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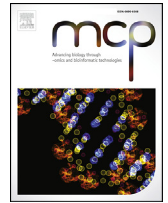

Production of stable *GFP*-expressing neural cells from P19 embryonal carcinoma stem cells

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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.

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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 1.5×10^6 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

antibodies.

2.10. Immunoflourescence

The primary antibodies including mouse anti-synaptophysin monoclonal antibody (Abcam, San Francisco, CA, ab8049), mouse anti β-III tubulin monoclonal antibody (Abcam, Cambridge, USA, ab7751) and rabbit polyclonal antibody to GFP (Abcam, Cambridge, USA, ab290) were used in this study. FITC-conjugatedanti-mouse IgG (Sigma, St. Louis, MO, F9137) and Cy5.29-conjugated anti-rabbit IgG (Abcam, Cambridge, USA, ab6564) were used as secondary antibodies. The cells were cultured on cover slip in 12-well plates, fixed and permeabilized with 0.2–0.3% Triton X-100 in PBS. To block nonspecific binding, the cells were then incubated with 10% NGS (normal goat serum, Sigma, G9023). For double labeling fluorescent, the cells were incubated with two primary antibodies together, for 60 min (anti-GFP and anti-synaptophysin, or anti-GFP and anti β-III tubulin) and then with the relevant secondary antibodies for 30 min. Several groups with no primary antibodies were considered as controls. Paraffin-embedded sections of chicken embryos were dewaxed, rehydrated, and washed. To quench endogenous formalin-related fluorescence, the slides were incubated in 2% sodium borohydride (Sigma, S9125) and 0.1 M glycine [20]. Antigen retrieval was performed enzymatically. Nuclei are counterstained by Hoechst for 5–10 min at room temperature.

3. Results

3.1. Stable transfection of eGFP gene

At the first part of this study undifferentiated P19 cells were transfected with pML8 and pB17 vectors. 48 h post transfection the cells were subjected to *puro* selection and expanded for 8 more days before analysis. Fluorescent intensity of $eGFP^+$ cells was detected under viable conditions by fluorescence microscopy, eight days post transfection (Fig. 1). The individual colonies with high *GFP* expression (Fig. 1A) were picked and expanded for at least 8 days before differentiation induction (Fig. 1B and C). The microscopic patterns of *GFP* expression were quantitatively assessed by FACS. Suspensions of single cells were prepared from the untransfected and transfected cells and subjected to FACS for determination of eGFP intensity. FACS analysis of pooled stable cells indicated that 82.87% of the cells were $eGFP^+$ (Fig. 2B), while only 0.56% were positive in the untransfected cells (Fig. 2A).

To confirm the finding of *eGFP* expression in living EC cells, we performed western-blot experiments using anti-GFP antibody (Fig. 2C). The results indicated sufficient expression of *eGFP* in transfected P19 cells. No eGFP protein was detected in untransfected cells.

3.2. Neuronal differentiation of stable P19-GFP^+ cells by selegiline

Based on our previous studies, the best selegiline concentration for neural induction of ES and P19 cells is 10^{-8} M [7,8]. Therefore, this concentration of the drug was used to induce neural differentiation of P19-GFP^+-derived EBs. Selegiline-treated EBs were plated and then allowed to attach on gelatin-coated dishes to form outgrowth of neuron-like cells. Fig. 3 represents the morphology of the differentiated P19-GFP^+ cells. The cells were cultured and expanded at undifferentiated state (Fig. 3A) and then subjected to suspension cuture to produce EBs (Fig. 3B). Proliferative cells expand around the EBs and form a monolayer of cells (Fig. 3B–D).

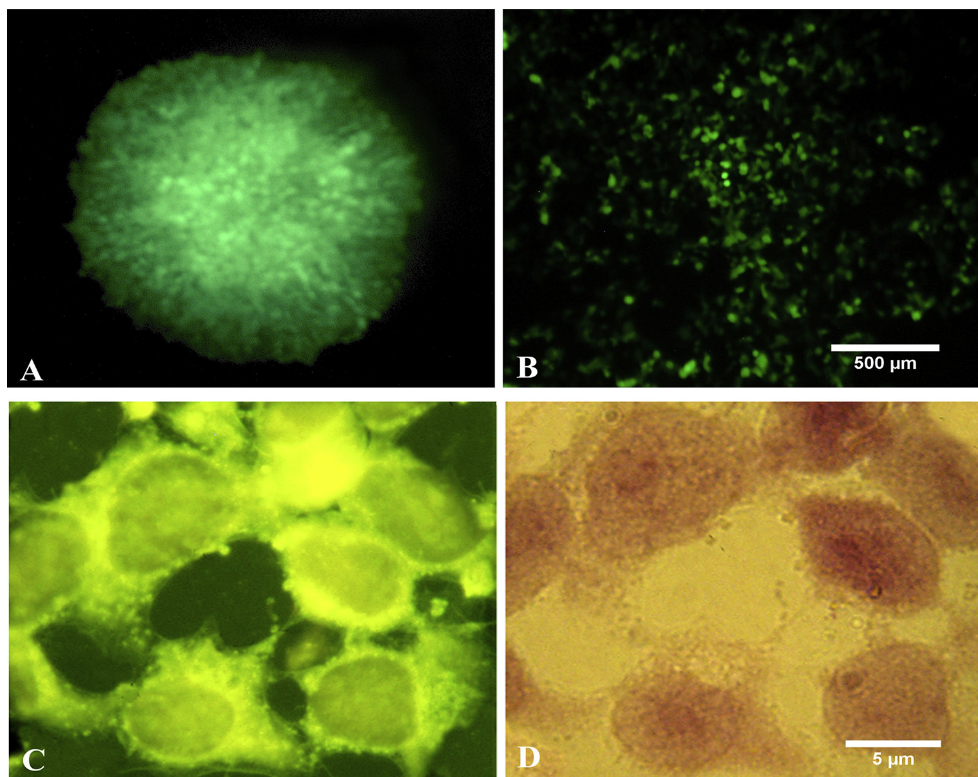

Fig. 1. Fluorescent intensity of undifferentiated P19-GFP^+ cells was demonstrated by fluorescence microscopy at 8 days post-transfection, in the cells transfected with pML8 and pB17. A: An individual colony with high *GFP* expression, subjected to *puro* selection 48 h post-transfection. B & C: The colonies were picked and expanded for at least 8 days before differentiation induction. D is hematoxylin-eosin staining of the same field shown in C. Tightly packed polygonal cells with high nucleus/cytoplasm ratio, represent undifferentiated cells.

After about 2 wk, the selegiline-treated cells showed characteristic morphology of neuronal cells (Fig. 3C and D). Aggregates of neurons have been formed with areas of lower density of neural cell bodies in between. The aggregates are interconnected by fine processes (arrowheads). Cresyl violet staining of the differentiated cell indicated that neuronal cells formed cellular networks on top of the cell monolayer (Fig. 3E and F). Fig. 3G shows P19-GFP^{+}-derived neurons under viable conditions by fluorescence microscopy after about 6 mo post transfection. Panel H is a bright field of the same image shown in panel G. Although, no quantitative analysis was carried out, overall, our observations showed that many of the differentiated GFP^{+} cells had morphology of neural cells, as well as complex patterns of branches.

Co-expression of neuronal markers and *GFP* in P19-derived neuron-like cells was analyzed using double immunofluorescence (Fig. 4). Expression of neuronal specific markers, synaptophysin and β-III tubulin were examined using specific antibodies, to characterize post-mitotic mature neurons. Immunoflourescence of the differentiated P19-GFP^{+}cells showed that they were immunoreactive to β-III tubulin (Fig. 4A–D) and synaptophysin (Fig. 4E–H), after selegiline treatment. This Figure highlights many cells that co-express both GFP (B) and β-III tubulin (C) or GFP (F) and synaptophysin (G). Panels A and E are the bright field of the same images shown in B and F respectively. Merged images of GFP and β-III tubulin (D) and GFP and synaptophysin (H) are also presented.

Data analysis of real-time PCR revealed that the differentiated cells expressed neuroepithelial and neural specific markers, nestin and synaptophysin (Fig. 5). Expression of nestin by selegiline-treated cells (211.6805) was significantly higher than that of P19 and RA (10.57669) groups (Fig. 5A). Synatophysin gene expression in these cells (147.8221) was also more than that of all the other groups (RA: 28.42384, B: 59.47915) (Fig. 5B). The difference was significant. Furtheremore, the proliferative marker of stem cells, Oct3/4 showed significant reduction in the differentiated cells (Fig. 5C).

3.3. *In ovo transplantation of P19-GFP^{+}cells*

To investigate the ability of selegiline treated P19-GFP^{+}cells to survive, differentiate, and migrate into central nervous system, the cells were transplanted into the brain vesicle of 3-days-old chick embryos (Fig. 6A). A clump of GFP-expressing cells is visible clearly in the living embryo, at the time of cell injection (Fig. 6B). Fluorescence analysis of whole chick embryos injected with P19-GFP^{+}cells, illustrated that the cells were capable of surviving in the embryos (Fig. 6C). By 3–5 days after transplantation, GFP-expressing cells migrated to the regions distant from the implantation site, in the host embryo. Fig. 6C shows that the cells migrated away from the transplantation site and could integrate into the surrounding tissues such as visceral arches, brain vesicles and neural tube. *In ovo* transplanted cells survived and underwent morphologic differentiation reminiscent of neurons (Fig. 7). Antibodies against neuronal specific markers and anti-GFP antibody were used simultaneously to determine whether the transplanted

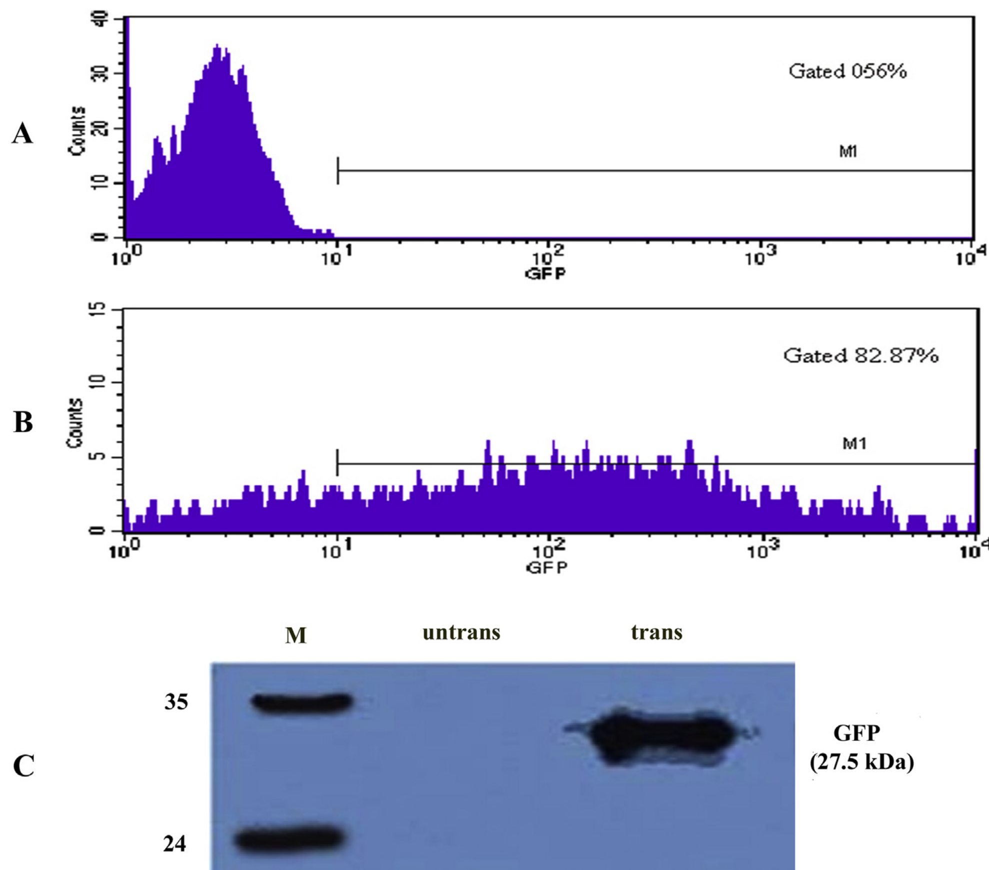

Fig. 2. *GFP* expression in undifferentiated P19 EC cells. A and B show FACS analysis of EC cells 8 d after initial *puro* selection at 48 h post-transfection. A: Untransfected P19 cells. B: The cells transfected with pML8 and pB17. The peak pattern represents fluorescent profile of the cells. The gated region for positive eGFP cells is indicated by the bar. The number reflects the positive cell percentage. C: Analysis of eGFP protein (MW: 27.5 kDa) expression in P19 cells, by western-blot. GFP was detected with anti-GFP antibody. M: marker, untrans: untransfected P19 cells, trans: transfected P19 cells with pML8 and pB17.

P19-GFP$^+$cells expressed differentiated markers within the environment of the chick embryo. We found that the donor cells differentiated *in ovo*, with morphological and molecular characteristics of neurons. In the neural tube, the migrating cells formed a continuous layer (Fig. 7 arrowheads). A subpopulation of these cells was clearly found to co-express both GFP and β-III tubulin (Fig. 7A) or GFP and synaptophysin (Fig. 7B). Nuclei are counterstained by Hoechst. Beside GFP expression, donor cells are easily identifiable from host cells due to denser heterochromatic masses and larger nuclei. Distinct clusters of transplanted cells are visible with the positive response to specific marker of differentiating neurons, β-III tubulin (Fig. 7A, arrows). Synaptophysin-expressing donor cells are apparently intermingled with host tissues (Fig. 7B, arrows).

4. Discussion

In the present study, differentiation of stable GFP$^+$ cells was verified *in vivo* by *in ovo* transplantation to developing CNS of chick embryos at stage 18–20. First of all, by utilizing P19 EC cell line and vectors containing e*GFP* gene and *pgk-1* promoter, we indicated the possibility of production of stable GFP-expressing cells, *in vitro*. *GFP* expression of P19 cells was observed by fluorescence microscopy, and then confirmed by western blots and FACS analysis. Additionally, P19-GFP$^+$ cells were successfully differentiated into neural cells by selegiline drug, as an inducer. The differentiated GFP$^+$ cells obtained in this culture indicated typical morphology of neuron and exhibited a number of neuronal specific markers such as synaptophysin and β-III tubulin. Cultures of GFP$^+$-P19 cells and GFP$^+$ neuronal cells continued for more than 6 mo. To investigate the ability of selegiline treated P19-GFP$^+$cells to maintain survival, differentiate, and migrate in CNS, they were transplanted into the brain vesicle of chick embryos. Our data indicated that by 3–5 days after transplantation the cells migrated away from the transplantation site and could integrate into the surrounding host tissues such as brain vesicles, visceral arches, and neural tube.

A number of studies have reported *GFP* expression by P19 cells [21,22]. In the previous studies, these cells were transfected with a construct containing murine Tert promoter (mTert: reverse transcriptase component of murine telomerase) combined with *GFP* gene. Down-regulation of mTert_*GFP* expression during differentiation was observed as a result of RA application [22]. Kim et al. (2001) transfected P19 cells with the gene for *GFP* under the control of the neuronal *T*_1 tubulin promoter. After four days of treatment with RA, *GFP* was specifically detected in cells undergoing neuronal differentiation [21].

P19 EC line provides an excellent model to investigate regulation of neuronal differentiation and development. These cells are highly available and usually do not require feeders and/or special growth conditions. Interestingly, they can be induced to differentiate by simple manipulation of the culture conditions [3]. Finally, P19 EC cells are suitable for genetic manipulations such as transfection and establishment of stable cell lines expressing inserted genes [23]. The stable expression of reporter genes has excellent applications in cell biology [15]. In the current study, P19 EC cells were stably transfected by a construct containing e*GFP*. To obtain stable cell population, *puro* was used and the individual GFP$^+$ colonies were picked and expanded. Culture of P19-GFP$^+$ EC cells continued for more than 6 mo. These cells were successfully freezed and thawed for several times. In line with previous studies [24,25], the results of this report showed that it is possible to produce long-term *GFP*-expressing cells. Stable gene expression is an indicator of successful gene transfection and insertion [15]. Taghizadeh et al. (2008) were able to get transient *GFP*-expressing cells for up to 72–96 h post-transfection, but they were unable to extend these clones as long-term, stable *GFP*-expressing cells [15]. Other researchers have obtained similar results by using different *GFP*-expressing vectors [26,27]. Recently, Mii et al. (2013) demonstrated that ND-GFP cells, nestin-expressing cells from transgenic mice were able to differentiate into neurons, glia, keratinocytes, and smooth muscle cells *in vitro* [25]. Since, *GFP* encodes a non-toxic marker [28] and P19 cells are excellent recipients of foreign DNA [3], the difference between our results and those of the others may be due to different

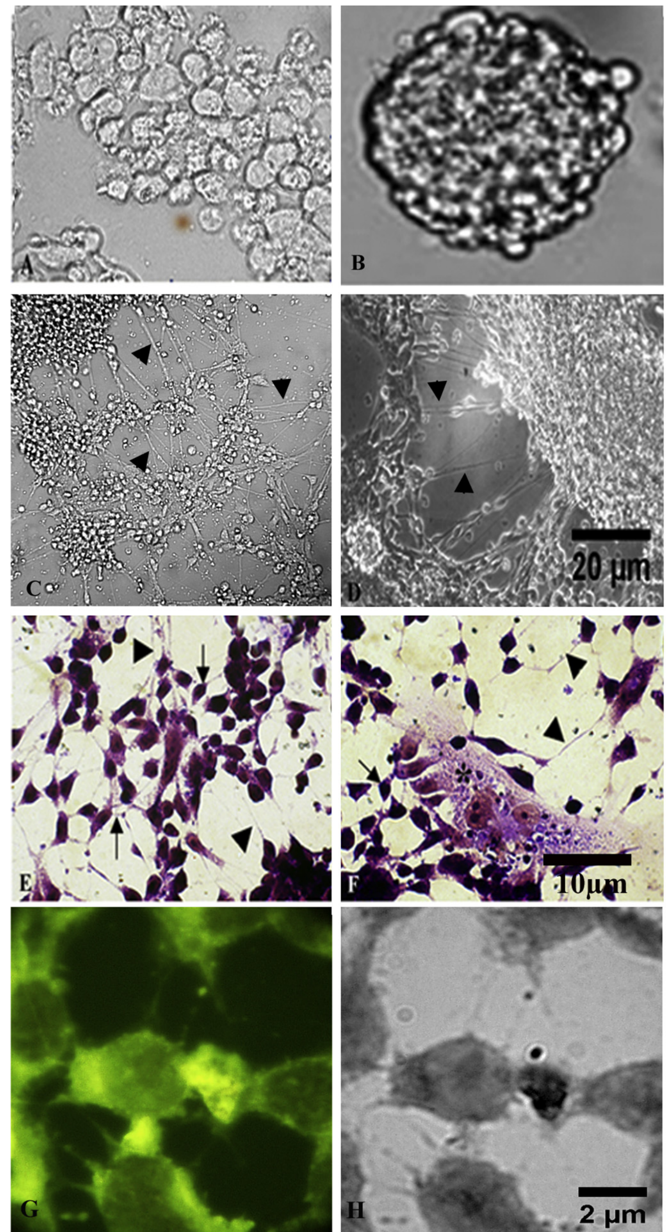

Fig. 3. Photomicrographs of undifferentiated and differentiated P19-GFP$^+$ cells into neuronal phenotype. A: An undifferentiated P19 clamp and C: A P19-derived EB before selegiline treatment. B and D: After about 2 wk, the selegiline-treated cells showed characteristic morphology of neuronal cells (soma and neurites). Aggregates of neurons have been formed with areas of lower density of neural cell bodies in between. The aggregates are interconnected by fine processes. Cresyl violet was used to stain the differentiated neurons. E & F: Neuronal-like cells (arrow) formed a cellular network on top of the monolayer. The arrowheads indicate the neuronal extension. The asterisk indicates a non-neuronal cell with distinctive nucleus and nucleolus (arrowhead). G. Fluorescence micrographs illustrating *GFP* expression in living neuronal-like cells derived from P19-GFP$^+$ cells after selegiline treatment. H is a bright field of the same image shown in G. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

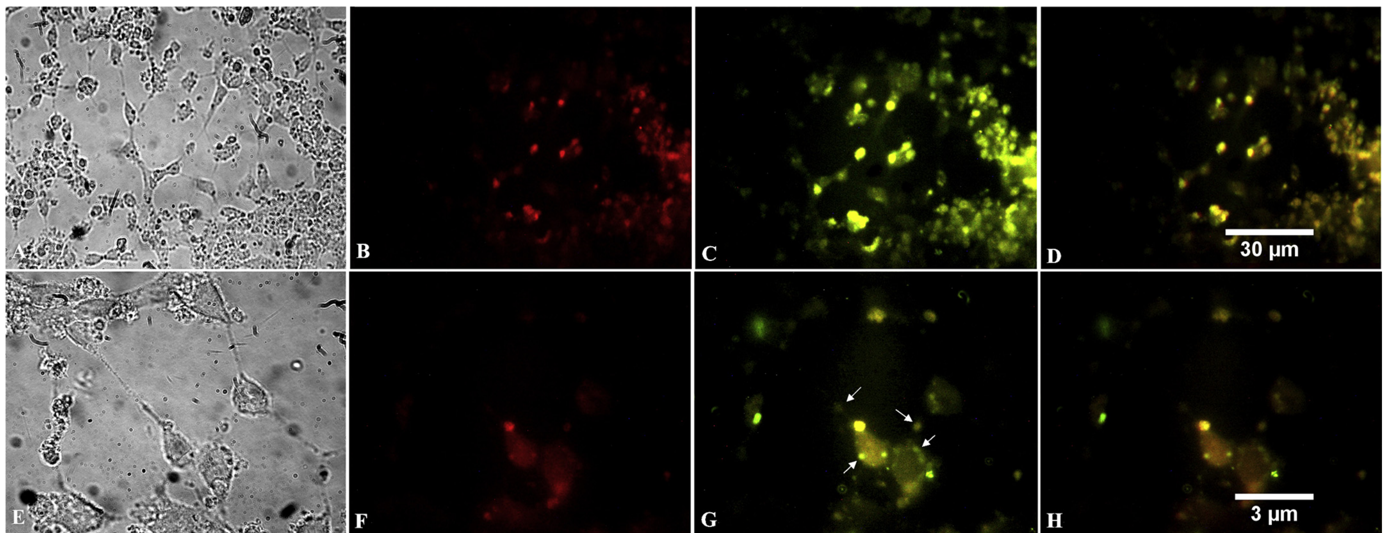

Fig. 4. Fluorescence micrographs illustrating the co-expression of GFP and specific neuronal markers in neuronal-like cells derived from P19-GFP^{+} cells after differentiation induction. Stable P19-GFP^{+} cells treated with selegiline show immunoreactivity for both GFP (B) and β-III tubulin (C), a classic marker for differentiating neurons, and both GFP (F) and synaptophysin (G), a reliable marker of nerve terminal differentiation. Synaptophysin concentrated in a brightly fluorescent spot (arrow). A and E are the bright field of the same images shown in B and F respectively. D: Merged image of GFP and β-III tubulin. H. Merged image of GFP and synaptophysin.

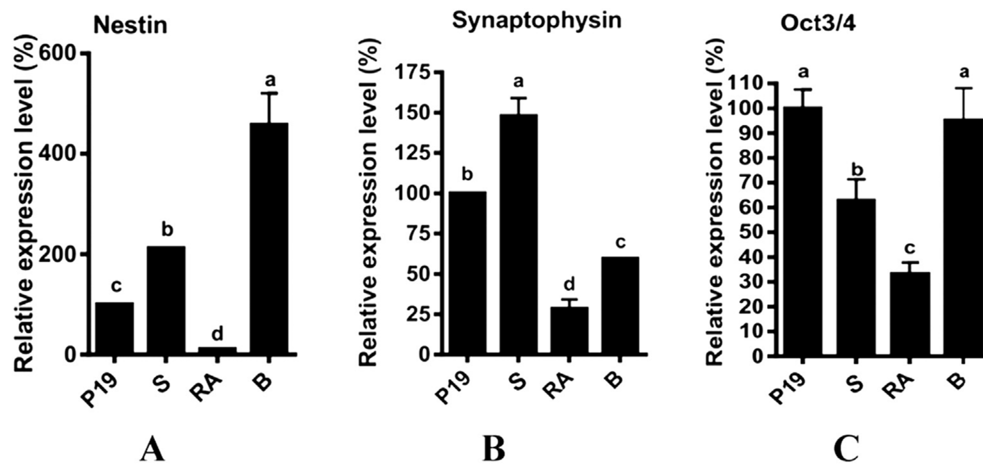

Fig. 5. Relative expression of neuroepithelial gene, nestin (A), neural specific gene, synaptophysin (B) and proliferative marker of stem cells, Oct3/4 (C). P19: untreated cells, S: selegiline-treated cells, RA: retinoic acid-treated cells, B: neonatal brain tissue.

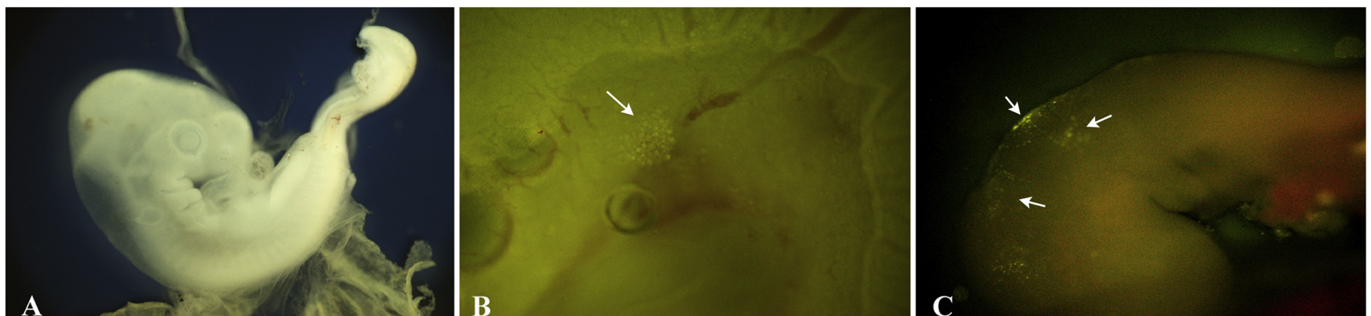

Fig. 6. *In ovo* transplantation of P19-GFP^{+}cells. Representative micrograph shows: A: A chick embryo in stage 19 (68–72 h) according to Hamburger and Hamilton. B: A clump of GFP-expressing cells, transplanted into the brain vesicle of 3-days-old chick embryos (arrow). The embryo was photographed in situ. C: By 3–5 days after transplantation, *GFP*-expressing cells migrated to surrounding tissues such as brain vesicles, neural tube and visceral arches (arrows).

transfection protocols, as well as using different stem cell lines. Furthermore, co-transfection of pB17 plasmid with exogenous expression plasmids greatly enhances the levels of transcript obtained from foreign DNA sequences. pB17 plasmid has a fragment of

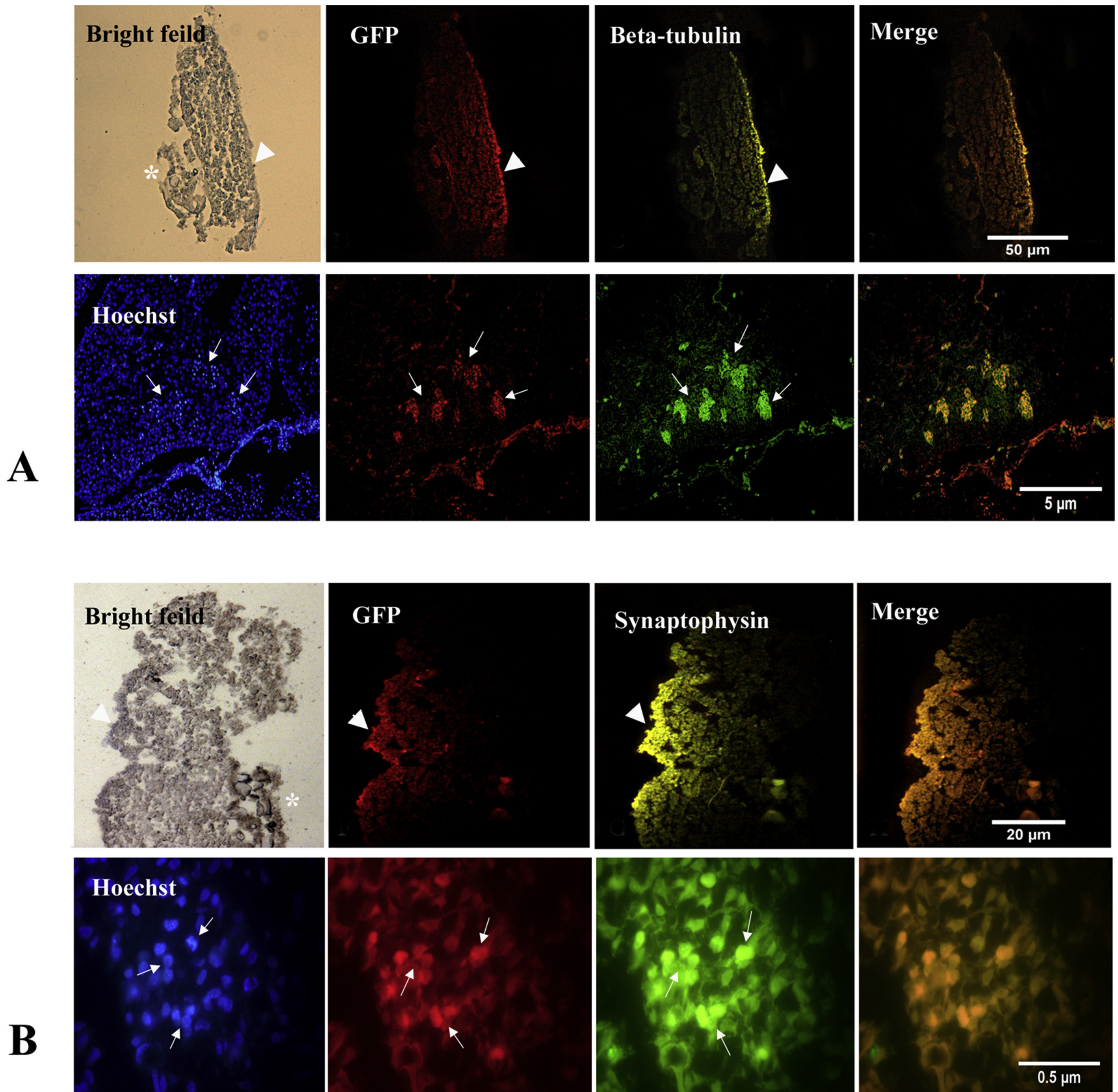

Fig. 7. Double immunofluorescent stainings of P19-GFP$^+$cells differentiated *in ovo*. Five μm parasagital sections were prepared and double stained with antibodies against neuronal markers and GFP. Stable P19-GFP$^+$ cells treated with selegiline show immunoreactivity for both (A) GFP and β-III tubulin and (B) GFP and synaptophysin. Nuclei are counterstained by Hoechst. In the neural tube the migrating cells formed a continuous layer (upper rows, arrowheads). Beside GFP expression, donor cells are easily identifiable from host cells due to denser heterochromatic masses and larger nuclei. Distinct clusters of transplanted cells are visible with the positive response to specific marker of differentiating neurons, β-III tubulin (A, lower rows, arrows). Synaptophysin-expressing donor cells are apparently intermingled with host tissues (B, lower rows, arrows). Asterisk point to optic vesicle in A and visceral arch and cleft in B.

murine *Pgk-1* gene and increases transfected gene expression, greatly [29]. Therefore, pB17 was included in all transfections, in the present study. This effect of pB17 is cell specific and occurs only in EC cells. Reporter genes can be used to purify populations of cells by FACS and to trace cells after transplantation [30,31]. The *GFP* gene was selected in the present study, because: 1) *GFP* is a reporter gene that encodes a non-toxic marker [28], 2) it is the most studied reporter gene and highly used in cellular and molecular biology [32], 3) it can be utilized in a variety of species [12,13,15], and most importantly, 4) the major advantage of *GFP* is its ability to exhibit endogenous fluorescence; a cofactor is needed for most fluorescent proteins [33].

Neuronal differentiation of P19 cells was previously reported [8,34,35]. In addition, the neuronal cells derived from P19 have been successfully applied in several transplantation studies [36]. Here, in the present study, we proposed a simple and efficient strategy for neuronal differentiation studies both *in vitro* and *in vivo*. As a basis for the next experiments, we first examined

whether the *GFP*-expressing cells also differentiate into a neural phenotype after treatment by a neuroprotective pharmacological drug, selegiline. Then the differentiated GFP^{+} cells were utilized in transplantation studies of our experiments. We should more realize the mechanisms of stem cell action and know how to control of stem cell survival, migration, proliferation and differentiation, post-transplantation [37]. Our results indicated that *in ovo* transplanted *GFP*$^{+}$ cells were able to survive, differentiate, migrate and integrate with host tissues. It is possible that the embryonic environment of the host could induce their differentiation. The chick embryo is able to serve as a unique and accessible experimental system for the investigation of inductive differentiation and interactions in development. The feasibility of labeling stem cells by *GFP* before transplantation has important and promising perspectives for their use in the future basic researches [16].

5. Conclusion

In the present study, using P19 EC cell line, stable and long-lasting *GFP*-expressing cells was made and characterized. Neuronal differentiation of these cells was induced by a neuro-protective drug, selegiline (deprenyl). Many of the differentiated *GFP*-expressing cells had key characteristics of neurons. In addition, we showed that *in ovo* grafted P19-GFP^{+} cells not only survived, but also migrated away from the transplantation site and differentiated into the cells with molecular characteristics of neurons. These neuron-like cells were apparently intermingled with host tissues. The chick embryo can be used as a unique and accessible experimental system for *in vivo* studies of stem cell development.

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Conflict of interest

The authors declare that there is no conflict of interest to disclose.

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