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Clonal analysis for elucidating the lineage potential of embryonic NG2$^+$ cells

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Abstract

Background aims. The widespread NG2-expressing neural progenitors in the central nervous system (CNS) are considered to be multifunctional cells with lineage plasticity, thereby possessing the potential for treating CNS diseases. Their lineages and functional characteristics have not been completely unraveled. The present study aimed to disclose the lineage potential of clonal NG2$^+$ populations *in vitro* and *in vivo*. *Methods*. Twenty-four clones from embryonic cerebral cortex-derived NG2$^+$ cells were induced for oligodendrocyte, astrocyte, neuronal and chondrocyte differentiation. The expression profiles of neural progenitor markers chondroitin sulfate proteoglycan 4 (NG2), platelet-derived growth factor-α receptor (PDGFαR); nestin and neuronal cell surface antigen (A2B5) were subsequently sorted on cells with distinct differentiation capacity. Transplantation of these NG2$^+$ clones into the spinal cord was used to examine their lineage potential *in vivo*. *Results*. *In vitro* differentiation analysis revealed that all the clones could differentiate into oligodendrocytes, and seven of them were bipotent (oligodendrocytes and astrocytes). Amazingly, one clone exhibited a multipotent capacity of differentiating into not only neuronal–glial lineages but also chondrocytes. These distinct subtypes were further found to exhibit phenotypic heterogeneity based on the examination of a spectrum of neural progenitor markers. Transplanted clones survived, migrated extensively and differentiated into oligodendrocytes, astrocytes or even neurons to integrate with the host spinal cord environment. *Conclusions*. These results suggest that NG2$^+$ cells contain heterogeneous progenitors with distinct differentiation capacities, and the immortalized clonal NG2$^+$ cell lines might provide a cell source for treating spinal cord disorders.

Key Words: *clonal analysis, differentiation, NG2$^+$ cells, transplantation*

Introduction

Observations made over the past few decades have revealed a fourth major glial cell population that has chondroitin sulfate proteoglycan 4 (NG2) expressed on the cell surface (NG2$^+$ cells) in the mammalian central nervous system (CNS) (1). The NG2$^+$ cells are widely distributed throughout the developing and mature CNS. They exhibit diverse morphology, differentiation capacity and function in both physiologic and pathologic conditions (2–4). But their inherent heterogeneity, especially their differentiation capacity, remains to be categorized.

The progenies of NG2$^+$ cells from different developmental stages and anatomical regions of the CNS have been demonstrated by double staining of NG2 proteoglycan with markers of neuron–glia lineages or *in vivo* fate mapping analysis. Specified by oligodendrocyte markers and transcription factors, NG2$^+$ cells are generally thought of as committed oligodendrocyte progenitors (5–9). However, recent studies have revealed that NG2$^+$ cells could also adopt astrocytes and neuronal fates. During development of the CNS, a few NG2$^+$ cells give rise to a subpopulation of protoplasmic astrocytes in the gray matter (5,9). Some NG2$^+$ cells may also be responsible for astrocyte generation in injured CNS (10–12). Interestingly, NG2$^+$ cells have been shown to generate neurons in some regions of the adult brain (13–17). Hence NG2$^+$ cells project themselves with high lineage complexity, and their neuronal lineage potential has raised considerable interest in NG2$^+$ cells for cytotherapy of CNS disorders.

Although transplanted NG2$^+$ cells could be manipulated to acquire neuronal fate epigenetically (18), the microenvironments of the adult spinal cord are still a restricted transplantation paradigm for them, where transplanted neural progenitors either remain undifferentiated or predominantly commit to a glial lineage, in most cases (19–22). The use of restricted neural progenitors for CNS transplantation is considered to

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have more beneficial effects (23). Therefore, in order to figure out the intrinsic lineage potential of NG2$^+$ cells and to look for promising transplants from them for repairing spinal cord disorders, it is necessary to investigate the lineages of amplified clonal NG2$^+$ cells *in vivo* via transplantation.

In this study, cell clonal analysis was performed to explore the lineage potential of NG2$^+$ cells. We provide evidence that embryonic clonal NG2$^+$ cells are able to differentiate into oligodendrocytes, astrocytes, neurons and chondrocytes at distinct compositions *in vitro*. Each type was found to have a distinct expression level for the neural progenitor markers chondroitin sulfate proteoglycan 4 (NG2), platelet-derived growth factor-α receptor (PDGFαR), nestin and neuronal cell surface antigen (A2B5), which may further specify the heterogeneity of NG2$^+$ cells. Transplantation studies further demonstrated the different migration patterns and differentiation capacity in individual clones *in vivo*. Collectively, our results provide some understanding of the differentiating properties of defined NG2$^+$ populations *in vitro*, together with their therapeutic potential.

Methods

Cell preparation and culture

The isolation and culture of primary NG2$^+$ cells followed previous protocols (24). Briefly, E16 embryos were removed from the maternal uterus of Sprague–Dawley pregnant rats and the cerebral cortex was dissected in cold phosphate-buffered saline (PBS). The dissociated cells were collected and immunopanned with primary NG2 antibody. The isolated primary NG2$^+$ cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 2% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, platelet-derived growth factor AA (PDGFAA; 10 ng/mL), B27 supplement (1:50), neural stem cell supplement (1:50) and N2 supplement (1:100) in a 37°C humidified atmosphere with 5% CO_2. Cultures were harvested when they reached 70–80% confluence and replated at a cell density of $1.5–2 \times 10^4$ cells/cm^2.

Human telomerase reverse transcriptase (hTERT) plasmid transfection

Cells (1×10^5) were seeded onto 60-mm tissue culture dishes and cultured overnight. human Telomerase Reverse Transcriptase (hTERT) plasmid (1 μg; MBA-141; Global Bioresource Center, Manassas, VA, USA (ATCC)) was transfected into NG2$^+$ cells by electroporation following the manufacturer's instructions. The cells were selected with hygromycin at 250 μg/mL. Single clones were picked up and expanded for further study (25).

Multilineage differentiation of NG2$^+$ cells

The clones were analyzed for their capacity to differentiate into oligodendrocyte, astrocyte and neuronal lineages. Cells were seeded at a density of 8000 cells/cm^2 onto poly-L-lysine (PLL)-coated coverslips for differentiation; cells cultured in regular culture medium were used as a control. The differentiating medium was changed every 2 days. All the experiments were repeated at least three times for individual clones.

Oligodendrocyte differentiation was induced by culturing cells in DMEM/F12 medium supplemented with 0.5% FBS and 40 ng/mL thyroid hormone (T3). For astrocyte differentiation, cells were cultured in DMEM/F12 medium supplemented with 10% FBS, insulin-like growth factor 1 (IGF1; 200 ng/mL; R & D systems, Minneapolis, MN, USA) and 40 ng/mL T3. Neuronal differentiation was performed by incubating cells in DMEM/F12 medium supplemented with 5% FBS and epidermal growth factor receptor (EGFR) inhibitor PD168393 (1 μg/mL) for 4 days, followed by withdrawing the serum and supplementing with PD168393 (0.5 μg/mL) for another 3–4 days.

Chondrocyte differentiation

To acquire chondrocyte differentiation, cells were plated in a pelleted micromass culture (10 μL) at a density of 10^6 cells/mL. The cultures were maintained in chondrocyte medium DMEM-low glucose with 1% FBS, supplemented with insulin (6.25 μg/mL), transforming growth factor β1 (TGF-β1; 10 ng/mL), and ascorbic acid-2- phosphate (50 nM), which was changed every 2 days for 3 weeks. Cells that grew in normal medium were used as a control.

Immunofluorescent staining

The oligodendrocyte, astrocyte and neuronal differentiated and control cells were fixed with 4% paraformaldehyde (PF; Sigma-Aldrich, St. Louis, MO, USA) for 15 min and permeabilized with Triton X-100/PBS for 30 min. After two washes with PBS, cells were blocked with 4% bovine serum albumin (BSA) for 1 h, followed by incubation with primary antibodies diluted in Triton X-100/PBS/1% BSA at 4°C overnight. After removal of primary antibodies, cells were washed with PBS twice and the appropriate secondary antibodies (Alexa Fluor 488/594/568 goat anti-mouse/rabbit; Invitrogen (Grand Island, NY, USA); diluted with Triton X-100/PBS) were added and incubated for 3 h at room temperature