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GENERATION OF DOPAMINERGIC NEURONS BY FUSION OF NEURAL STEM CELLS AND MIDBRAIN NEURONS

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Abstract

Neural stem cells (NSCs) are multipotent and self-renewing that can potentially generate various neurons for clinical purposes. However, the mechanism of regulating differentiation from NSCs to neurons is not well understood. In this report, we established a new method to obtain specific neurons from NSCs by cell fusion. NSCs were fused with midbrain neurons, resulting in hybrid cells that express an antigen pattern characteristic of dopaminergic neurons. Analysis of the marker enzyme tyrosine hydroxylase (TH) showed that hybrid cells were reprogrammed to somatic cells. This report represents a new significant contribution toward revealing the directional differentiation of NSCs and eventually providing the application in clinical transplantation therapy.

Key words: Neural stem cell, midbrain neuron, cell fusion, differentiation, dopaminergic neurons.

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Abbreviations: AD: Alzheimer's disease; **ESCs**: embryonic stem cells; **FITC**: Fluorescein isothiocyanate; **NPs**: neural precursors; **NSCs**: Neural stem cells; **PD**: Parkinson's disease; **PEG**: polyethylene glycol; **TRITC**: tetramethylrhodamine isothiocyanate; **TH**: tyrosine hydroxylase.

INTRODUCTION

Multipotent neural stem cells have the potential to differentiate into three lineages of cells in the central nervous system, including neurons, astrocytes and oligodendrocytes (6, 10). However, the mechanism of regulating differentiation from NSCs to neurons is not well understood. Due to their properties of self-renewal and multipotency, stem cell-based transplantation therapy has become an area of great interest in clinical research. This includes potential treatments for neural injury and neurodegenerative disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD) which are caused by the loss of dopaminergic and cholinergic neurons, respectively (2, 12). Therefore, the replacement of these cells as a therapeutic intervention will become more realistic and practical if these specific lost neurons can be obtained in the laboratory in a simple and efficient manner.

The neuronal replacement by embryonic stem (ES) cells as experimental therapies has recently received considerable attention(11). The key challenges of such therapies are the directed differentiation of stem cell-derived neural precursors (NPs) into specific neuronal types(9). Actually, NSCs must leave the cell cycle to become neurons and glia, however, the signals that regulate this transition remain largely

unknown. Mahairaki, et. al .reported that Wnt and Sox2 inhibit neural differentiation by Notch activation. Agathocleous, et al. described the activation of NeuroD1 and LINE-1 by a Wnt-mediated regulatory mechanism to generate neurons from NSCs in adult hippocampus, which is important for adult neurogenesis and survival of neuronal progenitors (1, 8).

Here, we report a new, simple and efficient method to generate specific sub-sets of neurons using chemical-induced cell fusion between differentiated neurons and the NSCs line C17.2 which is widely used as a cell transplantation candidate for the treatment of neurodegenerative disorders (15). We therefore anticipate that this method can facilitate the understanding of the mechanism of NSCs' differentiation and advance cell replacement therapy by potentially providing an unlimited source of neurons.

MATERIALS AND METHODS

Cell culture

The C17.2 murine neural stem cell line was kindly provided by Dr Evan Y. Snyder (Harvard Medical School) and cultured as previously described in ref. (15). The procedures for preparing primary cultures of hippocampal and midbrain neurons were also previously described in ref (3, 13, 14).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and were blocked with normal goat serum. Cells were then subjected to immunofluorescent staining using mouse anti-Nestin (1:500 Chemicon, Temecula, California, USA), mouse anti-β-tubulin III, rabbit anti-CD133 (1:200 Santa Cruz Biotechnology, Europe), mouse anti-MAP2 (1:1000, Abcam, Cambridge, UK) and mouse anti-TH (1:8000, Sigma) monoclonal antibodies. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies, TRITC-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse antibodies were added at a dilution of 1:100.

Plasmid transfection

Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. Hippocampal and midbrain neurons were tr**a**nsfected with plasmid EGFP-C1. All steps were performed as per manufacturer's instructions.

Cell fusion and subsequent culture

To induce cell fusion, NSCs and specific neurons (1:1) were mixed in presence of polyethylene glycol (PEG) (Roche, Nutley NJ) as per manufacturer's instructions. Following ten days in culture, G418 was used to select hybrid cells that were then subsequently picked and allowed to proliferate under standard conditions.

Phenotypic analysis of hybrid cells

Hybrid cells were fixed in 4% paraformaldehyde, stained with mouse anti-Nestin, rabbit anti-CD133, mouse anti-β-tubulin III, mouse anti-MAP2 (1:1000, Abcam, Cambridge, UK) and mouse anti-TH (1:8000, Sigma) monoclonal antibodies and X-gal. DNA content of hybrid cells was detected using Hoechst33342 (Sigma) staining.

RT-PCR analysis

Total RNA of C17.2 cell line, neurons, midbrain tissue and hybrid cells were extracted using Trizol Reagent (Invitrogen) and 2 μg of RNA was reverse transcribed. PCR conditions were optimized and linear amplification range for each primer was determined by varying annealing temperature and cycle numbers. Primers were as follows: β-tubulin III (530 bp), 5'-CTTCGCCCCACT TACAGC-3' and 5'-CTTCCGATTCCTCGTCAT-3'; TH (578 bp), 5'-AGCCAAAA TCCACCACTT-3' and 5'-GAAAATCACGGGCAGACA-3'; **β**-actin (590 bp) 5'-CCAAGGCCAACGCGAGAAGATGAC-3' and 5'-AGGGTACATGGTGGTGCC GCCAGAC-3'.

RESULTS

Properties of parent cells used in fusion

The C17.2 NSCs line used here is Nestin and X-gal positive. In order to confirm these properties, we stained with anti-Nestin antibody (Fig. 1A). Since the existence of LacZ loci in C17.2 cell line, it appeared the X-gal positive as shown in Fig. 1B. Hippocampal and midbrain neurons are MAP2 and TH positive, respectively. After culture *in vitro* for seven days, immunocytochemical analysis indicated that the majority of hippocampal neurons were MAP2 positive (Fig. 1C). Most midbrain neurons expressed TH (Fig. 1D). These observations indicated that these neurons retain their 'normal' characteristics as documented for hippocampal and midbrain neurons (4, 5, 7). In order to select hybrids, neurons were transfected with a plasmid containing green fluorescent protein (pEGFP-C1). After transfection, approximately 40% of hippocampal neurons (Fig. 1E, 1F) and midbrain neurons (Fig.1G, 1H) were GFP^+ after optimizing transfection conditions.

Morphological changes following cell fusion

After G418 selection for ten days (C17.2 carries *neo* selecting marker), hybrid cells displayed prominent morphological changes. Outgrowth of neurites, and a cell morphology resembling typical neurons were observed in hybrid cells from both hippocampal neurons (Fig. 2A, 2B) and midbrain neurons (Fig. 2C, 2D).

Hybrids display characteristics of both cell lines and neurons

To confirm drug-resistant cells arose through cell fusion between NSCs and specific neurons, DNA content was detected using Hoechst 33342 staining. Our result showed that

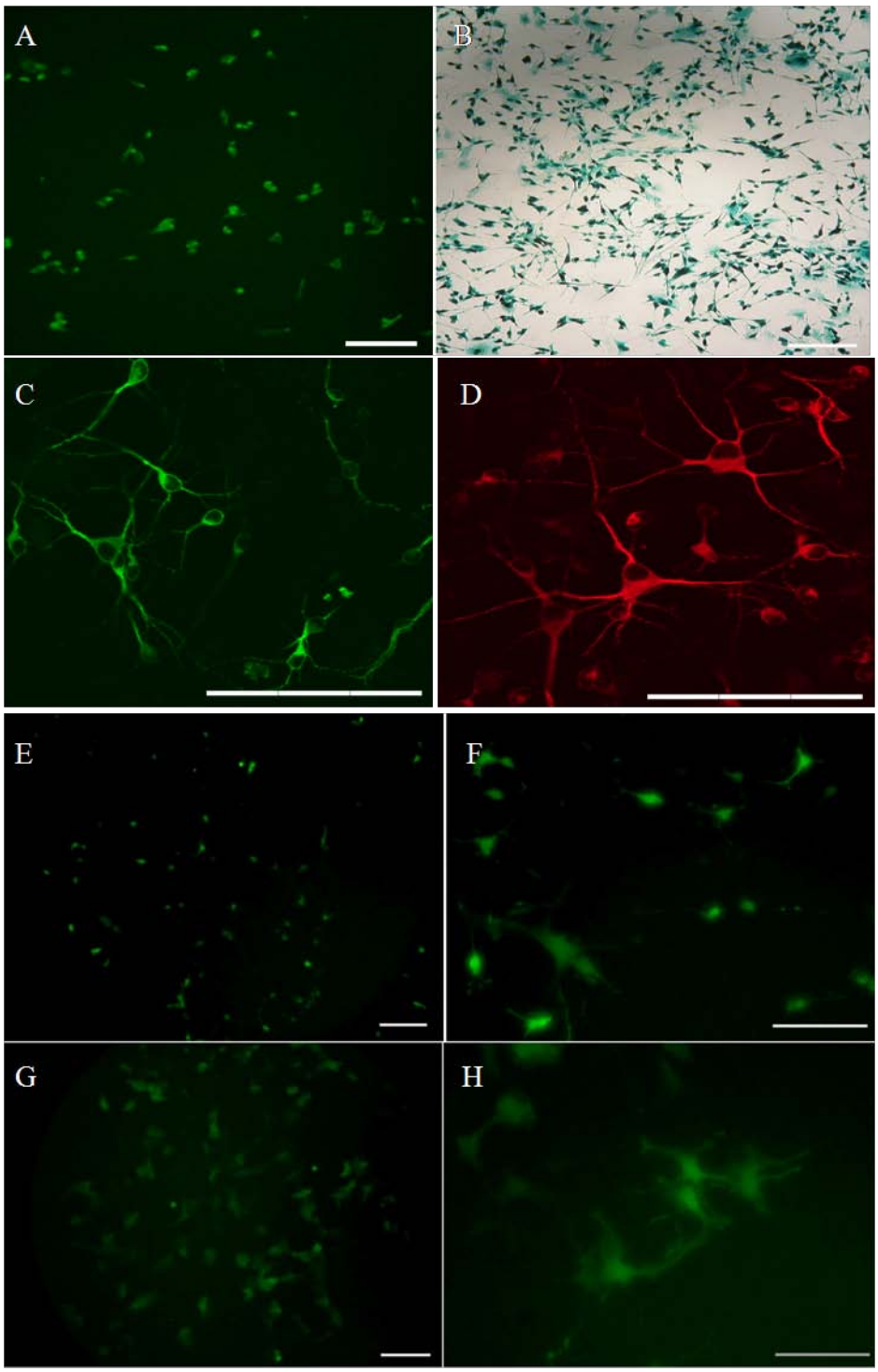

Figure1. Analysis of selective marker of parents used in fusion. C17.2 murine stem cell line identity. C17.2 can express the neural stem cell marker Nestin (A) and this cell line is also confirmed as X-gal positive (B). Immunofluorescent staining on terminally differentiated neurons with specific antibodies viewed using a Nikon fluorescent microscope. Hippocampal neurons were stained with MAP2 (C). Midbrain neurons were TH positive (arrowhead) (D). Photomicrographs of hippocampal neurons (E,F) and midbrain neurons (G, H) transfected with EGFP-C1 plasmids. Scale bars is 50 μm.

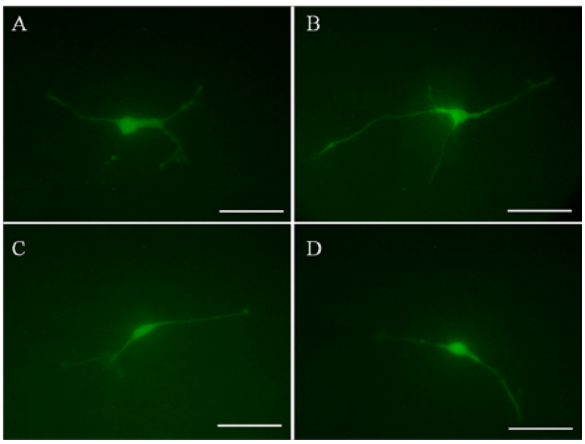

Figure 2. Resistance selection. Following treatment with G418, the soma of hybrid cells derived from hippocampal neurons became recognizable (A, B) while neurite outgrowth was extended from hybrid cells that were derived from midbrain neurons (C, D). Scale bars is 25 μm.

the DNA content in hybrids doubled in contrast to normal cells (Fig. 3A, 3B). Hybrid cells were GFP positive (Fig.3C) and also X-gal positive (Fig.3D), properties inherited from primary neurons and NSCs, respectively. Statistics showed that nuclei proportion of hybrids nearly doubled in contrast to that of parent cells (Fig.3E).

Hybrid cells derived from hippocampal neurons can express marker for NSCs and mature neurons

Following culture under standard conditions for five days, the phenotype of hybrid cells was analyzed by immunocytochemical staining. It was demonstrated that hybrid cells derived from hippocampal neurons expressed the mature neuronal marker Tuj1 (Fig.4C) and MAP2 (Fig. 4G), while the expression of Nestin (Fig.4B) and CD133 (Fig.4F), markers for NSCs, can also be detected in these hybrids. Furthermore, the expression of Tuj1 was located in the vicinity of nucleus (Fig.4D, arrowheads) and the expression of Nestin dominated other organelles (Fig.4D, arrows), which indicated that this hybird was in the initial stage of differentiation. In the other case, some hybrids were terminally differentiated since the whole neurite was MAP2 positive (Fig.4H, arrows) and the nucleus still maintained the characteristic of stem cell (CD133, Fig.4H, arrowheads).

Hybrid cells from midbrain neurons can express the phenotypic marker tyrosine hydroxylase

Hybrid cells derived from midbrain DA neurons expressed the important phenotypic transmitter tyrosine hydroxylase (Fig.4J) and also

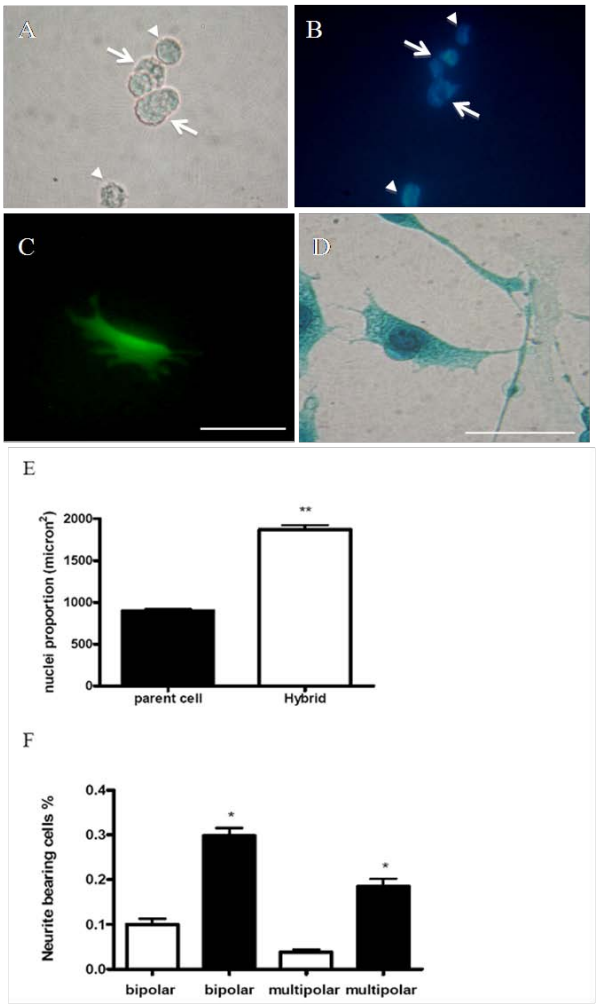

Figure 3. Analysis of nuclei fusions. Hoechst 33342 staining of normal C17.2 and hybrid cells. The DNA content of hybrid cells (arrow) was doubled in contrast to that of C17.2 (arrowhead) (B). (A) was a phase contrast image of (B). Hybrid cells derived from midbrain neurons were GFP (C) and X-gal positive (D). Nuclei proportion of hybrids nearly doubled in contrast to that of parent cells (E). The ratio of bipolar and multipolar cells in TH+ hybrids (black) and C17.2 (white). Values are expressed as means ± S.D., n =10. *p<0.01, **p < 0.001 (Asterisks indicate a significant difference between parent cells C17.2 and hybrids). Scale bars is 50 μm.

Nestin (Fig.4I). The expression of TH dominated the neurites of these hybrids (Fig.4L) while Nestin was only detected near the nuclei and the expression level was lower (Fig.4K). The ratio of neurite bearing cells in these TH^+ hybrids (Fig.3F, black column) was much higher than that of C17.2 (Fig.3F, white column), both for bipolar (30%±2% *v.s.* 9%±1%) and multipolar cells (18%±2% *v.s.* 4%±1%). RT-PCR analysis further confirmed the validity of these results suggesting that NSC-DAneuron hybrids displayed characteristics of a dopaminergic neuronal origin (Fig.4E). In other words, we

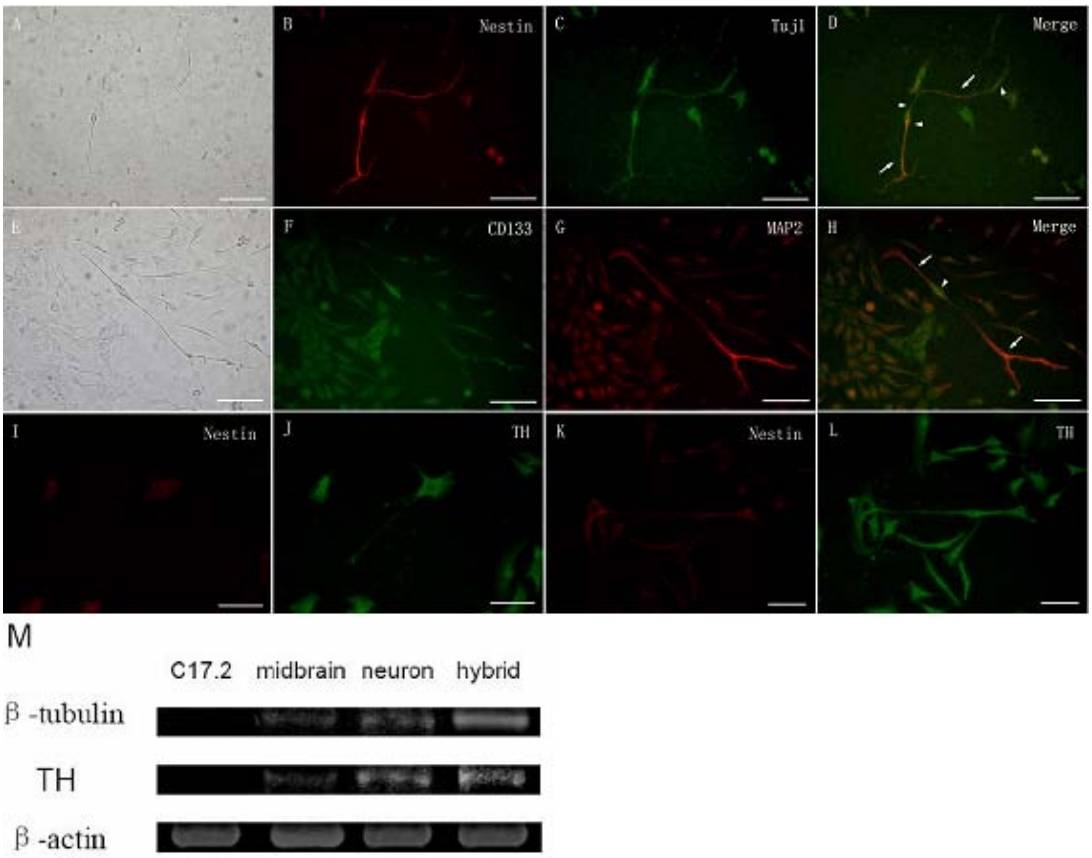

Figure 4. Immunocytochemical characterization of hybrid cells. Hybrid cells derived from hippocampal neurons were stained with Nestin(B), Tuj1(C), CD133(F), MAP2 (G). (A) and (E) were phase contrast images. Merges were shown in (D) and (H). Hybrid cells derived from midbrain neurons were TH positive (J, L) while these hybirds can also express Nestin (I, K), Scale bar is 50 μm. RT-PCR analysis of gene expression in NSCs, midbrain, specific neurons and hybrids are shown in (M). The fusion hybrids expressed markers that are characteristic of midbrain neurons, namely Tuj1 and TH, while C17.2 did not express these markers.

show that this NSCs line has the potential to differentiate into mature neurons or even dopaminergic neurons after fusion with specific terminally differentiated neurons.

DISCUSSION

In this study, we established a novel, simple and efficient method to obtain specific neurons from C17.2, a murine neural stem cell line. After cell fusion, hybrid cells express either the mature neuronal marker MAP2 or the phenotypic transmitter TH depending on what kind of neurons they were fused, such as hippocampal or midbrain neurons. In addition, hybrid cells inherited important properties from the C17.2 NSC line, such as the expression of Nestin, a marker for NSCs, as well as it's propagative ability and lacZ loci. The hybrid cells can be cultured under standard culture conditions of C17.2 line with their unique characteristics in several passages.

C17.2 NSCs has previously been shown to participate in neural development and is capable of differentiation into diverse neuronal and glial cell types as well as normal cytoarchitectural constituents (3, 13, 14). These results demonstrated the extraordinary capability of C17.2 NSCs to participate in regeneration. Therefore, this cell line is an ideal candidate for cell transplantation therapy. In this light, the hybrid cells we describe here, endowed with both the characteristics of the C17.2 line and specific neurons, has great potential to be used as an unlimited source of neurons for cell transplantation. More importantly, due to the nature of C17.2 (15) this hybrid cell has theoretically less chance to form astrocytomas, a major problem following cell transplantation therapies(16)

Previously, Schöler reported that embryonic stem cell karyoplasts could induce Oct4 expression in the somatic genome, yet cytoplasts lacked this ability (5). Kevin Eggan established

that human embryonic stem cells could reprogram the transcriptional state of somatic nuclei through cell fusion (4) We therefore hypothesized that the nuclei of NSCs may be reprogrammed through fusion with specific neurons. We found that hybrid cells expressed a mature neuronal marker or some important phenotype transmitters, such as TH. That is, hybrid cells have the potential to differentiate into specific neurons for clinical purposes. We presume that NSCs' differentiation pattern is dominated by their nuclei and the nuclei of specific neurons. After several passages, the hybrid cells produced also displayed characteristics of both C17.2 and specific neurons. In other words, a new cell line was established using this methodology (7).

We anticipate that our findings could facilitate the study on the committed differentiation of NSCs, and thus advance cell therapy for neural injury and degenerative disorders. It also provides a broader thinking for other cell directed differentiation.

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