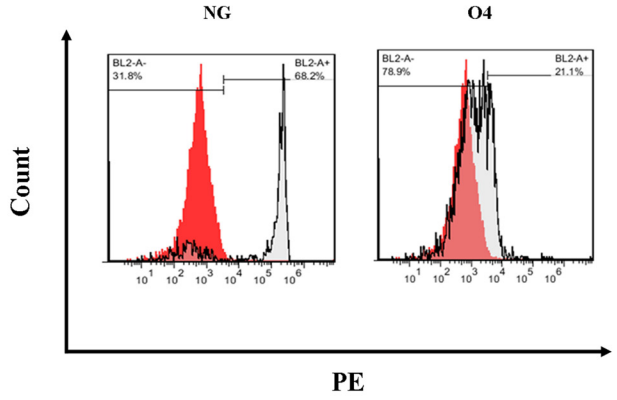

Figure 4. The level of released antibody against O4 and NG2 surface markers after the end of differentiation process. The vertical axis shows the fluorescent changes and horizontal axis shows the wavelength of the laser light which read the color. The gray light in emission spectrum has a pick in area of 10^4-10^5. The red light was used as an isotype of negative control.

embryonic stem cells derived from naturally fertilized embryos. According to the protocol which was reported by William et al. (14), b-mercaptoethanol and LIF can be used to prevent the differentiation of ESCs in culture medium. LIF is a subunit of IL-6 family that help myelin regeneration by means of its inhibitory effect on the cell differentiation in a desired stage (9, 15). Furthermore, treatment of EBs with RA can lead to suppress the mesodermal gene expression and commitment of EBs to neuronal precursor cells (16). Neuronal precursor cells produce a homogeneous population of neurons that have synaptic connection with each other. Several studies reported that EBs of ESCs in the presence of growth factors such as PDGF and FGF-2 can produce homogenous population of glial cells, and these cells differentiate into OPCs. RA is one of the stimulating factors for creating cells with neuronal cells morphology (16) and induce differentiation of stem cells into CNS cells (17, 18). In this study, OPCs differentiation of ntESCs was investigated using gene expression and flow cytometry analysis. Four important OPCs related gene markers including Nestin, MBP, Olig2 and NG2 were investigated after the period of study. OPC differentiation of ntESCs was confirmed by the expression of these genes in differentiated cells. To confirm the differentiation process,expression of NG2 and O4 surface markers were detected in differentiated cells using flow cytometry. The OPCs are classified as a type of the CNS cells that make myelin. OPCs maintain their ability for myelin regeneration but some post-meiotic cells such as mature and immature oligodendrocyte have not this ability (19). Several studies reported that transplantation of OPCs accelerates the improvement of motor function and enhances remyelination in SCI animal models (20-22). Kang et al. demonstrated that OPCs derived from rat adipose tissue-derived stromal cells survived and migrated into the injured region after intravenous injection in SCI rat model and the behavioral evaluation showed that the locomotors functions of OPC auto-grafted SCI rats were significantly restored (23). In another study, OPCs derived from hESC were engrafted into adult rat SCI and the results revealed that transplanted cells survived, redistributed over short distances, and differentiated into oligodendrocytes. Although time of transplantation plays critical role in the rate of improvement, as enhanced remyelination and substantially improved locomotors ability observed in rats that received OPCs 7 days after injury and in contrast, no enhanced remyelination or locomotors recovery was observed in rats that received these cells 10 months after injury (24).

In conclusion, ntESCs can well be differentiated into OPCs. These stem cells may have potential for application in the treatment of diseases caused by destruction of myelin. It may be considered as a solution for decreasing cell rejection.

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