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Production of stable *GFP*-expressing neural cells from P19 embryonal carcinoma stem cells

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ABSTRACT

Murine P19 embryonal carcinoma (EC) cells are convenient to differentiate into all germ layer derivatives. One of the advantages of P19 cells is that the exogenous DNA can be easily inserted into them. Here, at the first part of this study, we generated stable *GFP*-expressing P19 cells (P19-GFP$^+$). FACS and western-blot analysis confirmed stable expression of *GFP* in the cells. We previously demonstrated the efficient induction of neuronal differentiation from mouse ES and EC cells by application of a neuroprotective drug, selegiline In the second part of this study selegiline was used to induce differentiation of P19-GFP$^+$ into stable *GFP*-expressing neuron-like cells. Cresyl violet staining confirmed neuronal morphology of the differentiated cells. Furthermore, real-time PCR and immunoflourescence approved the expression of neuron specific markers. P19-GFP$^+$ cells were able to survive, migrate and integrated into host tissues when transplanted to developing chick embryo CNS. The obtained live *GFP*-expressing cells can be used as an abundant source of developmentally pluripotent material for transplantation studies, investigating the cellular and molecular aspects of early differentiation.

1. Introduction

Embryonic stem (ES) and embryonal carcinoma (EC) cells are pluripotent cells which can differentiate into all the germ layer derivatives. Examples of undifferentiated EC cell lines are the human Tera-2 [1], and mouse F9 [2] and P19 EC cells [3]. Similar to the other EC cells, P19 is immortal and rapidly proliferates in culture [4]. On non-adherent surfaces the EC cells stick to each other and form small aggregates, embryoid bodies (EBs). Non-toxic concentrations of some drugs can be added easily to P19 cultures to induce efficient differentiation of the cells. The most effective drugs in differentiation induction of P19 cells are retinoic acid (RA) [5] and dimethyl sulfoxide (DMSO) [6]. Our previous studies showed that neural differentiation of a mouse ES line (CCE) and P19 EC cells can be efficiently induced by a pharmacological neuroprotective drug, selegiline (deprenyl) [7,8]. Selegiline is a good candidate in treatment of neurotoxicity which is used clinically in Parkinson's disease [9].

One of the major advantages of P19 cells is that they are excellent recipients of DNA transfected by calcium phosphate or electroporation procedures [3]. Green fluorescent protein (*GFP*) as a reporter gene encodes a non-toxic protein [10,11]. This protein exhibits intrinsic fluorescence, does not dependent on cofactors or substrates and can be utilized in a variety of species [12,13]. A mutant version of *GFP*, enhanced *GFP* (*eGFP*) is optimized for microscopy and flow cytometry detection [14]. Labeling stem cells

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with reporter genes such as *GFP* before transplantation has important applications in basic researches and stem cell biology [15,16].

In this study, we generated stably transfected P19 cells (P19-GFP$^+$) with a vector containing *eGFP*. Selegiline was then used to induce cell differentiation into stable *GFP*-expressing neuron-like cells. Finally, to investigate cell ability to survive, migrate, integrate and differentiate in host tissues, the selegiline treated P19-GFP$^+$ cells were transplanted to the developing chick embryo central nervous system (CNS).

2. Materials and methods

2.1. Plasmids

The following plasmids were used: pML8 (encods *puromycin* resistance gene and *eGFP* under *Pgk-1* promoter control) and pB17 (containing a fragment of *pgk-1* gene). All plasmids were kindly donated by Dr. McBurney's laboratory (Ottawa Regional Cancer Center, Ottawa, Canada).

2.2. Culture of P19 cells and their transfection

P19 cells were plated at concentration of 1.5 $\times$ 10^6 count and transfected with 5 μg of circular plasmid by calcium phosphate ($CaPO_4$) co-precipitation method [17]. Briefly, the cells were expanded 24 h before transfection and solution of $CaPO_4$–DNA was added dropwise onto them. They were incubated for 7–9 h at 37 °C in 5% CO_2 and then the growth medium was replaced by fresh α-MEM (Minimum Essential Medium, Gibco-BRL, Carlsbad, CA, 11900073). A medium containing 2 μg/ml *puromycin* (*puro*) was used for stable transfection. After 8 days post transfection, individual GFP$^+$ colonies were picked and expanded for more than 6 month (mo). Fluorescent intensity was visualized by fluorescence microscopy in living P19-GFP$^+$cells, 8 days post transfection.

2.3. Hematoxylin and eosin staining

The adherent cells were fixed, rehydrated and rinsed in PBS. They were then incubated in hematoxylin for 15 min and counterstained in 1% Eosin for 30 s. The cells were dehydrated and cleared in Xylene and finally mounted for bright light imaging.

2.4. Fluorescent-activated cell sorting (FACS) of P19-GFP expressing cells

For flow cytometry, suspension of single cells was prepared to analyze *GFP* expression, directly. Untransfected cells were used to adjust detector settings. Fluorescent intensity determination was performed on FACS (LSR, Becton Dickenson) with 10,000 events per sample. The cells were gated for GFP$^+$ signals. The data acquisition and analysis were carried out with CELLQUEST software (Becton Dickenson, USA).

2.5. Western-blot of GFP protein in P19 cells

Protein concentration of the cell extracts was determined according to the Bradford (DC Protein Assay; Bio-Rad, Canada). Equal amounts of protein were transferred to nitrocellulose membranes and then blocked and incubated with anti-GFP antibody (rabbit anti-GFP antibody, dilution 1/2000, Sigma, G1544, USA). Detection of secondary antibody was carried out using goat anti-rabbit horseradish peroxidase (HRP)-conjugated (Santa Cruz, USA). The membranes were finally exposed to X-ray film (Kodak MR-1) in a range of 30 s to 5 min.

2.6. Neuronal induction of P19-GFP+cells

Undifferentiated stable P19-GFP$^+$ cells were dissociated and cultured in 20 μl hanging drops to produce EBs. The resulting EBs were then cultured in α-MEM supplemented by 3% fetal bovine serum (FBS, Gibco, 10270-106). To induce neuronal lineage differentiation of EBs discrete concentration of selegiline (10^{-8} M, Zahravi Pharmaceutical Company, Tabriz, Iran) was applied [7,8].

2.7. Cresyl violet staining

The adherent cells were fixed, dehydrated, washed and then incubated in stain solution (0.25% cresyl violet, 0.8% glacial acetic acid, 0.6 mM sodium acetate) [18] for 3–10 min. The stained cells were washed and then mounted for examination.

2.8. Real-time polymerase chain reaction

Pluripotency stem cell marker, Oct3/4 (POU class 5 homeobox 1), as well as neuroepithelial (nestin) and neural specific (synaptophysin) markers, were evaluated by QPCR. Total RNA was extracted from the cells using Qiazol lysis reagent (Qiagen, 79306). cDNA synthesis was carried out by PrimeScript™ RT reagent Kit (Takara cDNA kit, RR037A_e.v1112Da) using SYBR Premix Ex Taq (TaKaRa, RR081Q) and specific primers (Table 1). To perform RT–PCR assays StepOnePlus™ Real-Time PCR System was used. RT samples, negative controls (no primers or no template) and β-2 microglobulin (β-2M, as housekeeping gene) were run together. To analyze the PCR reaction efficiency for each gene tested, standard curves were used. A melt-curve analysis was performed at the end of each reaction. Expression levels were normalized to individual β-2M (as internal control). The profile was obtained by plotting relative gene expression levels comparing to undifferentiated P19 EC cells.

For statistical analysis the differences between the mean values were compared using Student's t-test for two groups and one-way analysis of variance (ANOVA) and Duncan's Test for more than two groups. Results are reported as mean ± SD (standard deviation) and $p < 0.05$ was considered to be statistically significant.

2.9. In ovo surgery and cell transplantation

Fertile White Leghorn chick eggs were provided from a local supplier (Zagros, Shahrekord, Iran) and incubated at 38 °C in a humidified incubator for about three days. Staging of the embryos were carried out according to Hamburger and Hamilton [19]. Under sterile conditions, a window was made in the egg shell. About 200 cells were transplanted into the brain vesicle of chick embryos at stage 18–20. The eggs were sealed and then incubated for additional 1–3 days. At the appropriate time the chick embryos were fixed, dehydrated in ethanol and embedded in paraffin. Five μm parasagital sections were prepared and double stained with

Table 1
Real time PCR Primer Sequences.

Gene	Primer sequences	Genbank accession
Nestin	F: 5′-TCCGGGCCCCTGAAGTCGAG-3′ R: 5′-CCAGGGCTTCCACAGCCAGC-3′	NM_016701
Synaptophysin	F: 5′-CATTCATGCGCGCACCTCCA-3′ R: 5′-TTGCTGCCCATAGTCGCCCT-3′	NM_009305
Oct3/4	F: 5′-TTTCTGAAGTGCCCGAAGCCCT-3′ R: 5′-TTCCATAGCCTGGGGTGCCAAA-3	NC_000083
β-2M	F: 5′-AGTCGTCAGCATGGCTCGCT-3′ R: 5′-TGAGGCGGGTGGAACTGTGT-3′	NM_009735