ABSTRACT

TULSI PATEL
Directed Differentiation of Human Embryonic Stem Cells into Glial Cells
(Under the Direction of DR. STEVEN L. STICE)

Human embryonic stem cells are pluripotent cells that have the potential to differentiate into all cell types found in the human body. The Stice Lab has previously derived neural progenitor (hNP) cells, which can be further differentiated into neurons, from human embryonic stem cell lines. Currently, the media used for hNP proliferation is comprised of neural basal media, Leukemia Inhibitory Factor, Fibroblast Growth Factor 2, L-glutamine, and an undefined B27 supplement. To better understand the role of B27 on neurons, hNP cells were grown in various concentrations of B27 ranging from 0 to 1XB27. Our results showed 0.1X B27 was sufficient for hNP proliferation. Additionally, RNA analysis data from differentiated neurites that were proliferated in 0.5X B27 and 0.1X B27 media showed a significantly higher expression of Glial Fibrillary Acidic Protein (GFAP), a marker used to identify glial cells, when compared to our control cells. These observations indicate that hNP cultures proliferating in lower concentrations of B27 differentiate into glial-like cells. Immunofluorescent experiments to detect glial protein levels indicated that the cells did not express GFAP or A2B5, a glial precursor marker, at significant levels. As a result, it was concluded that decreasing the levels of B27 induces transcription of glial genes. However, these genes are not translated into proteins. Glial cells provide support and nutrition for neurons in the central nervous system. Defining a uniform culturing condition for these cells could help understand and cure glial-degenerative diseases like Multiple Sclerosis, Alzheimer's, and Alexander's Disease.

INDEX WORDS: Stem Cells, Differentiation, Glial Cells, B27, Glial Fibrillary Acidic Protein

DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO GLIAL CELLS

by

TULSI PATEL

A Thesis Submitted to the Honors Council of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

BACHELOR OF SCIENCE

in GENETICS

with HIGHEST HONORS

and CURO SCHOLAR DISTINCTION

Athens, Georgia

2009

DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO GLIAL CELLS

by

TULSI PATEL

Approved:

Dr. Steven L. Stice _____ 04-28-2009_____
Dr. Steven L. Stice Date
Faculty Research Mentor

Approved:

Dr. Daniel E. L. Promislow _____ 04-28-2009_____
Dr. Daniel E. L. Promislow Date
Reader

Approved:

Dr. David S. Williams _____ 05-08-2009_____
Dr. David S. Williams Date
Director, Honors Program, Foundation Fellows and
Center for Undergraduate Research Opportunities

Approved:

Dr. Pamela B. Kleiber _____ 05-08-2009_____
Dr. Pamela B. Kleiber Date
Associate Director, Honors Program and Center for
Undergraduate Research Opportunities

DEDICATION

I would like to dedicate this thesis to all the couples who donated their left over IVF embryos to research. Thank you for believing in the healing potential of stem cell research.

ACKNOWLEDGEMENTS

I would like express my deepest gratitude to Dr. Steven Stice, who has been my research mentor for two years. I have been inspired by his dedication to science and to society at large. This work would not have been possible without his support and guidance.

I am greatly indebted to Dr. Brian Gerwe, my graduate mentor for over a year. He taught me many of the techniques that were essential in finishing this project. I also want to acknowledge that he prepared some of the figures that are presented in this thesis.

I would like to thank all the members of the Stice Lab, especially, Amber Young, Jennifer Mumaw, Dr. Franklin West, and Dr. Mahesh Dodla, for answering my questions and helping me with various experiments.

I also want to expresses my deepest appreciation for Dr. Daniel Promislow for serving as my reader and for all his advise. Dr. Promislow has also been an excellent advisor and mentor to me.

Finally, I want to thank Dr. Kleiber for guiding me throughout my undergraduate career.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1
INTRODUCTION

Cellular degenerative diseases occur when specific cells are either programmed incorrectly or damaged such that they cannot perform their jobs adequately. In such diseases, normal bodily functions are lost because the damaged cells cannot be naturally replaced with healthy ones. One method of curing degenerative diseases and injuries would be to artificially replace these damaged cells with healthy ones. Therefore, producing healthy human cells *in vitro*, in order to elucidate their normal functioning and usefulness in cell-based therapies, has become the focus of much ongoing research.

Neurodegenerative diseases such as Parkinson's, Huntington's and epilepsy are caused by the death or loss of function of neural cells in the brain and the spinal cord. Functional neurons transmit electrochemical impulses that control activities like movement, sensation, and learning. Essential to the function of neurons are glial cells that provide support and nutrition to neurons, maintain homeostasis, and assist in signal transmission (Wilson and Stice, 2006). When any of these central nervous system cells are damaged, some of this important brain activity is lost.

To understand and cure neurodegenerative diseases, insight into normal neural and glial function is essential. While animal cells are currently used to understand the development and function of neurons, there is no adequate source of human neural cells that could be used to translate basic findings in animal cells to clinical applications for human patients.

Embryonic stem cell research may bridge this gap. Human embryonic stem cells (hESC) derived from human blastocysts are pluripotent cells that undergo prolonged self-renewal and

can differentiate into derivatives of all three embryonic germ layers (Thomson et al., 1998). The in vitro derivation of specific cell lineages from these cells can lead to cell replacement therapies for cellular degenerative diseases. Studying the basic development and differentiation of various cell types, like neurons and glia in the central nervous system (CNS), from human embryonic stem cells is therefore extremely important.

The purpose of this study is to develop culturing conditions that will allow us to differentiate human embryonic stem cells into astrocytes. Astrocytes are glial cells that play a vital role in the normal functioning of the CNS. They regulate the blood-brain barrier formation, homeostasis, neurotransmission, synaptogenesis, and neurogenesis. During neuronal injury, astrocytes mediate neuronal regeneration by direct activation and regulation of various growth factors and receptors. Additionally, astrocytes also regulate the amount of inflammatory mediators formed by brain macrophages called microglia. Increased brain inflammation is shown to be a risk factor in the onset of neurodegenerative diseases, and by regulating brain inflammation, astrocytes play an indirect role in the response to neuronal injury (Min et al., 2006).

Important to this study are three concepts: characterization of astryocytes, *in vivo* development of astrocytes and other central nervous system cells, and current methods used for hESC differentiation into neuronal cells. Firstly, it is crucial to be able to have reliable methods to identify astrocytes in a pool of different central nervous system cells, for without them, we would never know if we have successfully differentiated stem cells into astrocytes. Secondly, an understanding of the factors that are important in the development of neurons and glia in the brain would help us determine the adequate culturing conditions *in vitro*. And lastly, the proposed culturing conditions for astrocytes are derived from already established differentiation

conditions used to derive neurons from human embryonic stem cells. Therefore an overview of this process is also helpful.

Characterization of Astrocytes

Astrocytes are restricted to the CNS and have two known morphologies: in the white matter, they are present as "fibrous" cells with long unbranched cellular processes, and in the gray matter they are "protoplasmic" cells with short and highly branched cellular processes. It is not clear whether the difference in morphology results from the difference in the physical environments, or if the two are different cell types with independent developmental pathways (Kessaris et al., 2008). Because of this discrepency, cell morphology is not adequate in identifying astrocytes and another method is necessary.

It has been shown that astrocytes have a high content of cytoplasmic filaments, especially the intermediate filament, glial fibrillary acidic protein (GFAP). Numerous immunofluorescence studies done in rat, rabbit and human cells have shown that GFAP is present in astroglia filaments, but not in neuronal filaments (Bignami and Dahl 1973, 1974, 1977, Antanitus et al., 1976). Immunoreactivity of GFAP is therefore considered characteristic of astrocytes. However, GFAP is not exclusively found in astrocytes. Bergmann glia in the cerebellum, Muller glia in the retina, radial glia in the embryonic neural tube, and subependymal astrocytes in adults are also GFAP positive (Kessaris et al., 2008). But because more specific proteins or functional characterizations for astrocytes have not yet been discovered, presence of GFAP protein is used as an identifying marker of mature astrocytes in this study.

Additionally, A2B5 antibody is also used to stain for glial precursor cells. The A2B5 antigen is a complex ganglioside that was discovered in the plasma membrane of the retinal neuron cell body, and is not present in the dendrite or axons (Eisenbarth et al., 1979). A2B5 is

found in the precursors for type 2 astrocytes and oligodendrocytes (Raff et al 1983) and specifies glial precursor cells in culture from other CNS cells (Hunter and Bottenstein, 1991, Fok-seang and Miller, 1992). While not specific to astrocytes, this antibody is none the less helpful in identifying glial differentiation from neuronal differentiation.

Development of Astrocytes in the Central Nervous System

Neurons and most glia differentiate from neuroepithelial cells that emerge from the ectoderm germ layer of a gastrulated embryo (Wilson and Stice, 2006). Neuroepithelial cells are multipotent and have the potential to differentiate into all neuronal and glial cell types in the central nervous system (CNS). Neuroepithelial cells first proliferate to reach a critical mass, so that enough neurons and glia are generated for normal CNS activity and then begin to differentiate. Once differentiation begins, neurogenesis precedes gliogenesis (Wilson and Stice, 2006, Kessaris et al., 2008).

The differentiation potential of neuroepithelial cells at different times during development is defined by signaling factors and morphogens like sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) (Kessaris et al., 2008). The triggers that regulate the switch from neurogenesis to gliogenesis are not entirely known. However, it is known that once gliogenesis begins, the formation of oligodendrocytes and astrocytes is controlled on the dorsal-ventral axis by levels of BMPs and Shh. BMPs, which induce astrocyte specification and inhibit oligodendrocyte specification are found in high concentration on the dorsal side, while Shh, which induce oligodendrocyte specification and inhibit astrocyte specification, are expressed most on the ventral side (Kessaris et al., 2008). This leads to mutually exclusive interactions between Shh and BMPs and forms a gradient in which precursor cells either differentiate into

oligodendrocytes or astrocytes, depending on how much exposure to Shh and BMP they experience.

The switch from the generation of neurons to astrocytes is controlled by the Jak/Stat pathway, which is inhibited in early development by neurogenic transcription factors like Ngn1 early in development. As Ngn expression decreases, the repression of Stat is relieved and transcription of GFAP and other astrocytic genes is activated. Other cytokines like LIF and the Notch signaling pathway also play different roles in the development of astrocytes (Kessaris et al., 2008).

HESC Differentiation into Neurons

The Stice lab has successfully derived a self-renewing population of neuroprogenitor (hNP) cells, which are the *in vitro* equivalent of neuroepithelial cells found in the brain. We have also been able to further differentiate these cells into neurons. The media used for hNP proliferation is comprised of neural basal media, Leukemia Inhibitory Factor, Fibroblast Growth Factor 2, L-glutamine, and an undefined B27 supplement. FGF2 is removed from this media for an additional two weeks of cell culturing to induce motor neuron differentiation (Shin et al., 2005). The proliferation media is referred to as Prolif and the differentiation media without FGF2 is called L-Diff. Nestin and beta III Tubulins are the primiary markers that are used to distinguish hNPs from differentiation neurons. Proliferating hNP cells are positive for Nestin, an intermediate filament expressed transiently in various cells during development, including neural precursor cells. Nestin is down regulated once the cells start to differentiate and is therefore used to identify neural progenitor cells (Wiese et al., 2004). Once hNP cells are exposed to the differentiation media, L-Diff, they start to express neuron specific Beta III Tubulin, which

indicates their development from progenitor cells to differentiating neurons. TuJ 1 antibody is used to stain for Beta III Tubulin.

Like neuroepithelial cells, we expect the hNP cells to have the ability to differentiate into all cell types found in the CNS, including astrocytes. We have shown that adding 5% fetal bovine serum to the differentiation media induces GFAP expression (Gerwe et al., unpublished data), indicating that these hNP cells are developing towards an astrocytic lineage instead of differentiating into neuronal cells.

All components of the hNP culturing media are defined except B27. B27 used in the lab is purchased from Invitrogen®, which does not reveal the exact components of the supplement. Defined media supplements have become a norm in cell culturing because they reduce the use of biologically derived serums and increase consistency in cell culturing (Bottenstein and Sato, 1979). The supplement B27 has been widely used in successfully culturing various neuronal cell lines. However, inconsistencies in the effect of B27 on cultured cells have been apparent in recent years. These inconsistencies have been attributed to the presence of components like bovine serum albumin and transferrin (Chen et al. 2008), which are isolated from biological sources and can therefore vary according to the age, sex, nutrition, and physiological state of the donor animal (Bottenstein and Sato, 1979). Numerous investigators have made their own formulation of B27 and the most recent of these is described in Chen *et al*. (2008). The main difference between this B27 formula and the ones defined earlier is that Chen *et al.* clearly define the vendor and product for the bovine serum albumin and use halo-transferrin instead of apo-transferrin (Chen et al., 2008). Podratz *et al.* also defined their own formula for B27, which consists of another defined supplement, N2 and other serums and anti-oxidants. **Table 1** is comprised of a detailed list of these B27 components (Podratz *et al.,* 1998). It is currently

unknown whether all these components are essential or even advantageous for the proliferation of hNPs and for uniform differentiation to functional neurons. However, one of the components, retinyl acetate, is known to have an active role in CNS development.

Retinyl acetate, which is used in cells as retinoic acid (RA), is a signaling molecule that induces early neurogenesis. Retanoic acid is derived from Vitamin A, which is an essential dietary component. Vitamin A deficiency has long been associated with numerous defects, including nerve degeneration in the spinal cord, which results in loss of coordination and other functions indicative of motor neuron diseases in pigs and chicken (Maden, 2002). Other experiments have showed that embryonic carcinoma cell cultures differentiate into neurons in the presence of RA (Jones-Villeneuve et al., 1982). Since these initial experiments, much more is known about the specific role of RA in CNS development.

Table 1: Components of B27, as defined by Podratz et al, 1998. B27 is composed of N2, various antioxidants and other factors and serums.

N2 Components	**Amount**		**Antioxidants**	**Amount**
Linoleic Acid	1 µg/ml		Catalase	2.5 µg/ml
Linolenic Acid	1 µg/ml		Superoxide Dismutase	2.5 µg/ml
Progesterone	0.0063 µg/ml		DL-α-tocopherol acetate	2.5 µg/ml
Albumin, bovine	2.5 µg/ml		DL-α-tocopherol	1 µg/ml
Putrescine	16.1 µg/ml		Glutathione (reduced)	1 µg/ml
Selenium	0.016 µg/ml		**Other Components**	**Amount**
Insulin	4 µg/ml		Corticosterone	0.02 µg/ml
Transferrin	5 µg/ml		Biotin	0.1 µg/ml
			L-Carnitine	2.0 µg/ml
			D (+)-galactose	15 µg/ml
			Retinyl acetate	0.1 µg/ml
			Ethanolamine	1 µg/ml
			T3 (triodo-1-thyrosine)	0.002 µg/ml

RA is a caudalizing molecule that mediates sequential specification of the neural tube into the forebrain, midbrain, hindbrain, and spinal cord, the four major subdivisions along the anterior-posterior axis (Wilson and Stice, 2006). It has been shown that RA works early in embryonic development and functions upstream of patterning genes and proneural genes in the embryo (Maden, 2002). Hundreds of genes involved in the processes of neural differentiation and neurite extension have shown to be regulated by RA. These genes include transcription factors, structural proteins, enzymes, cell-surface glycoproteins, extracellular proteins, neurotransmitters, neuropeptide hormones, growth factors and cell-surface receptors. Specifically, RA has been shown to play an important role in the specification of the hindbrain in chick and mouse embryos. In the absence of RA the hindbrain does not develop, while the midbrain and forebrain do develop and are enlarged. Alternatively, if RA is over expressed, the hindbrain is enlarged, while the anterior structures are absent or compressed (Maden, 2002). As for the in vitro differentiation of neurons, recent studies have indicated that efficient motor neuron differentiation from neuroprogenitors in neural rosettes requires both the caudilizing effects of RA and ventralizing effects of Shh. It has also been demonstrated that motor neuron differentiation is restricted by a specific window of time during which RA and Shh have to be applied. The presence of retinyl acetate in B27 may therefore be important for the specification of the neuronal fate during proliferation and differentiation. Decreasing the concentration of B27 may therefore decrease the ability of hNPs to differentiate into neuronal cells.

In an effort to better understand the role of the commercial B27 (Invitrogen®) supplement in cell proliferation and differentiation, we grew cells in varying concentrations of B27 and monitored our cells for resulting effects. We observed that hNP cells do not survive in the absence of B27. However, a spike in the RNA expression of the GFAP gene is seen when

hNP cells are cultured in decreased concentrations of B27. Additionally, when cells grown in varying concentrations of B27 are differentiated in the presence of 5% FBS, cells in lower concentrations of B27 expressed GFAP at higher levels than did control cells grown in 1X B27. Therefore, there is reason to believe that proliferation of hNP cells in low concentration of B27 followed by differentiation in 5% FBS might induce specification of astrocytic cells. However, when cells grown in lower concentrations of B27 were differentiated with and without 5% FBS, immunofluorescent staining experiments did not show significant expression of GFAP or A2B5 protein in differentiating cells. As a result, it is proposed that decreasing the concentration of B27 in cell culturing induces transcription of glial genes. However, these transcripts may not be translated into proteins due to negative regulation.

CHAPTER 2
MATERIALS AND METHODS

Human Neural Progernitor (hNP) Culturing

hNP cells were derived from WA09 human embryonic stem cell line as described in past reports by our laboratory (Mitalipova *et al.*, 2003). Cells were grown on plates coated with polyornithine (20 μg/ml) and laminin (5 μg/ml). The control 1X B27 proliferation media consisted of neurobasal medium (Gibco) supplemented with 2mM L-glutamine, 1X penicillin/ streptomycin, 1X B27 (Gibco), 20ng/ml of bFGF (Sigma-Aldrich) and 10ng/ml of Leukemia inhibitory factor (LIF, Chemicon). Cells were also proliferated in 0.5X B27, 0.25X B27, 0.125X B27. Cells were kept under 5% CO_2 atmosphere at 37°C and media was changed every third day. When cells on plates became 90% confluent, they were passaged 1:2 by manual dissociation.

Human Neural Progenitor Differentiation

Cells were differentiated on polyornithine (20 μg/ml) and laminin (5 μg/ml) coated plates. The differentiation media consists of the same components as the proliferation media without bFGF. Cells were differentiated in differentiation media with and without 5% FBS. Cells were passaged 1:2 on Day 3 of differentiation and were differentiated for a total of 35 days in 5% CO_2 at 37°C.

Quantitative Real Time-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to determine the relative gene expressions of Nestin, beta III Tubulin and GFAP in preliminary experiments in which cells proliferated in 0.5X B27 were differentiated in L-Diff with 1X B27 and L-Diff with 0X B27. RT-PCR was also used to

determine the relative GFAP expression during the differentiation of cells proliferated in 1X B27, 0.5X B27 and 0.125X B27 Prolif.

Qiashredder and RNEasy Mini kits (Qiagen) along with manufacturere's RNA extraction protocols were used to extract mRNA from cells. The quality and quantity of RNA was verified by using a RNA 600 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies) and the total RNA was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.). One microliter of the cDNA sample (diluted 1:7.5) was added to 1X primer/ probe solution (stock 20X, Applied BioSystems) along with 2X Taqman PCR Master Mix into 384 well plate and sterile water was added (v_f = 10µl). Real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc.) under standard cycle conditions. Preliminary experiments for 0.5X B27 cell differentiation were replicated in duplicates and differentiation experiments in L-Diff with 5% FBS were carried out with quadruple replications. All failed (undetermined) reactions were excluded when ΔCt values were calculated. For calculation of relative fold change values, samples were normalized against their respective endogenous 18S ribosomal RNA utilizing the ΔΔCT method of quantification (Applied Biosystems Inc.) (14). Then, the average fold changes from four independent runs were calculated as $2^{-\Delta\Delta CT}$. Statistical significance was determined via 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons focusing on temporal GFAP expression.

Immunofluorescence Staining for Intracellular Proteins

Immunofluorescence was used to determine expression of nestin and A2B5 in cells proliferated in 1X B27, 0.5X B27, and 0.125X B27 at day 56. 1X B27, 0.5X B27, 0.25X B27, and 0.125X B27 cells differentiating with or without 5% FBS for 35 days were also stained weekly for nestin, A2B5, GFAP, and TuJ 1.

Cells placed on 4 chamber well slides coated with polyornithine (20 μg/ml) and laminin (5 μg/ml) were washed once with PBS and fixed with 2% Paraformaldehyde for 15 minutes. After fixation cells were washed three more times in PBS and permeabilized in High Salt Buffer with 0.05% Tween 20. Blocking was carried out for 45 minutes in 6% Donkey Serum in High Salt Buffer. Primary antibodies against GFAP (Sigma-Aldrich), Nestin (Neuromics), beta III tubulin (Neuromics) and A2B5 were diluted in blocking solution and placed on cells for 1 hour at room temperature. Cells where then washed 4 times with high salt buffer and then placed in secondary antibody for 1 hour at room temperature or overnight at 4˚C. Secondary antibody donkey α-mouse IgG Alexa-conjugated-488 fluorophor was used against primary antibody for GFAP; secondary antibody donkey α-chicken IgY Texas-Red was used against beta III tubulin and Nestin primary antibodies; and secondary antibody goat α-mouse IgM Alexa-conjugated-488 fluorophor was used against A2B5 primary antibody. The Alexa Fluora antibodies were diluted 1:1000 and Texas-Red was diluted 1:250 in blocking solution. Cells were then washed three times in PBS and nuclei were stained with mounting solution containing 4',6'-diamidino-2-phenylindole (DAPI). Cells were observed under an Olympus Ix81 with Disc-Spinning Unit and pictures were taken using Slide Book Software (Intelligent Imaging Innovations) and analyzed by way of Image-Pro Plus (Media Cybernetics).

CHAPTER 3
RESULTS

Role of B27 Supplement in Neuroprogenitor Proliferation

Cells were proliferated in Prolif media with 1X B27, 0.5X B27, 0.25X B27, 0.125X B27, or 0X B27, with and without N2. These cells were regularly monitored and light microscopy pictures were taken weekly. Our data from this experiment showed that cells die in the absence of B27, even if N2 is present. In fact, the presence of N2 did not affect cell growth at any of the B27 conditions. Additionally, cells proliferating in 0.5X and 0.25X B27 grew just as well as the cells growing in 1X B27. When a direct switch was made from 1X B27 to 0.125X B27, most cells did not survive the first passage. However, if cells cultured in 0.5X B27 or 0.25X B27 were switched to 0.125X B27, they proliferated well after an initial shock period (**Fig. 1**). These observations indicate that cells go through a shock period when the concentration of B27 is decreased, and populations of cells that can adapt to a lower concentration of B27 are being selected for in this experiment.

Expression of Differentiation Markers During hNP Proliferation

At 21 days of proliferation, hNP cells proiliferating in 1X B27, 0.5X B27, 0.25X B27 and 0.125X B27 were immunostained for Nestin, an hNP marker, and TuJ 1, a neuronal marker. It was observed that cells proliferating in Prolif media with 1X B27, were positive for Nestin and negative for TuJ 1 (**Fig 2a**). However, cells proliferating in prolif with 0.5X B27, 0.25X B27, and 0.125X B27 were positive for both Nestin and TuJ 1 (**Fig 2a**). This demonstrates that a lower concentration of B27 induces early differentiation.

Cells proliferated for 56 days were immunostained for Nestin and A2B5, a glial precursor marker. It was seen that a small percentage of cells in 0.5X B27 and 0.125X B27 show faint expression of A2B5, which is absent in cells proliferating in 1X B27 (**Fig 2b**). These data suggest that cells in lower concentrations of B27 might be transitioning into a glial phenotype.

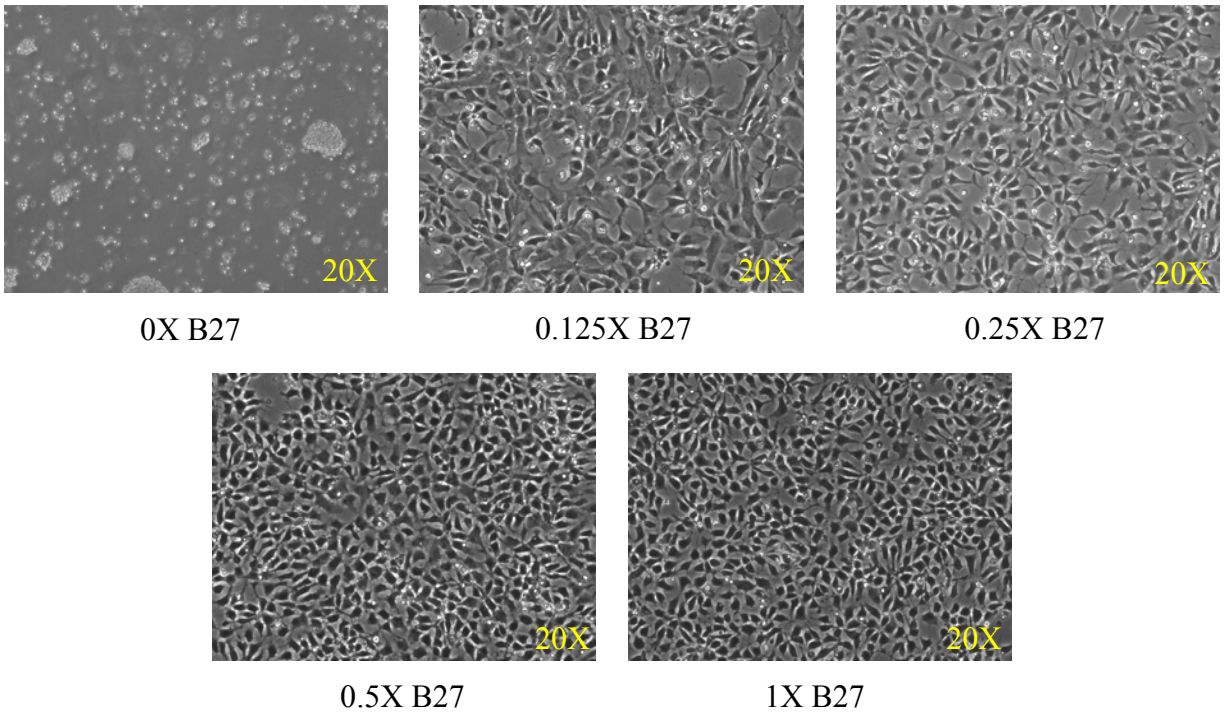

Figure 1: **Light Microscopy Pictures of Proliferating Cells at Day 19.** hNPs die in the absence of B27, even when 1X N2 is present. Cells in 0.25X, 0.5X, 1X B27 grow equally well. Cells in 0.125X B27 go through a longer shock period, but remain proliferative. Cell morphology is different in lower concentrations of B27, especially in 0.125X B27 where a lot of flat cells are seen.

Gene Expression in Differentiating Cells

In a preliminary experiment, cells proliferated in 0.5X B27 and 1X B27 were differentiated in media without FGF2. Our differentiation media (L-Diff) is also supplemented with B27, so to see if B27 played a major role in differentiation, we differentiated cells in L-Diff with both 1X B27 and 0X B27. RNA collected from these differentiating cells at day 14 and day 35 of differentiation was analyzed using real time polymerase chain reaction (RT-PCR) to identify the temporal expression pattern of Nestin, Beta III Tubulin, and GFAP. The preliminary

results from this experiment (**Figure 3a)** show that relative change in mRNA levels at Day 14 and Day 35 of differentiation in L-Diff with and without B27 when compared to the basal level of RNA expression in hNPs proliferating in media with 1X B27. Statistical analysis could not be performed on these data, since we only had 2 replicates.

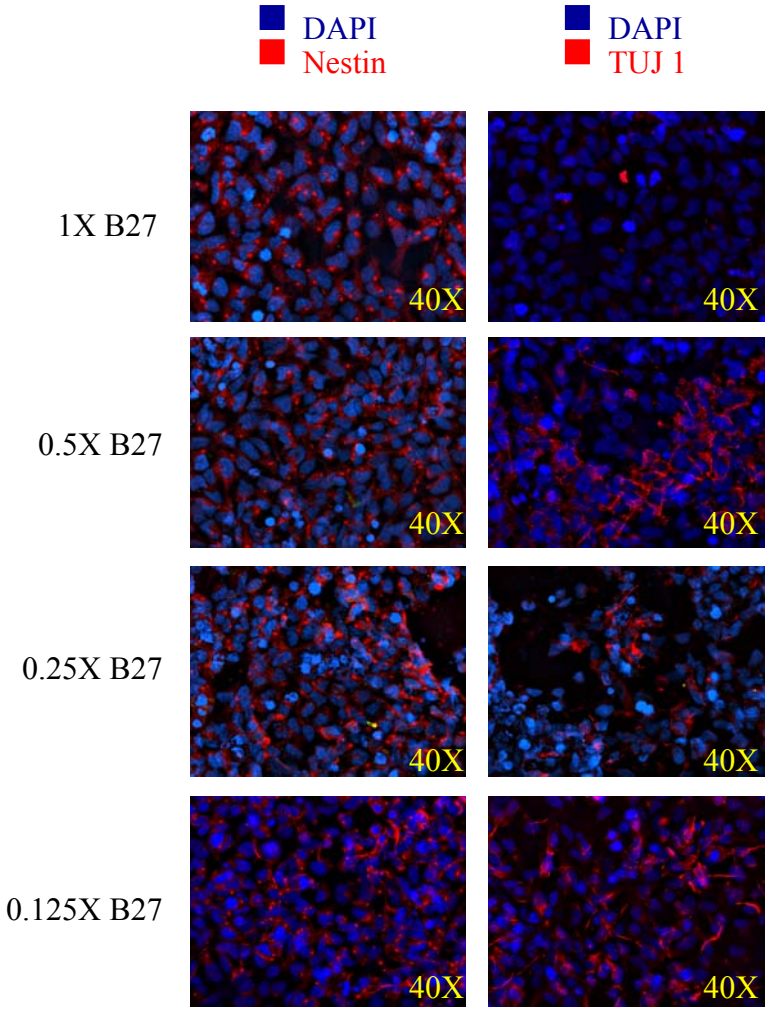

Figure 2a: **Immunofluorescence Staining for Nestin and TuJ 1 at Day 30**. Cells in all concentrations of B27 are positive for Nestin, the progenitor marker. Cells in 0.5X B27, 0.25X B27, and 0.125X B27 are also positive for TuJ 1, a neuronal differentiation maker.

The preliminary RT-PCR analysis showed that the expression of GFAP increased in differentiating 0.5X B27 cells (cells that were proliferated in 0.5X b27) at Day 35 (**Figure 3a**). This suggests that 0.5X B27 cells might be differentiating into glial cells instead of neurons. Removal of B27 may therefore induce glial differentiation. The data also illustrated that the

expression of nestin and beta III tubulin was similar in hNP cells proliferated in 1X B27 and in 0.5X B27 cells differentiated with or without B27. Nestin expression usually decreases in hNP cells as differentiation begins, however the presence of nestin in differentiating 0.5X B27 cells suggests that cells are retaining some of their progenitor quality. Beta III tubulin expression, on the other hand, increases as hNP cells are differentiated into neuronal cells. However, the RT-PCR data showed that beta III tubulin expression did not increase when 0.5X B27 cells were differentiated, which could mean that the cells are not differentiating into neurons (**Figure 3a**).

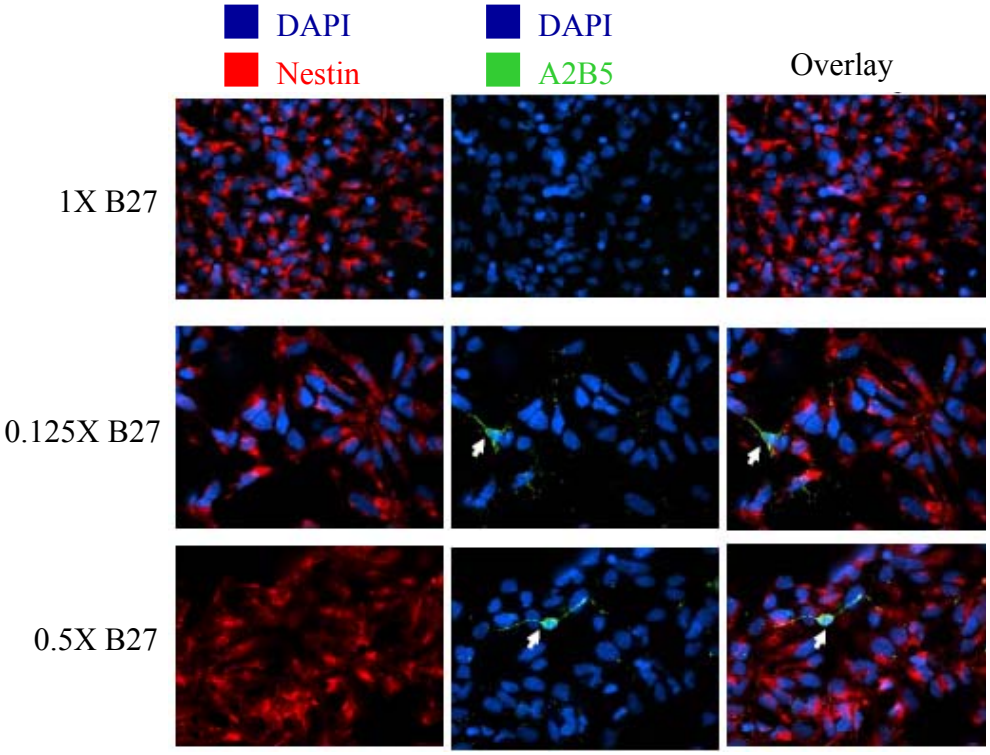

Figure 2b: **Immunofluorescence Staining for Nestin and A2B5 at Day 56.** Lowering the concentration of B27 increases A2B5 expression. Cells in all concentrations of B27 are positive for Nestin, a progenitor marker. Cells in 0.5X B27, and 0.125X B27 are also positive for A2b5, a glial progenitor marker (arrows point to A2B5 expression).

Additionally, these preliminary data also showed that there was a similar pattern of gene expression in cells differentiating in both 1X B27 L-Diff and 0X B27 L-Diff (**Figure 3a**). Additionally, cells differentiating with B27 showed a relatively higher expression of GFAP than

did cells differentiating without B27. For these reasons and because cells differentiating without B27 do not adhere well to the laminin on cell culture plates, 0X B27 L-Diff was not used in further experiments.

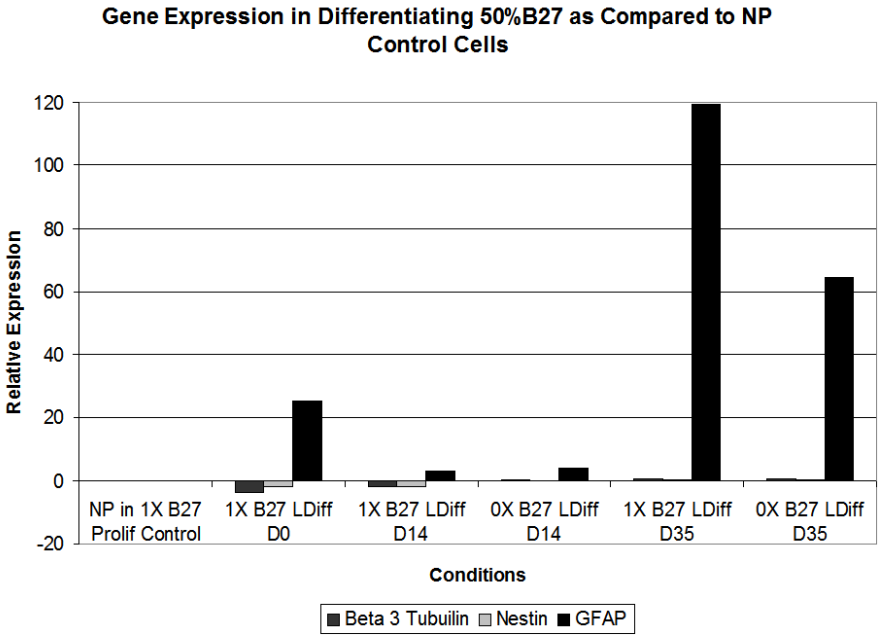

Figure 3a: **RT-PCR Analysis of GFAP, TuJ 1, and Nestin Expression in Differentiating 0.5X B27 Cells.** GFAP expression increases in differentiating 0.5X B27 cells. This analysis demonstrates the relative expression of Nestin, Beta III Tubulin, and GFAP in differentiating 0.5X B27 cells when compared to hNP cells proliferating in 1X B27. There is no apparent difference in the expression of Nestin and Beta III Tubulin. RNA expression of GFAP increases during differentiation in both 0X B27 L-Diff and 1X B27 L-Diff.

Adding 5% fetal bovine serum to the differentiation media (1X B27 L-Diff) has been shown to increase GFAP expression in differentiating hNP cells (Gerwe et al., unpublished data). hNP cells were therefore proliferated in 1X B27, 0.5X B27, and 0.125X B27 and then differentiated in L-Diff with 5% FBS. RNA samples collected at day 0, day 21, and day 28 of differentiation were subjected to an RT-PCR analysis and the relative expression of GFAP was measured. The results, demonstrated in **Figure 3b**, showed that cells in lower concentrations of B27 expressed GFAP at a higher level than did cells proliferated in 1X B27. All day 21 treatments were significant ($p<0.05$) when compared to day 0 undifferentiated hNP cells with 3.6 +/- 1 relative fold change for 0.5 X B27 and 6.3 +/- 0.4 for 0.125 X B27 treatments. Expression

of GFAP on day 28 was also significantly higher than expression on day 21. Additionally, there was a ~10.5 fold difference between day 28 hNP cells differentiated in –bFGF media with 5% FBS (4.35 +/- 1 relative fold change) and the cells proliferated in 0.125 X B27 and then differentiated in –bFGF media with 5% FBS (14.84 +/- 2).

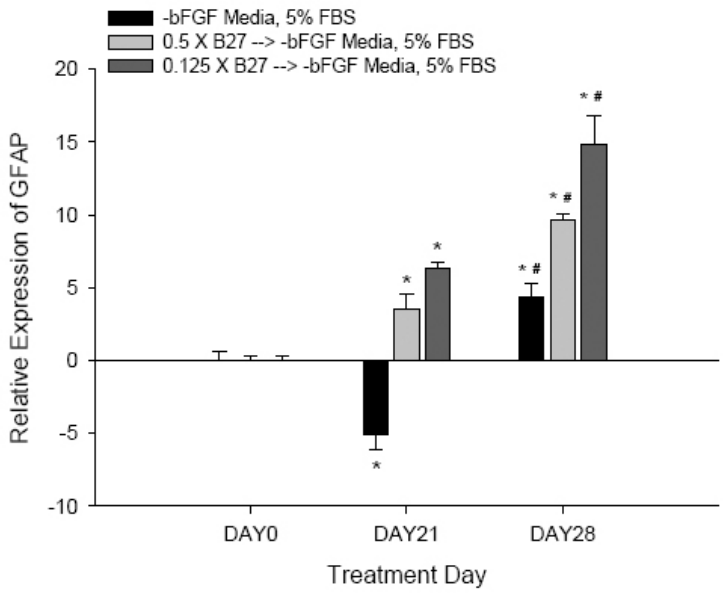

Figure 3b: RT-PCR Analysis of GFAP Expression in 1X b27, 0.5X B27, and 0.125X B27 Cells Differentiating in 5% FBS. hNPs proliferated in lower concentration of B27 express GFAP at higher levels when differentiated in L-Diff + 5% FBS. GFAP expression in cells proliferated in all conditions increased significantly from day 0 to day 21 and day 28. On both days, GFAP expression is higher in cells proliferated in lower concentrations of B27 than in 1X B27.

Immunofluorescence Data from Differentiating Cells

The relatively high RNA expression of glial genes and low expression of neuronal genes seen in the RT-PCR suggested that cells proliferated in media with lower B27 concentration and further differentiated in L-Diff might be developing towards an astrocytic lineage. However, the RNA data on its own does not show that GFAP transcripts are being translated into proteins. So in order to support the idea that glial markers are present in these cells, immunofluorescent studies were performed on differentiating cells.

Cells proliferated in 1X B27, 0.5X B27, 0.25X B27, and 0.125X B27 for 30 days were differentiated in L-Diff media and L-Diff media with 5% FBS. Cells were immunostained with

antibodies against Nestin, a neural progenitor marker, Beta III Tubulin (TuJ 1), a neuronal marker, GFAP, an astrocytic marker, and A2B5, a glial progenitor marker. The immunostaining data was collected on days 14, 21, 28, and 35 of differentiation. Data from days 14, 28, and 35 are shown here.

Day 14 of Differentiation

Figure 4 contains the immunostaining pictures of differentiating cells at day 14. Cells proliferated in varying concentrations of B27 were differentiated both with and without FBS. However, at this point, FBS does not seem to have a significant effect on differentiation, as there is not a significant difference in the protein expression patterns seen in the cells cultured with or without FBS. All cells are positive for Beta III Tubulin, seen by the high TuJ 1 fluorescence, signifying that cells are starting to differentiate. It is also seen that cells proliferated in 0.25X B27 and 0.125X B27 are positive for Nestin, suggesting that they may still retain some progenitor qualities. Some GFAP expression is also visible in 0.125X B27 and 0.25X B27. However, this is not very significant. Some A2B5 expression is also seen in 0.5X B27, 0.25X B27, and 0.125X B27 cells. While glial markers are more visible in lower concentrations of B27 than in 1X B27, neither GFAP nor A2B5 is expressed at a sufficiently high level to suggest that these cells are in fact glial cells.

Day 28 of Differentiation

Figure 5 contains the immunostaining pictures of differentiating cells at day 28. The presence of FBS seems to make 1X B27 cells express more GFAP and A2b5, which is consistent with previous unpublished data. However the presence of FBS does not significantly change the protein expression patterns of the cells proliferated in any of the lower concentrations of B27. All cells with lower concentrations of B27 are Nestin positive, showing that these cells may still

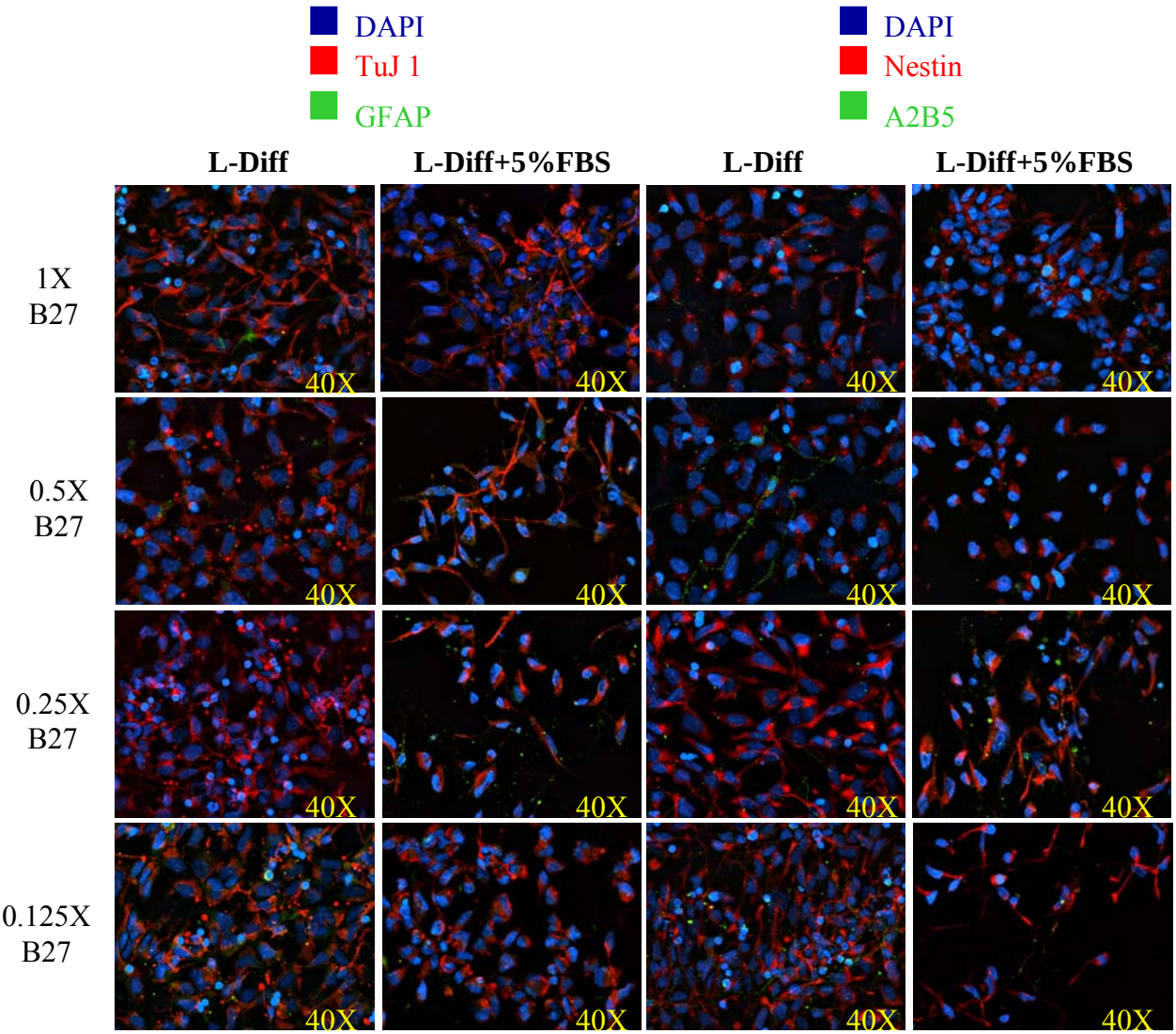

Figure 4: **Immunofluorescence Staining for Nestin, TuJ1, GFAP, and A2B5 at Day 14 of Differentiation.** Some expression of glial markers, GFAP and A2B5, is seen; however, this expression is not significant. The presence of FBS does not make a significant difference in protein expression.

be proliferative. Cells that were grown in 0.5X B27 do not express high levels of TuJ 1, which is consistent with the preliminary RT-PCR data presented above (**Figure 3a**). And, while some GFAP expression is seen in 0.125X B27 cells, there is still no significant expression. A2B5 is expressed at higher levels than it was on day 14. Based on these data, it cannot yet be said that a significant percent of these cells are astrocytes or glial cells.

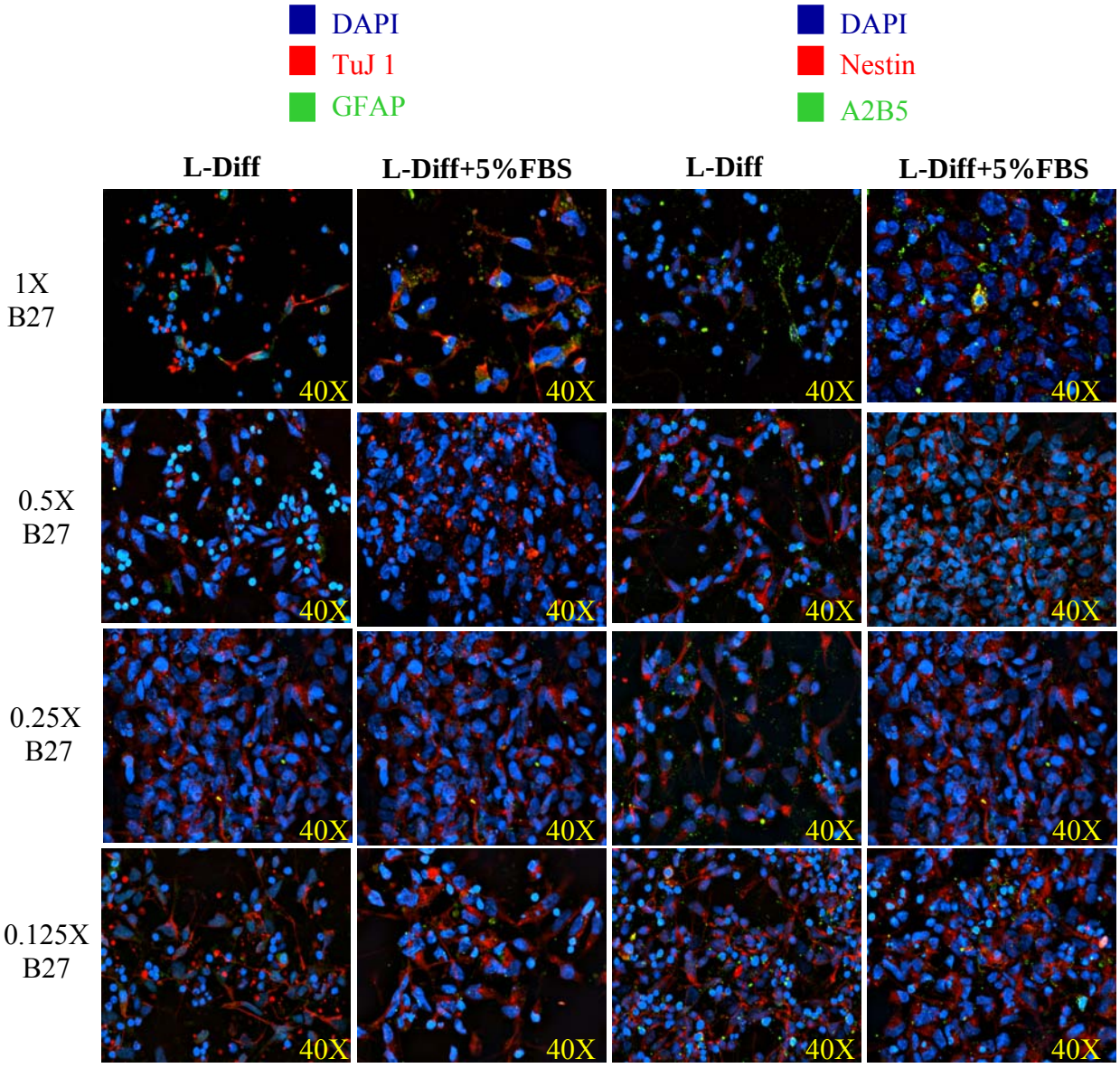

Figure 5: Immunofluorescent staining for Nestin, GFAP, TuJ 1, A2B5 and DAPI in cells proliferated in different concentrations of B27 at Day 28 of differentiation. Some expression of glial markers, GFAP and A2B5 is seen, however this is not highly significant.The presence of FBS increases expression of GFAP and A2B5 in 1X B27 cells, however, it does not yet play a significant role in cells proliferated in lower concentrations of B27.

Day 35 of Differentiation

Figure 6 contains the immunostaining pictures of differentiating cells at day 35. All the 0.125X B27 cells differentiating without FBS sheeted off and immunofluorescence data could therefore not be collected. Some expression of A2B5 is seen in differentiation cells in almost all conditions. However, this expression is not yet significant. Almost no expression of GFAP is

visible in any of the conditions. 0.5X B27 cells differentiating with or without FBS, 0.25X B27 cells differentiating without FBS, and 0.125X B27 cells differentiating in the presence of FBS still show high expression of nestin, indicating that these cells have progenitor qualities, even 35

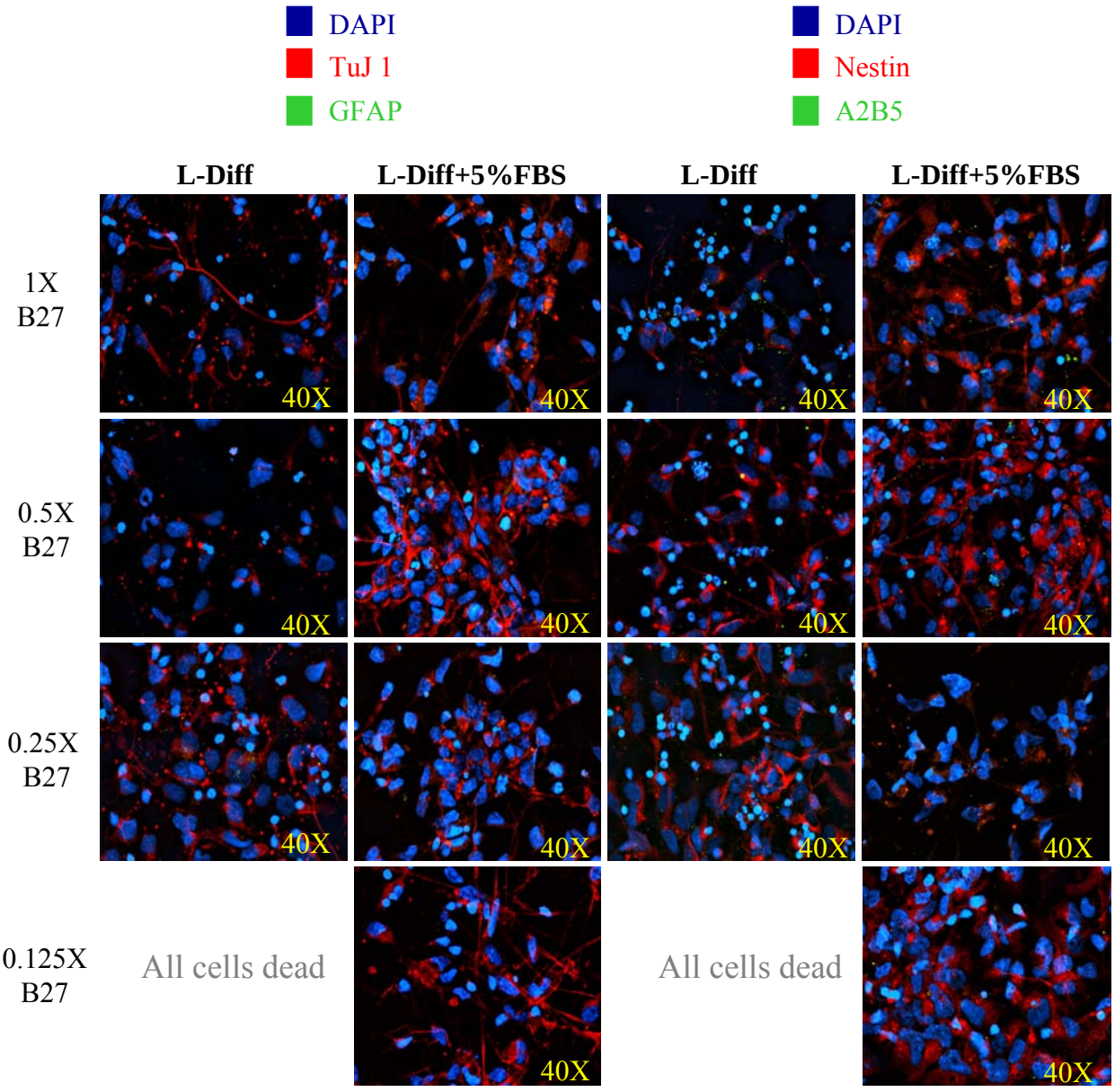

Figure 6: **Immunofluorescent staining for Nestin, GFAP, TuJ 1, A2B5 and DAPI in cells proliferated in different concentrations of B27 at Day 35 of differentiation in L-Diff and L-Diff + 5%FBS media.** Some expression of glial marker, A2B5 is seen, however this is not highly significant and is lower than it was on Day 28. Almost no GFAP expression is visible. The presence of FBS increases expression of nestin in all cells; however, it does not yet play a significant role in increasing A2B5 or GFAP expression.

days into differentiation. 0.5X B27 cells differentiating without FBS do not show significant TuJ 1 expression, showing that these cells may not be neuronal. These results do not clearly suggest that cells proliferated in lower concentrations of B27 are differentiating into a glial lineage.

Cell Morphology During Differentiation

Before differentiation, hNP cells do not have extended neurite projections and have a roughly circular cell morphology. Once differentiation begins and cells start to differentiate into neurons, they develop extended neurite projections, lose their circular morphology, and start to look elongated. Light microscopy pictures of the differentiating cells used for immunostaining were taken every week during differentiation (**Figure 7**). A comparison of these pictures shows that cell morphology varies between cells that differentiate in the presence and absence of FBS. Cells differentiating without FBS obtain neurite projections and look more like motor neurons. Cells differentiating in the absence of FBS have these neurite projections, yet some of the cells still maintain their circular morphology. Some cells differentiating in 5% FBS also start to obtain a flat morphology. Additionally cells proliferated in lower concentrations of B27 become flat earlier than cells growing in 1X B27 do (**Figure 7**). It was also observed that a lot more cells were present in differentiation media with 5% FBS than in differentiation media without FBS. This could be because cell death is decreased in the presence of FBS or because cells remain proliferative for a longer time or both.

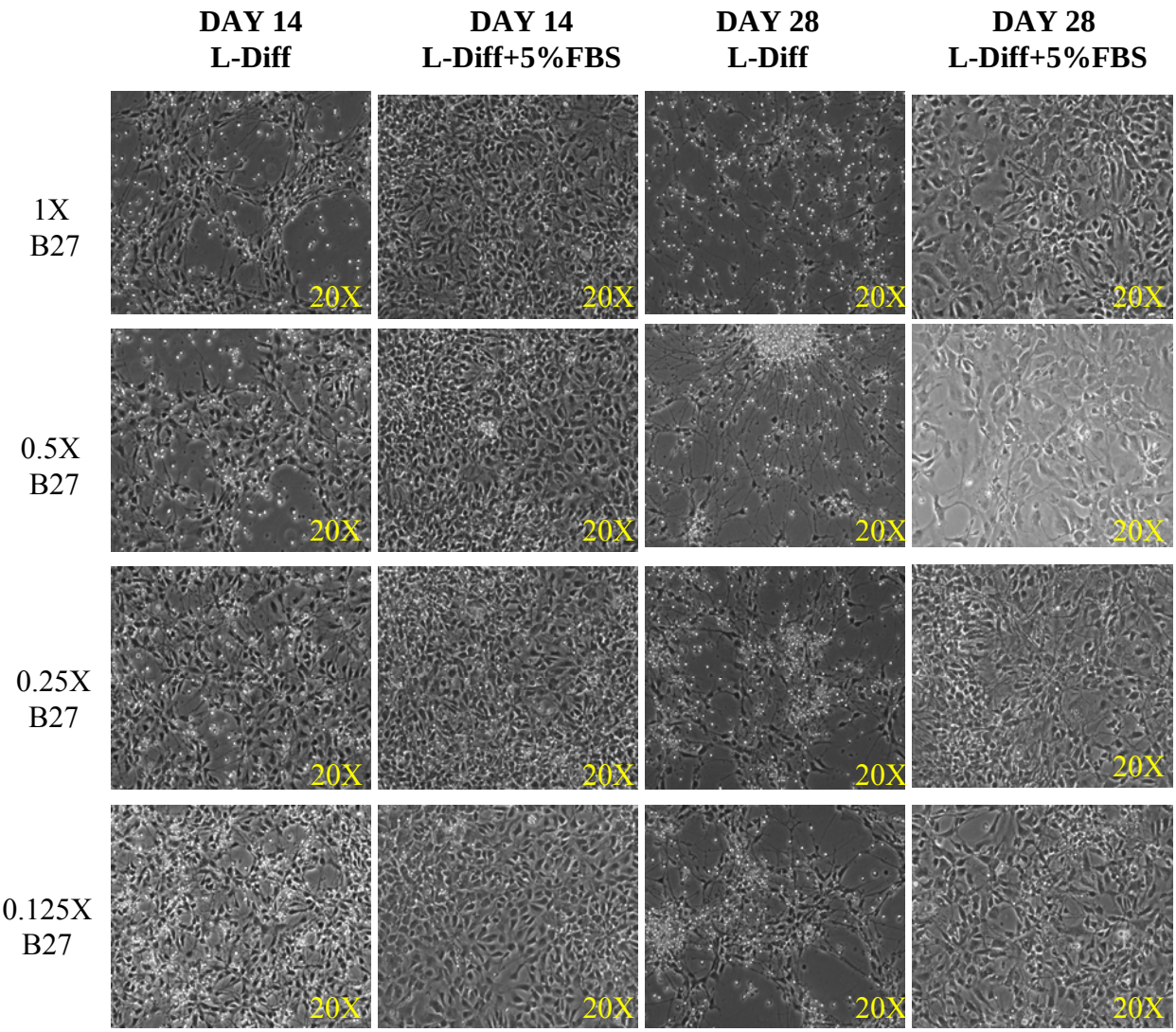

Figure 7: **Cell Morphology During Differentiation.** Cells in lower concentrations of B27 obtain a different morphology while they are proliferating. An apparent difference in morphology is seen between cells differentiation with and without FBS. Cells in 5% FBS show a flat morphology and have neurite projections, while cells in just L-Diff are long with neurite projections. Additionally cell density is much higher in 5% FBS.

CHAPTER 4
DISCUSSION, CONCLUSION, AND FUTURE DIRECTIONS

Role of Supplement B27 in Culturing hNP Cells

In the absence of B27, cells die at around Day 19 of proliferation (**Figure 1**). Therefore, it is clear that some of the components of B27 are crucial for cell survival. In lower concentrations of B27, cells have been grown for up to 56 days. These cells retain the ability to proliferate at day 56 and probably would be able to self renew for a long time. After an initial shock period, which is longer for cells in 0.125X B27, these cells proliferate at about the same rate as cells in 1X B27. In fact, populations growing in 0.25X B27 proliferated at a higher rate than cells in 1X B27. All these cells were also frozen and thawed back into a proliferative stage without difficulty. This shows that a small concentration of B27 is sufficient to retain the proliferative characteristics of hNPs. It also seems that after the initial shock period, cells that have the ability to survive in lower concentrations of B27 are selected for and these cells do not necessarily retain all the characteristics of hNPs proliferating in 1X B27. An observation of cell morphology using light microscopy revealed that cells in lower concentrations of B27 are flat and develop short neurite projections, indicating that these cells may be differentiating away from an hNP cell type (**Figure 7**).

Expression of Differentiation Markers During hNP Proliferation

When a difference in morphology was observed between cells proliferating in different concentrations of B27, these cells were immunostained for Nestin, a neural progenitor marker, Beta III Tubulin, a neuronal maker, and A2B5, a glial progenitor marker, and GFAP, an astrocytic marker. At Day 30 of proliferation, all cells were positive for Nestin, cells in lower

concentrations of B27 were positive for Beta III Tubulin, and there was no visible A2B5 or GFAP staining in any of the cells (**Figure 2a**). When cells were stained at day 56 of proliferation, a small percent (about 5%) of the cells showed A2B5 expression (**Figure 2b**). Nestin is transiently expressed in precursor stages during the development of various cell types, its expression in these proliferating cells is therefore expected. Beta III Tubulin is present in differentiating neurons, and onset of its expression begins when cells are placed in differentiation media (L-Diff). Beta III Tubulin expression is not visible in hNP cells in proliferation media with 1X B27. The early onset of Beta III Tubulin activity, especially when cells are still proliferating is only viewed when cells are cultured in lower concentrations of B27.

HNP cells are the in vitro equivalent of neuroepithelial cells, which differentiate into all cell types (neurons and glia) found in the CNS. Based on this, we suspected that cells in lower concentrations of B27 might be developing towards a glial lineage. While proliferative cells did not express high levels of GFAP, an astrocytic marker, some A2B5 expression was viewed, which indicated that at least a small subset of these cells might be differentiating into glia or glial precursor cells. To further test this hypothesis, cells proliferated in varying concentrations of B27 were differentiated.

Expression of CNS Cell Markers During Differentiation

Differentiation experiments were initially performed using L-Diff with 1X B27 and L-Diff without B27. However, it was noticed that cells differentiating in the absence of B27 started to sheet off, making data collection difficult. Additionally, the preliminary data collected from cells in both differentiating conditions showed that both populations had similar patterns of gene expression (**Figure 3a**).

Later differentiating experiments were performed with L-Diff media and L-Diff with 5% FBS. Other members of the labs have shown that hNP cells differentiating in L-Diff with 5% FBS show a significantly higher expression of GFAP in RT-PCR analysis than do cells differentiating in L-Diff (**Figure 3b**). We hoped that lowering the concentration of B27 during proliferation and adding 5% FBS during differentiation would induce glial differentiation.

The expression patterns of four different markers were studied in the differentiation experiment and are summarized here.

Nestin

As mentioned earlier, Nestin is a neural progenitor marker. It is expressed at high levels in hNPs cultured in proliferation media. Expression of Nestin was studied in preliminary RT-PCR analysis of differentiating 0.5X B27 cells and in immunofluorescence studies done on differentiating cells.

The RT-PCR analysis showed that Nestin expression in proliferating and differentiating 0.5X B27 cells was similar to expression seen in hNPs proliferating in 1X B27 (**Figure 3a**). Similarly, in immunostaining experiments, cells that were proliferated in 0.5X B27, 0.25X B27, and 0.125X B27 remained positive for Nestin during proliferation and differentiation. Cells proliferated in 1X B27, however, did not express high levels of Nestin by day 14 of differentiation. Differentiation media with 5% FBS induced a higher expression of Nestin in 1X B27 cells, however, this expression was not as high as expression in lower concentrations of B27 (**Figures 4,5,6**).

These results suggest that a lower concentration of B27 helps retain progenitor characteristics in cells, even when they are placed in differentiation media.

Beta III Tubulin

The expression of beta III Tubulin was also studied in preliminary RT-PCR analysis of differentiating 0.5X B27 cells and in immunofluorescence staining of differentiating cells.

The RT-PCR analysis showed that, like Nestin, expression level of Beta III Tubulin was similar in differentiating 0.5X b27 cells and proliferating 1X B27 cells (**Figure 3a**). This suggests that the differentiating cells were not expressing Beta III Tubulin at significant levels. The data from the immunofluorescence experiment showed that all cells were positive for TuJ 1 at 14 days of differentiation. However, on day 28 and day 35, 0.5X B27 and 0.25X B27 did not have high expression of Tuj 1 when compared to 1X B27 cells (**Figures 4,5,6**). Beta III Tubulin is specific to neuronal cells and a decrease in expression could mean that these cells are not differentiating into neurons.

One of the components of B27 is retinoic acid (RA), which, as discussed earlier, has been shown to play an important role in the development of neurons in the CNS. Perhaps decreasing the concentration of B27 lowers the concentration of RA enough to reduce neuronal differentiation. NPs are precursors to both neuronal and glial cells, when neuronal differentiation is halted by the lack of RA, the cells might differentiate into glial like cells by default. It is also possible that RA negatively regulates genes that play a key role in glial differentiation, and some of these genes may be up-regulated in the absence of RA. However, numerous other factors also effect the development of neurons and decreasing the concentration of B27 may also alter some of these other pathways.

Glial Fibrillary Acidic Protein

In this study, GFAP was used to identify astrocytic cells. GFAP expression was studied in RT-PCR analysis of 0.5X B27 cells differentiating in L-Diff and of 0.125X B27, 0.5X B27,

and 1X B27 cells differentiating in L-Diff with 5% FBS. Immunostaining experiments were also performed to visualize the presence of GFAP in differentiating cells.

Both the RT-PCR analyses showed that GFAP was expressed at significantly higher levels in differentiating cells that had been proliferated in lower concentrations of B27 than in cells proliferated in 1X B27 (**Figures 3a,b**). However, in the immunofluorescence experiments, GFAP expression is not significant at day 28 or day 35 of differentiation (**Figures 5,6**). Some expression is seen in 0.125X B27 cells and 1X B27 cells at day 28 of differentiation. However, this expression is not as high as was suggested by the RT-PCR analysis.

This discrepancy in the data could be explained by gene regulation at the translation stage. Lowering the concentration of B27 during proliferation and adding 5% FBS during differentiation might increase the transcription of the GFAP gene into mRNA. However, this may not be enough to ensure that the mRNA gets translated into protein. Gene expression is controlled at the translation level by numerous mechanisms including regulation of transport of mRNA from the nucleus to the ribosome, binding of repressors to the 5' end of the mRNA to inhibit initiation of RNA translation, and by binding of repressors to the 3' end of the mRNA and inhibiting initiation of translation by interfering with communication between the 5' and 3' end (Alberts et al., 2008 p. 488). Such mechanisms could be playing a role in stopping the translation of high levels of GFAP RNA detected in the RT-PCR analysis into protein that would be visible in immunostaining experiments. In any case, these results suggest that these cells have not differentiated into astrocytes.

A2B5

A2B5 is a cell surface marker for precursor cells to oligodendrocytes and astrocytes. Expression of A2B5 was studied by immunostaining in this project.

Cells proliferating in 0.5X B27 and 0.125X B27 showed some expression of A2B5 at day 56, while cells proliferating in 1X B27 did not show any A2B5 expression (**Figure 2b**). In the differentiation experiments, A2B5 expression was seen in 0.5X B27, 0.25X B27, and 0.125X B27 cells at Day 14 of differentiation. At Day 28, all cells, including ones proliferated in 1X B27 showed some expression of A2B5 (**Figures 4,5**).

While there is not a very high expression of A2B5, the expression that is seen indicates that some of the cells might be turning into glial precursor cells. Additionally, in lower concentrations of B27, some of the cells that show some A2B5 expression are also positive for Nestin, which could indicate that these cells might have progenitor qualities and the ability of self renew. These cells could be isolated and cultured separately to see if they can self renew into A2B5 positive populations. If this is possible, it would provide us with a preliminary technique for generating glial precursor cells. However, the expression of just A2B5 is not enough evidence to definitely indicate that these cells are glial precursor cells. Testing these cells for the presence of other markers would help support the hypothesis.

Conclusion and Future Directions

Cells proliferating in low concentrations of B27, when differentiated, show some expression of glial markers, GFAP and A2B5. However, this expression is not significant enough to suggest that a high percent of the cell population is differentiating into glial cells.

Cells cultured in lower concentrations of B27 are still interesting because they show an early expression of the neuronal marker Beta III Tubulin, and retain expression of a progenitor marker Nestin during differentiation. Gaining further understanding of why B27 has this effect on hNP cells would be interesting. One method in which this could be pursued would be by using a defined formulation of B27 in cell culturing and eliminating some of the components to

see how they individually effect cell proliferation and differentiation. This would indicate which components of B27 play important roles in cell culturing. Additionally, it would be interesting to add RA to cells growing in lower concentrations of B27. If the presence of RA decreases the differences between control 1X B27 cells and cells grown in lower concentration of B27, this would help understand the role of RA in hNP growth and differentiation.

In vitro differentiation of neuronal and glial cells will help understand the development of these cells and provide us with cells that can be used for therapeutic purposes. Therefore, a clearer understanding of the factors that play a role in glial differentiation is crucial.

WORKS CITED

1. Wilson, P. G., and S. L. Stice. (2006). Development and Differentiation of Neural Rosettes Derived From Human Embryonic Stem Cells. *Stem Cell Review 4*, 67-77.

2. Thomson, J. A., Itskovits-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. V., Jones, J. M. (2003) Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391): 1145-1147.

3. Min, K., Yang, M., Kim, S., Jou, I., Joe, E. (2006). Astrocytes Induce Hemeoxygenase-1 Expression in Microglia: A Feasible Mechanism for Preventing Excessive Brain Inflammation. *The Journal of* Neuroscience 26(6):1880 –1887.

4. Kessaris, N., N. Pringle, and W. D. Richardson. (2008). Specification of CNS glia from neural stem cells in the embryonic neuroepithelium. *Philos Trans R Soc Lond B Biol Sci 363*:71-85

5. Bignami, A., and D. Dahl. (1973). Differentiation of astrocytes in the cerebellar cortex and the pyramidal tracts of the newborn rat. An immunofluorescence study with antibodies to a protein specific to astrocytes. *Brain Research 49*: 393-402.

6. Bignami, A., and D. Dahl. (1974). Astrocyte-specific Protein and Neuroglial Differentiation. An Immunofluorescence Study with Antibodies to the Glial Fibrillary Acidic Protein. *J. COMP. NEUR. 15 (3):* 27-38.

7. Bignami, A., and D. Dahl (1977). Specificity of the Glial Fibrillary Acidic Protein for Astroglia. *The Journal of Histochemistry and Citochemistry 25 (6):* 466-469.

8. Antanitus DS, Choi BH, Lapham LW. (1975). Immunofluorescence staining of astrocytes *in vitro* using antiserum to glial fibrillary acidic protein. *Brain Res 89(2)*:363-367.

9. Eisenbarth, G. S., Walsh, F. S., Nirenber, M. (1979). Monoclonal antibody to a plasma membrane antigen of neurons. *Proc. Natl. Acad. Sci. U.S.A. 76:* 4913-4917.

10. Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R., Noble, R. (1983). Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. *Journal of Neuroscience 3*: 1289-1300.

11. Hunter, S. F., and J. E. Bottenstein. (1991). 0-2A Glial Progenitors from Mature Brain Respond to CNS Neuronal Cell Line-Derived Growth Factors. *Journal of Neuroscience Research 28*: 574-582.

12. Fok-Seang, J., and R. H. Miller. (1992). Astrocyte Precursors in Neonatal Rat Spinal Cord Cultures. *Journal of Neuroscience 12(7)*: 2751-2764.

13. Shin, A., Dalton, S., Stice, S. L. (2005). Human Motor Neuron Differentiation from Human Embryonic Stem Cells. *Stem Cells and Development 14*:1-4.

14. Wiese C, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, Wersto RP, et al. (2004). Nestin expression—a property of multi-lineage progenitor cells? *Cell Mol Life Sci 61*:2510–2522.

15. Bottenstein, J.E., Sato, G.H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc Natl Acad Sci USA 76*:514–7.

16. Chen, Y., Stevens, B., Chang, J., Milbrandt, J., Barres, B. A., Hell, J.W. (2008). NS21: Re-defined and Modified Supplement B27 for Neuronal Cultures. *Journal of Neuroscience Methods 171*: 239–247

17. Podratz, J. L., Rodriguez, E. H., Dinonno, E. S., Windebank, A. J. (1998). Myelination by Schwann Cells in the Absence of Extracellular Matrix Assembly. *GLIA 23*:383–388.

18. M. Maden. (2002). Retinoid Signalling in the Development of the Central Nervous System. *Nature Reviews Neuroscience 3*: 943-953.

19. Jones-Villeneuve, E. M. V., McBurney, M. W., Rogers, K. A., Kalnins, V. I. (1987). Retinoic Acid Induces Embryonal Carcinoma Cells to Differentiate into Neurons and Glial Cells. *Journal of Cell Biology 94*: 253-262.

20. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2008). *Molecular Biology of the Cell, 5th ed.* Garland Science, Taylor & Francis Group: New York.