ANALYSIS OF NEURAL STEM CELLS AND NEUROSPHERES

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Shivani Palanivel

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ANALYSIS OF NEURAL STEM CELLS AND NEUROSPHERES

Approved by:

Dr. Yuhong Fan, Advisor
School of Biological Sciences
Georgia Institute of Technology

Dr. Mirjana Milosevic-Brockett
School of Biological Sciences
Georgia Institute of Technology

Date Approved: May 2020

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NOMENCLATURE

NSC	Neural Stem Cell
NS	Neurosphere
DIV	Days in Vitro
NSC-SR	Neural Stem Cell - Self-Renewal Culture Medium
NSC-DN	Neural Stem Cell - Differentiation Medium
PBS	Phosphate Buffered Saline
FBS	Fetal Bovine Serum

ABSTRACT

Neural stem cells (NSCs) are important multipotent adult stem cells capable of self-renewal and differentiation into various cell types present in the central nervous system. Studying the characteristics of NSCs and their differentiation offers insights into developmental mechanisms and allows us to assess their utility *in vitro* disease modeling and neurotoxicological research.

This study investigates the properties of NSC during propagation and differentiation in culture. Neurospheres, formed from NSC aggregates, were cultured and examined for morphology and growth rate by cell counting and size analysis. Neurospheres were induced to differentiate toward neural network formation. The expression of markers of NSC and astrocytes in neurospheres during differentiation was detected by immunostaining methods. NSC multiplied with an exponential proliferation rate accompanied by a steady increase in neurosphere diameter. The neurosphere cells expressed Nestin, an NSC/ progenitor cell marker, when cultured in self-renewal media, and displayed pronounced GFAP expression after induction of differentiation indicating a prevalence of astrocytes. Further studies will provide better understanding of NSC characteristics that may be applied for the advancement of stem cell therapy to help combat neurodegenerative diseases.

CHAPTER 1

INTRODUCTION

Stem cells possess the unique ability to self-renew and differentiate into multiple cell types. These characteristics yield them a critical role in the maintenance of tissue integrity, homeostasis and the repair of tissue damage. Their therapeutic potential has evoked great interest in researching their dynamics, function and regulation in both normal and pathological conditions.

Neural stem cells (NSCs) are self-renewing multipotent cells that can give rise to specialized neural populations in the central nervous system, including neurons, astrocytes and oligodendrocytes (Ladiwala et al., 2012). In the adult rodent brain, NSCs persist in the subventricular zone (SVZ) lining the lateral ventricles of the forebrain and the subgranular zone (SGZ) in the hippocampus along the dentate gyrus. They contribute to brain plasticity throughout life, and thus are of potential therapeutic value (Bond et al., 2015).

Neurons are responsible for the transmission of electrochemical signals, while glial cells provide critical functional support that helps maintain the signaling ability of neurons (Williams et al., 1991). Glial cells include astrocytes and oligodendrocytes. Astrocytes maintain an appropriate chemical environment for neuronal signaling. They control neurotransmitters around synapses, control ion concentrations and provide metabolic support. Oligodendrocytes provide axonal support. They produce fatty myelin sheath which insulates a neuron's axon and allows faster travel of electrical signals (Bull et al., 2005).

NSCs cultured with Epidermal Growth Factor (EGF) and Fibroblast Growth Factor (FGF) aggregate to form large, spherical neurospheres (NS) *in vitro*. Neurospheres can be mechanically separated to assist in propagation. It has been found that neurospheres and neurosphere-forming cells are both morphologically and functionally heterogeneous (Zhao, 2018). Biological events including apoptosis, mitosis and phagocytosis, are dependent on NSCs localization within the architecture of the neurosphere (Bez et al., 2003). Because of the differentiation potential, neurosphere structure is complex, and the cells within neurospheres are often heterogeneous (Han et al., 2017; Obernier, 2019). The mixture of cells present can serve as an *in vitro* model that mimics brain development (Shimada et al., 2017). NSCs generate all three neural lineages when propagated in a culture concentrated with specific growth factors, thus the *in vivo* niche may determine NSC potential (Bond et al., 2015).

NSCs express the neuroepithelial stem cell protein (Nestin) that functions as a cytoskeletal intermediate filament. Unlike general cytoskeletal elements, intermediate filaments are expressed in a cell-type specific manner such that differentiation processes are marked by a transition from one intermediate filament subtype to another (Bull et al., 2005). Differentiated cells of neuronal and astrocytic lineages depict a downregulation of Nestin expression detected by an anti-Nestin antibody in immunostaining assay (Bernal & Arranz, 2018). Glial fibrillary acid protein (GFAP) is a protein commonly associated with astrocytes. Astrocytes play crucial roles in synaptic activity by removing glutamate at the synapse and releasing modulators that interact with synaptic receptors. Increasing evidence suggests that glial cells (particularly astrocytes and microglia) play a critical role in the neuropathology of several diseases (Xapelli et al., 2020). Astrocytic function includes calcium signaling and inflammatory molecule dissemination, both of which are crucial in the body's immune response. Microglia are crucial in maintaining neuronal apoptosis, neurogenesis, synaptic interactions and cytokine related immune-response (Xapelli et al., 2020).

In the developing cortex, NSCs undergo symmetric self-renewal to expand the stem cell population, which is followed by asymmetric neurogenic cell division resulting in neuron formation and subsequently asymmetric gliogenic cell division for glial cell production (Bonaguidi et al., 2011). In the SGZ, new neurons and astrocytes are constantly produced. *In vitro* evidence indicates that precursor cells isolated from CNS are responsive to specific growth factors allowing them to differentiate into neural lineages.

This study examines the growth and differentiation of neural stem cells and neurospheres. I investigate the properties of NSCs during self-renewal and differentiation through a neurosphere based assay for NSC culture and neural differentiation. NSCs isolated from mouse fetal brains were cultured to form neurospheres, propagated, and analyzed for growth and morphology. Neurospheres were induced to differentiate and analyzed for the expression of NSC marker (nestin) and astrocytes marker (GFAP) by immunostaining assay.

NSCs have significant therapeutic potential for patients with CNS related disabilities. Understanding NSC growth and differentiation characteristics can assist us better targeting NSCs for future therapeutic approaches.

CHAPTER 2

MATERIALS AND METHODS

NSC Culture

NSCs used in study were previously isolated from E12.5 mouse fetal brains in Fan Lab. Frozen NSCs were thawed and cultured in Neural Stem Cell Self Renewal culture (NSC-SR) medium (StemCell Technologies, CA). The media included Neurobasal media (stemcell technologies) supplemented with N2 (Gibco) supplement, rhEGF (20ng/mL, Invitrogen), rmFGF (20ng/mL, Gibco), B27-vitamin A (1:50, Gibco), Beta-Mercaptoethanol (of 55mM) and Pen Strep (Sigma Aldrich). The NSCs were cultured to form neurospheres in 6-well Ultra-Low attachment plates (Corning Cat#3471). Approximately $5x10^5$ NSCs were resuspended in 2mL of NSC-SR media and seeded in one well of the 6-Well plate. Fifty percent of the media was changed every 2-3 days. For propagation, Neurospheres were mechanically dissociated through pipetting, and re-plated after counting cells using Trypan Blue staining and a hemocytometer under a phase-contrast microscope. The NSCs were incubated at 37ºC in a humidified incubator with 5% carbon dioxide.

Neurosphere Differentiation

The neurospheres were cultured to obtain $1.2x10^6$ NSCs. The neurospheres were induced to differentiate into neural networks in culture using Neural Stem Cell Differentiation (NSC-DN) culture medium (StemCell Technologies, CA). A glass slip was placed in each well of the 24-well plate that was pretreated with 200mg/mL Laminin (Thermo Fischer Scientific) overnight. The cultured neurospheres were transferred to a 15mL tube and centrifuged for 1min at 200rpm. The supernatant was aspirated and the neurospheres pellet was resuspended in 100uL of NSC-SR media and triturated to achieve a single cell suspension. The cells were counted and seeded at 2-4x10^5 cells 1mL of NSC-DN media per well of 24-well cell culture plate. The plate was incubated in the carbon dioxide incubator and differentiating neurospheres were examined at days 2 and 4 (D2, D4) post seeding.

Immunostaining

Immunostaining was performed following a procedure established by Zhang et al. (Zhang et al., 2012). Briefly, the glass slides (mounted with cells) were transferred to a new 24 well cell culture plate, washed with PBS (3x5min), fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 20 min, washed with PBS (3x5min), and permeabilized with 0.2% Triton-X-100 (Sigma-Aldrich) for 20 min. Cells were subsequently washed with PBS (3x5min) then incubated for 30 minutes at 4°C in 10% FBS/PBS blocking solution. The cells on glass plates were subsequently incubated in 200uL of primary antibody diluted in 10% FBS/PBS (overnight, 4°C) according to their respective dilutions as listed below. The cells were rinsed the next day with 0.2% Tween 20/PBS (3x5min) before incubation with the secondary antibodies (specific to their primary antibodies) diluted in 10% FBS/PBS. The cell nuclei were counterstained with Hoechst (1:1000). Secondary antibody staining was done by placing drops of 25uL secondary antibody diluted in 10% FBS/PBS on a piece of parafilm kept in a petri dish with wet paper. The glass slips were then placed cell side down on the antibodies, the plate was wrapped in foil and placed in the 4°C fridge. Following antibody staining, the slides were rinsed with 0.2% Tween 20/PBS (3x5min), mounted with Fluoromount G on glass coverslips. Images were taken with an Olympus Fluorescence Microscope.

Primary antibodies (dilution):	Secondary antibodies (dilution):
anti-GFAP (1:300) (Abcam)	Cy3-coupled donkey anti-rabbit (1:200) (Jackson)
anti-Nestin (1:20) (Millipore)	Alexa Fluor donkey anti-mouse (1:200) (Jackson)

CHAPTER 3

RESULTS

NSCs were isolated and cultured in NSC-SR medium and monitored as they formed free floating neurospheres *in vitro* in 6-well Ultra-Low attachment plates, as depicted in Figure 1. NSC propagation procedures were repeated 3 times, with 3 different frozen primary cell lines of NSCs previously isolated in Fan Lab from mouse fetal brains. Neurospheres were dissociated mechanically using a pipetting technique, and single cell suspensions were visible as well as larger spherical aggregates (Figure 1).

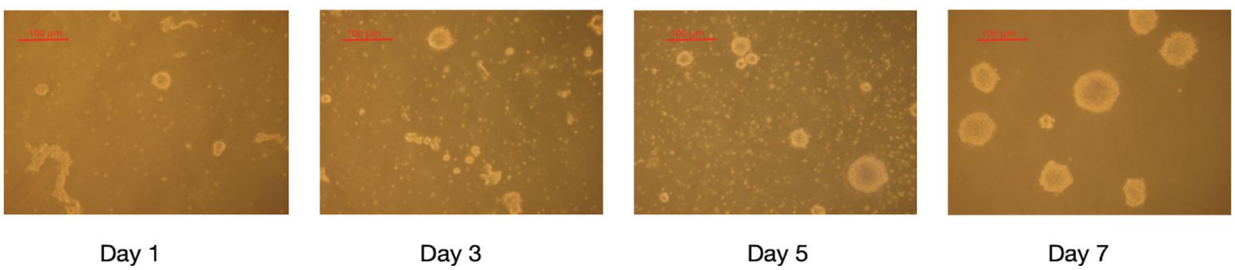

Figure 1. **Phase contrast images of cultured neurospheres**. NSCs were cultured to form neurospheres and passaged. Representative images of neurospheres of indicated days in culture are shown.

The total cell count was calculated for each batch of NSCs propagated. Trypan blue dye and a hemocytometer were used to conduct cell counting assay. The data was compiled and standard deviations (SD) were calculated to analyze the variance of cell growth of the 3 batches of NSCs cultured over a seven day period. The growth curve of NSCs was graphed (Figure 2).

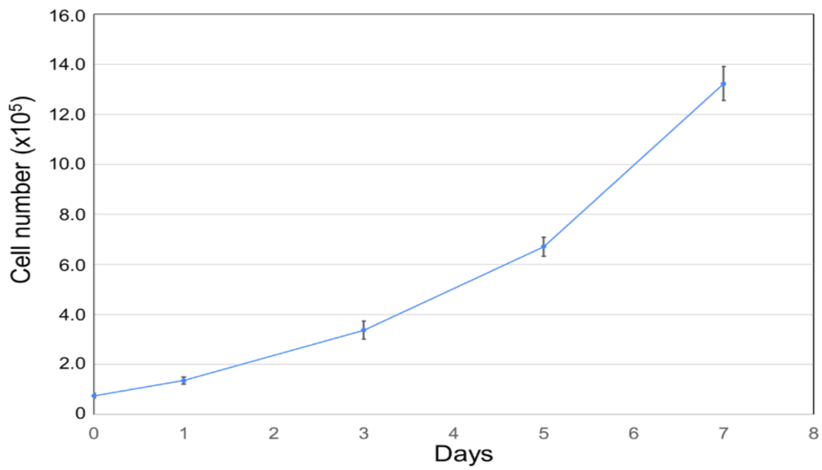

Figure 2. Growth curve of cultured neurospheres. Neurospheres were dissociated, counted and passaged. 7.5×10^3 cells were seeded and cultured in NSC-SR medium to form neurospheres. An aliquot of cultured neurospheres were counted to obtain the total number of cells present at indicated days post seeding. N=3. Bars, SD.

Next, the sizes of neurospheres were analyzed for their diameters using microscopy images and ImageJ software. Five neurospheres were randomly selected and measured every two days of culture, and their average diameters and standard deviations were measured (Figure 3, Table 1). The diameter of neurospheres increased steadily in culture, and by day 7 of cell culture, the neurospheres grew to about 100µm in diameter (Figure 3, Table 1). The standard deviations for each set of measurements were within 5 to 7 micrometers, indicating a fairly uniform size distribution of neurospheres of the culture.

Table 1. Sizes of neurospheres.

Days in culture	NS 1	NS2	NS3	NS4	NS5	Average Diameter (µm)	St. Dev. (µm)
1	36	43	28	40	33	36.0	5.9
3	47	65	54	57	60	56.6	6.7
5	76	87	84	74	85	81.2	5.8
7	93	106	97	102	95	98.6	5.3

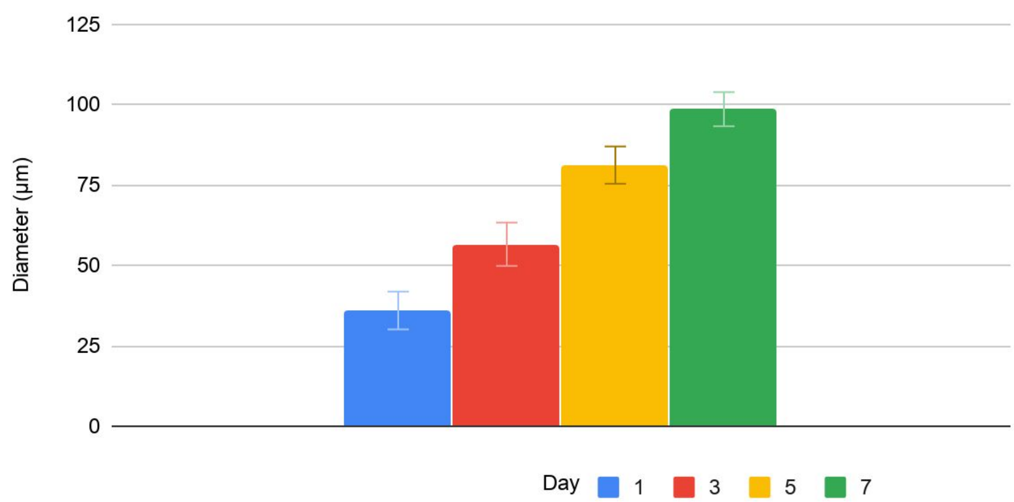

Figure 3. Neurosphere Size Assay. Graph depicting average diameter of neurospheres over a seven day culture period. Diameter was measured using microscopy images and the ImageJ software.

To gauge the expression of Nestin, a molecular marker for NSCs, in the neurosphere culture, on day 7 of propagation, neurospheres were immunostained with an anti-Nestin antibody and imaged using an immunofluorescence microscope (Figure 4). Green fluorescence signals imaged using the Olympus Fluorescence Microscope revealed the presence of Alexa Fluor conjugated to the secondary antibody bound to the anti-Nestin antibody. These results indicate that cultured neurospheres exhibit Nestin expression characteristics of NSCs. The blue fluorescence signals are from the Hoechst counterstain of cell nuclei (Figure 4).

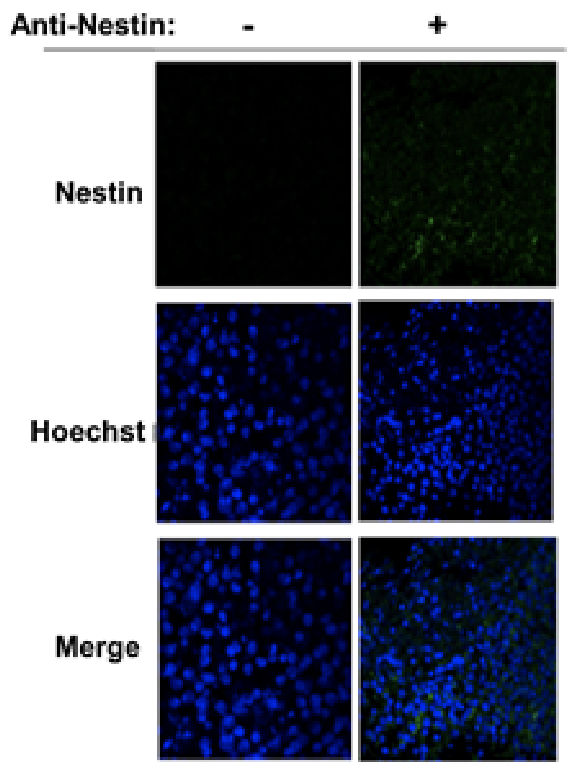

Figure 4. Nestin expression in neurospheres. Neurospheres cultured on day 7 of propagation were immunostained for expression of Nestin. Nuclei were stained with Hoechst 33342. Representative images are shown. Images were taken using a Confocal Fluorescence Microscope at 60x magnification. Immunostaining without a primary antibody was included as assay controls.

To determine their capacity of differentiation, neurospheres of day 7 culture were induced to differentiate for two and four days (D2, D4) using NSC-DN media following the scheme shown in Figure 5. NSC-DN media were replaced as necessary to ensure differentiation of the neurospheres. Neurospheres on both differentiation days (D2 and D4) had neurite outgrowth and cells migrating outside of neurospheres as shown in Figure 6.

NSC culture	**D1**	**D2**	**D3**	**D4**
Days:	7	7+2		7+4

Figure 5. NSC culture and differentiation scheme. NSCs cultured to form neurospheres and subsequently differentiated into neural networks.

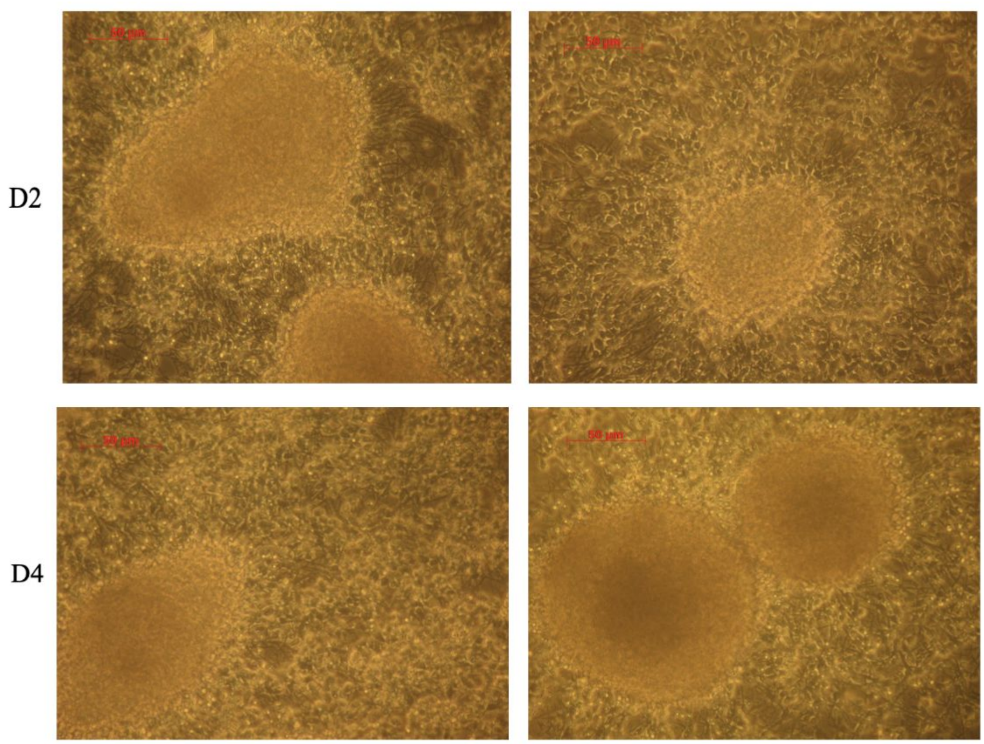

Figure 6. Phase-contrast microscopy images of differentiating neurospheres. Neurospheres undergoing differentiation for 2 and 4 days (D2, D4) were imaged using a Phase-Contrast microscope. Scale bar: 50µm.

To further investigate the differentiation extent of these neurosphere cultures, an immunostaining assay using an Anti-GFAP antibody and a Cy3 (red) conjugated secondary antibody was carried out to gauge the expression of GFAP protein, which is present predominantly in astrocytes. GFAP positive cells were present in both D2 and D4 culture but GFAP appeared to be more greatly expressed in D4 cells (Figure 7). The fluorescence images also reveal the formation of dendritic connections between NSCs, with these connections being more numerous and visible on D4. Hoechst counterstaining for cell nuclei showed the expression of GFAP in cytoplasm as expected (Figure 7).

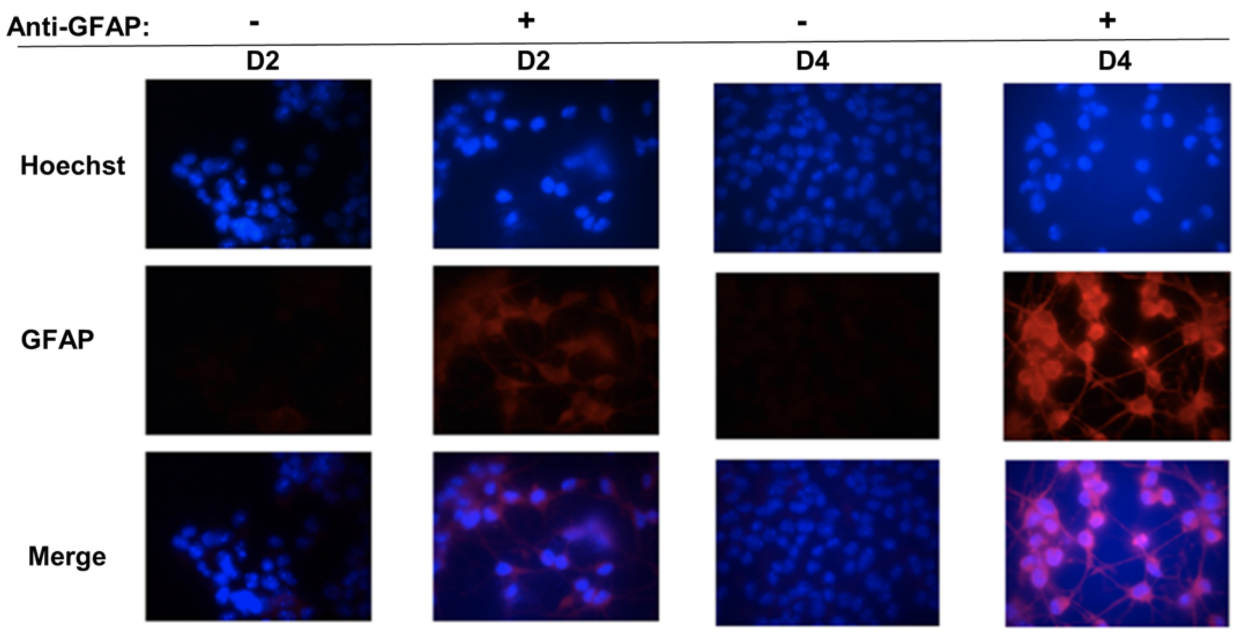

Figure 7. GFAP expression in differentiating neurospheres. Neurospheres at day 2 (D2) and day 4 (D4) post induction of differentiation were immunostained for expression of GFAP. Nuclei were stained with Hoechst 33342. Representative images are shown. Immunostaining without primary antibody was included as assay controls.

CHAPTER 4

DISCUSSION AND FUTURE WORK

The NSCs, cultured and propagated as neurospheres, had an exponential growth rate with the sizes of neurospheres increased steadily. On average, among all three NSC batches that were thawed and cultured, I was able to obtain a culture density of about 55,000 cells/mL by Day 7 of treatment with NSC-SR medium and with mechanical dissociation every two days. This observation is consistent with results from previous studies of NSC growth.

I induced the differentiation process on the cells of Day 7 in propagation and performed immunostaining assays to detect the expression of Nestin, a cytoskeleton intermediate filament characteristic of NSCs, and GFAP, the intermediate filament proteins present in mature astrocytes. The results confirmed the expression of Nestin in neurosphere cells in propagation and revealed dramatic expression of GFAP in D2 and D4 of differentiating neurospheres in this differentiation scheme. Because immunostaining was only conducted on one batch of cells, the experiments would need repeats to strengthen these findings.

Through analyses of growth curve of growing neurospheres and size comparisons of neurospheres, I determined that the neurospheres reached about 100μm in diameter after 7 days in culture, which is smaller than previously recommended neurosphere sizes with diameters ranging between 200 and 400μm for differentiation. Future studies should more closely monitor neurosphere sizes to determine the optimal sizes for inducing specific lineages in the differentiation process. The work conducted here provides preliminary information on NSC growth rates when cultured with NSC-SR medium and the increased GFAP expression as these neurospheres differentiate. Future studies with immunostaining to analyze the expression of other molecular markers for neural differentiation, such as β-III tubulin for neuronal cells, will help to better characterize these cells and their differentiation conditions.

Furthermore, it would be interesting to look at the effects of epigenetic agents on NSC differentiation. An accumulation of data from recent research suggests that methylation and demethylation of specific genes may play a key role in the development of the CNS. DNA Methyltransferases (DNMTs) are critical for neural differentiation. Using small molecules to modulate DNMT activity to investigate the effects of DNA methylation in NSCs and neural differentiation under the differentiation schemes in the present study should lead to interesting findings.

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