CHARACTERIZATION OF ADULT HUMAN NEURAL PROGENITOR CELL DIFFERENTIATION *IN VITRO* AND *IN VIVO*

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Arts in Biological Sciences with Distinction.

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ABSTRACT

The stem cell cancer hypothesis has suggested that stem and progenitor cells may be the likely source of accumulation of oncogenic factors that lead to specific cancer cell types. Additional insight into this mechanism may be garnered by studying the differentiation of adult human neural progenitor (AHNP) cells and the role of L1, the cell adhesion molecule that has been previously shown to increase glioma cell motility and invasion. Previous studies have shown that adult human neural progenitor cells can differentiate into neurons and astrocytes both *in vitro* and *in vivo*. This also suggests that these cells can potentially be used to treat individuals with nervous system disorders that specifically involve neurons and astrocytes. No work had shown that oligodendrocytes had been isolated in culture or *in vivo*.

Immunocytochemical characterization of adult human neural progenitors was performed in order to assess L1 levels and the progenitor nature of the cells being cultured. Vimentin immunostaining suggested that the cells were expressing progenitor markers during *in vitro* experiments. The astrocyte marker, glial fibrillary acidic protein, was also expressed in cell culture. L1 ectodomain was detected in the progenitor cells, as well as Pax-6 transcription factor for L1. In co-culturing experiments using both monolayer co-cultures and aggregate co-cultures, the AHNPs were cultured with chick embryo brain cells and evaluated for differentiation. Monolayer co-cultures proved to be difficult as AHNPs minimally interacted with the chick embryo brain cells. Aggregate co-cultures improved cell-to-cell interactions and positive oligodendrocyte immunostaining was found.

Though improvements to the co-culturing methods can be developed, the AHNPs did show a potential plasticity to induce differentiation. The progenitor cells were cultured without growth factors and neuronal markers were detected. Overexpression of L1 ectodomain in the AHNPs did not produce any changes, though the future investigation of cell motility and invasion will require understand if any L1-mediated signaling is involved.

Chapter 1

INTRODUCTION

1.1 Adult Human Neural Progenitor (AHNP) cells and differentiation

1.1.1 Stem cells, progenitor cells, and the stem cell cancer hypothesis

Scientists study cancer in order to develop effective preventive therapies targeted to suppress the progression of cells toward future malignancies. Stem and progenitor cells have been identified as likely candidates as the cells of origin for certain cancers, particularly in cases of leukemia, glioblastoma, and prostate cancer. In particular, stem cells possess inherent abilities for self-renewal and longevity; these traits allow for the propagation and accumulation of genetic mutations that may lead to oncogenesis and thus have led to the development of the stem cell cancer hypothesis (Visvader, 2011). According to Canoll and Goldman (2008) and depicted in Figure 1, pluripotent stem cells are at the top of the normal cellular hierarchy that can yield more restricted and thenceforth committed progenitor cells. These cells can then further yield the mature cell types that constitute a particular tissue. Neural stem cells, for example, differentiate into three neural cell types: (1) neurons, (2) oligodendrocytes, and (3) astrocytes during the development of the adult human nervous system.

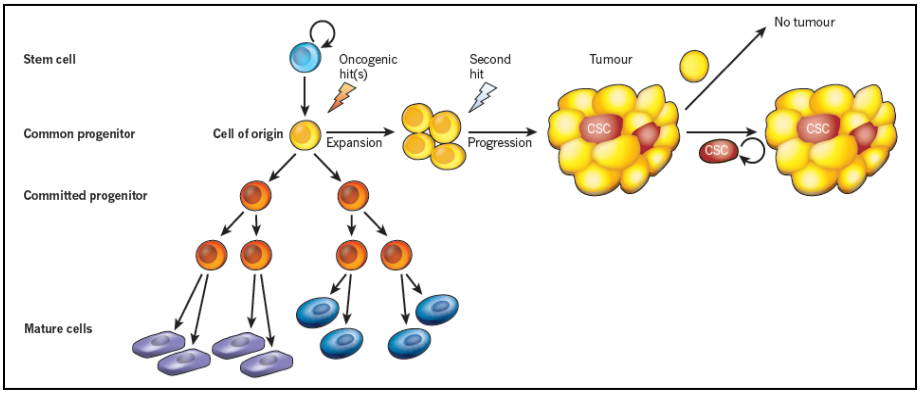

Figure 1. **Cellular hierarchy and progression of a cancer stem cell.** Normal cellular hierarchy beginning with stem cells progressing toward restricted progenitors and the more common mature cell types of a particular tissue. Possible accumulation of oncogenic mutations leading to tumor development are also depicted in the model. Source: Visvader (2011).

1.1.2 Adult Human Neural Progenitor (AHNP) cells

In contradistinction to stem cells, progenitors are multipotent, or more restricted in that they can differentiate into fewer cell types than stem cells and they are migratory (Canoll and Goldman, 2008). Progenitor cells of the neural type are very important in the study of regenerative neurobiology because progenitor cells can differentiate into the two major cell types of the central nervous system: neurons and glial cells. Glial cells are further divided into astrocytes or oligodendrocytes; particular attention has been given to studying glial cells because they are extremely numerous in the nervous system and play an integral role in development and recovery from injury (Lie et al., 2004).

Adult human neural progenitor cells (AHNPs) are progenitor cells derived from the hippocampus or multiple forebrain regions that can differentiate into neurons

and astrocytes depicted in Figure 2 (Walton et al., 2006). AHNPs display astrocyte-like morphology and like other progenitors, display immature markers of glial cell types, such as glial fibrillary acidic protein (GFAP) for example (Visvader, 2011). For this reason, adult human neural progenitors are characterized as "type B" progenitors (Stiles and Rowitch, 2008). These cells are maintainable in adherent conditions and are highly expandable, allowing progenitors to grow in culture and undergo multiple population doublings. Walton et al. (2006) reported 60-65 passage doublings before observing significant cell senescence and death. Neural progenitors maintain their ability to generate glial and neuronal cell types *in vitro* and *in vivo* while also being amenable to genetic modifications.

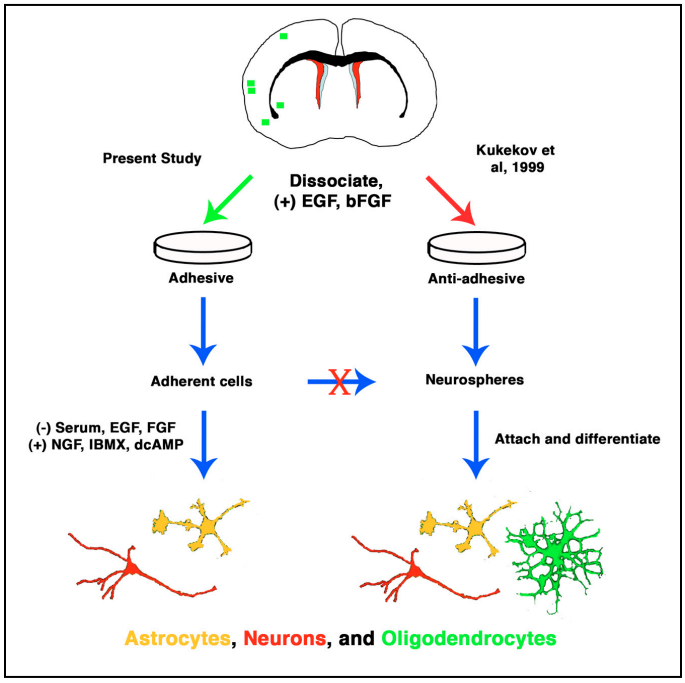

Figure 2. Isolation and culture of progenitor cells for study and differentiation. Source: Walton et al. (2006).

These characteristics suggest plasticity in the central nervous system that may allow for AHNP application in treating neurodegenerative diseases (depicted in Figure 3). Migrating progenitors have been shown to be responsive to injury; cell proliferation noted shortly after seizures and ischemia suggest this prospect for repair that may be manipulated *ex vivo*. The use of neural stem and progenitor cells as an endogenous source of cells for nervous system cell replacement therapies promises to aid in central nervous system diseases such as Parkinson's and Alzheimer's (Lie et al., 2004).. A major advantage provided by this hopeful source of treatment includes avoiding immunological reactions.

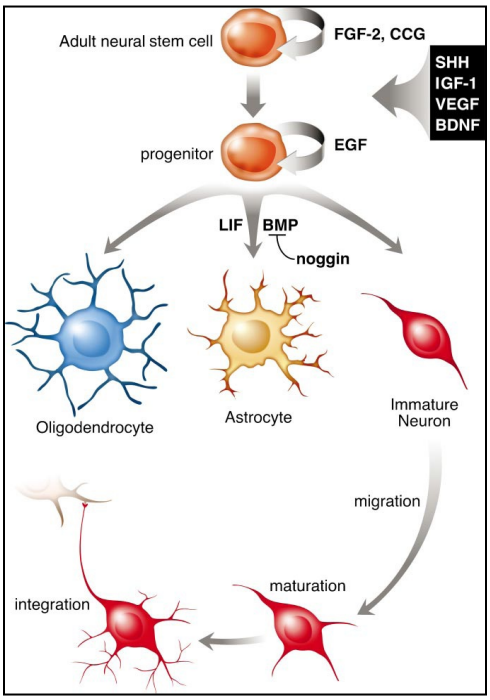

Figure 3. Molecular regulation and integration of human adult neural progenitors. Source: Lie et al. (2004).

1.1.3 AHNPs isolated from hippocampus and subventricular zone of brain

The adult human neural progenitors used for this study were isolated from the primary tissue of individuals who underwent surgery related to temporal lobe epilepsy. The hippocampus, temporal cortex and subventricular zone (SVZ) were sources of the AHNPs because adult neurogenesis has consistently been found in these zones of the brain (Lie et al., 2004); Figure 4 shows the different progenitor cells isolated from the subventricular zone.

AHNPs are not an immortalized cell line, permitting a certain number of passages or population doublings. Published data by Walton et al., 2006, indicates a maximum of 60-65 population doublings, but our lab results indicate only approximately 20 doublings were possible. AHNPs also require the use of serum and growth factor supplementation to their media to prevent cell senescence and differentiation *in vitro.*

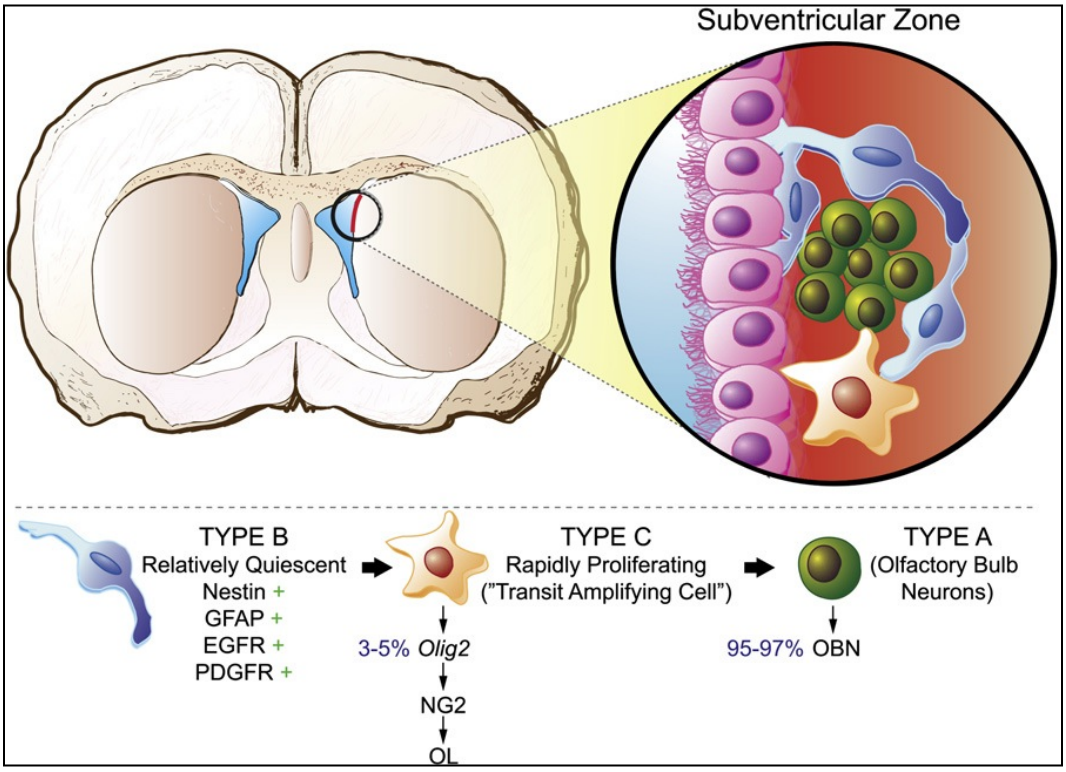

Figure 4. **Conceptual schematic for isolating human neural progenitors from the subventricular zone.** The different subtypes of progenitor cells indicate the potential of cells to produce the neural precursor cells. Source: Stiles and Rowitch (2008).

1.2 L1CAM and AHNPs

1.2.1 L1 Cell Adhesion Molecule (L1CAM)

L1 is a 200-220 kDa cell adhesion molecule (CAM) belonging to the neuronal immunoglobin (Ig) superfamily of CAMs. L1 consists of a large extracellular domain comprised of six Ig-like and five fibronectin-type III repeats (FNIII-like) linked to a single transmembrane intracellular cytoplasmic tail (Schafer and Altevogt, 2010). L1 structure is depicted in Figure 5. Posttranslational processing of the molecule affects the molecular weight range of L1. The 220-kDa form represents mannose glycosylation of the full length of the extracellular domain of the molecule, whereas the 200-kDa form suggests incomplete processing (Schäfer et al., 2010). This molecule undergoes homophilic (L1-L1) binding as well as heterophilic binding with other integrins via interactions through the RGD motif. This motif is present in the Ig-like domain six and is made up of the amino acids Arginine-Glycine-Aspartate (Mechterscheimer et al., 2001). L1 plays a large role in neuronal migration, axon growth and bundling, axonal fasciculation, and synapse formation in the developing and adult brain as studied in L1-deficient mouse mutants and individual patients suffering from CRASH syndrome (Schäfer and Altevogt, 2010).

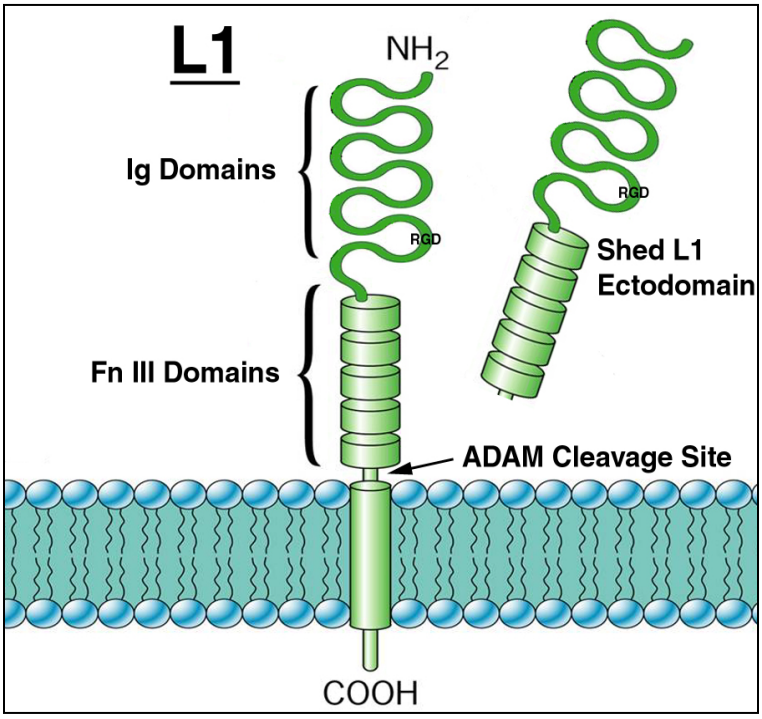

Figure 5. **Structure of L1.** L1 cell adhesion molecule showing the six immunoglobin domains with the RGD sequence, the five fibronectin domains, and A Disintegrin And Metalloproteinase (ADAM) cleavage site. Source: Dr. Deni S. Galileo, edited from Karp (2004).

The role of L1 in neurological conditions further suggests its relevance to this study. The L1 gene is on the X chromosome in humans; mutations to this gene underlie neurological conditions collectively called L1 Syndrome or CRASH syndrome (Corpus callosum hypoplasia, Retardation, Adducted thumbs, Spastic paraplegia, and Hydrocephalus) (Raveh et al., 2009). Furthermore, recent studies have suggested several cancers show unregulated L1 expression. Presence of L1 in cancerous tissue and cells was correlated with poor prognosis in colon cancer as well as melanoma, Schwannoma, glioma, and breast cancer. L1 was also detected in less common cancer types, including neuroblastoma and some examples of renal

carcinoma (reviewed in Raveh et al., 2009). L1 expression is not expressed in these normal tissues and cells.

L1 can be cleaved and released abnormally from cancer cell membranes by two members of the Disintegrin and Metalloproteinase (ADAM) family of proteins, ADAM10 and ADAM17, producing a 190-kDa ectodomain. When cleaved, the soluble L1 ectodomain has been suggested to stimulate glioma cell motility and migration (Yang et al., 2009, 2011). Soluble L1 has been detected in serum samples of patients with ovarian and uterine tumors, suggesting a role in cancer cell metastasis as well (Fogel et al., 2003).

1.2.2 AHNPs made to express green fluorescent protein and L1CAM

L1 ectodomain fragment was generated from the pCDNA3-L1 vector provided by Dr. Vance Lemmon (The Miami Project to Cure Paralysis, University of Miami, FL). The fragment was inserted into a lentiviral vector #1879 (provided by Dr. John C. Kappes, Univ. of Alabama, Birmingham) containing a CMV promoter. The vector construct was transfected into HEK293T cells with the helper plasmid pMD.G and packaging plasmid pCMVΔR8.2 by ratio of 4:3:1 (20 μg:15 μg: 5 μg for a 10 cm plate) using a standard calcium phosphate method (Galileo et al., 1999). Supernatant containing virus was collected 48 hours and 72 hours after transfection and AHNPs were infected using 10 μg/mL polybrene. Stably infected cells with the encoded vector were selected by 2 μg/mL puromycin resistance and later confirmed

by Western blot. AHNPs with the full-length L1 ectodomain are referred to as AHNP/L1LE (L1 long ectodomain) (Li and Galileo, 2010).

Green fluorescent protein (GFP) labeling of the AHNPs was accomplished by using a recombinant lentiviral vector #72 that encoded GFP and puromycin resistance (provided by Dr. John C. Kappes, Univ. of Alabama, Birmingham) (Fotos et al., 2006). The lentiviral vector constitutively expresses GFP (Yang 2011).

1.3 Objectives and Specific Aims

The effects of the L1 ectodomain on AHNP cell differentiation were of interest because there is a correlation between cancer cells and L1 expression and cleavage. The specific aims for this project were: 1) characterizing the AHNPs *in vitro*, 2) determining if the chick embryo model is suitable for studying AHNPs *in vivo* and investigating if any differentiation occurred therein, and 3) investigating and comparatively studying the difference between AHNP-GFP and AHNP-L1LE cells in differentiation studies that simulate brain development.

1) In order to characterize the AHNPs *in vitro*, the cells were cultured for multiple passage doublings and tested via Western blot analysis and immunochemistry for the presence of specific proteins known as progenitor markers as well as proteins of interest (i.e. L1CAM). This included analysis also for Pax-6, vimentin, and differentiation markers for neurons, astrocytes, and oligodendrocytes.

2) With the purpose of studying AHNPs *in vivo*, the chick embryo model was suggested because of its use previously in the Galileo lab for glioma and other

differentiation experiments (Galileo, 2003). Initially, a large number of AHNPs ($2x10^6$ cells/mL per embryo) were injected into the optic tectum (midbrain) and hindbrain regions of the developing chick embryo brain. Upon maturation, embryos were sacrificed to examine any differentiation in the brain via whole brain dissection; those brains used were sectioned for easier observation. If any AHNPs were observed in sections, immunostaining was then pursued for L1 expression and other neural cell type differentiation.

3) In order to simulate brain development, a co-culturing technique was developed for AHNPs to be studied in a method that simulate brain development *in vitro*. AHNP-GFP and AHNP-L1LE cells were each cultured simultaneously with dissociated whole brain cells from chick embryo brains in culture dishes coated with Poly-HEMA in order to prevent attachment to the dishes and encourage cell interactions. Aggregates that formed were embedded and sectioned for immunostaining to detect any differentiation.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture

Adult human neural progenitor cells (Steindler Neural Stem Cell lab, McKnight Brain Institute, University of Florida) were maintained in growth media made up of 1:1 Dulbecco's Modified Eagle's Medium (DMEM) and F12 (Gibco), supplemented with 5% fetal bovine serum (FBS), 2mM L-glutamine (CellGro), 200mM stock N2 supplement (1% of total media volume, Gibco), and 100μg/mL stock antibiotic-antimycotic (1% of total media volume was anti-anti, comprised of penicillin, streptomycin, and amphotericin B, Gibco). Cultures were given growth factor supplements of bFGF (basic fibroblast growth factor, 20μg/mL, BioSource) and EGF (epidermal growth factor, 20μg/mL, Invitrogen) every other day. The cells were incubated in a humidified atmosphere which was maintained at 5% CO_2 and 37^{o}C. Two different cell lines were made. One line of AHNPs was infected with lentiviral vector #1879 encoding for full-length L1 ectodomain (or L1LE for long ectodomain). Both AHNP lines were infected with lentiviral vector #72 encoding green fluorescent protein (GFP) and puromycin resistance (provided by Dr. Deni S. Galileo).

2.2 Chick Embryos

Fertilized White Leghorn chicken eggs were provided by the Department of Animal and Food Sciences at the University of Delaware. Eggs were incubated in a humidified atmosphere, at 37.5^{o}C until the eggs had reached the desired embryonic stage, starting at E0. Every 24 hours of incubation made up an embryonic day. Once embryos were at the desired embryonic day (E5 for injection, E12 to E15 for dissection of brain), a hole was cut at the top of the blunt end of the egg, exposing the air pocket of the embryo. The hole exposed the embryo and embryonic blood vessels for injection experiments. After providing antibiotics to ward off infection, the hole was sealed with tape and the embryo was then incubated to later stages.

2.3 Antibodies and Reagents

Many antibodies were used for this characterization study. AHNPs were immunostained for multiple proteins and therefore required many primary antibodies. Primary antibodies used for immunostaining of the AHNP cells, brain sections, monolayers, and co-cultures included: H5 (monoclonal mouse anti-vimentin anitbody, Developmental Studies Hybridoma Bank), Pax-6 (monoclonal mouse anti-Pax-6 antibody, Developmental Studies Hybridoma Bank), GFAP (DAKO polyclonal rabbit antibody for glial fibrillary acid protein), O4 (monoclonal mouse anti-O4 oligodendrocyte marker), MAG (mouse monoclonal anti-myelin associated glycoprotein, clone 513), and TUJ1 (mouse monoclonal anti-β-III-tubulin from Santa Cruz Biotechnology). A glutamine synthetase antibody (GS) was received as a gift

from Dr. P.J. Linser from the Whitney Laboratory for Marine Bioscience at the University of Florida (a mouse monoclonal anti-GS antibody). An additional beta-tubulin antibody (Developmental Studies Hybridoma Bank) was used as a control during Western blot analysis. Two different anti-human L1 antibodies were used both during immunostaining and Western blot analysis. NCAM-L1 UJ127 is a mouse monoclonal anti-L1 antibody (Santa Cruz Biotechnology, sc-53386) recognizing the fourth fibronectin repeat within the ectodomain of the L1 molecule. NCAM-L1 C-20 is a goat polyclonal antibody (Santa Cruz Biotechnology, sc-1508) that was raised against a peptide within the C-terminus of human NCAM- L1, located in the cytoplasmic domain.

Secondary antibodies were used for immunostaining as well. Only red secondary antibodies were used because the AHNPs were GFP positive and therefore fluoresced green. The secondary antibodies used were Alex Fluor 594 donkey anti-goat, goat anti-rabbit, and goat anti-mouse (Molecular Probes and Invitrogen).

Furthermore, during immunostaining, antibodies were diluted using blocking buffer solution, composed of 0.03% Triton X-100 and 5% normal goat serum (NGS) (unless otherwise indicated) diluted in phosphate buffered saline (PBS). For the antibody NCAM-L1 C-20, NGS was replaced with 5% bovine growth serum (BGS).

Table 1. **AHNP differentiation marker profile.** A list of different markers AHNPs were found to express either *in vitro*, after induced differentiation experiments performed by Walton, et al. (2006), and results in the Galileo lab. Positive results are indicated with a "+" and negative with a "-". Blank spaces are meant to be interpreted that the lab did not test for the respective marker. Information about vimentin and nestin as biological markers (denoted with *) was obtained from Bramanti, et al. (2010).

Marker	Normal Expression	Walton's Findings	My Findings
GFAP	Astrocyte marker	+ *in vitro* - after cortical injections	+ *in vitro*
NeuN	Neuronal marker	- *in vitro*	
PSA-NCAM	Neuronal marker	- *in vitro* + after inducing neurogenesis	
CNPase	Oligodendrocyte marker	- *in vitro*	
O4	Oligodendrocyte marker	- *in vitro*	+ in co-culture/aggregate experiment
Nestin	Neural stem cell marker*	+ *in vitro*	
A2B5	Immature neuronal marker	+ *in vitro*	
NG2	Glial marker	+ *in vitro*	
S100β	Astrotypic marker	+ *in vitro*	
Glutamine Synthetase (GS)	Astrotypic marker	+ *in vitro*	- *in vitro* after growth factor starvation experiment - in co-culture/aggregate experiment
β -III-Tubulin (TUJ1)	Neuronal marker	+ after cortical injections into ventricles of NOD-SCID mice + after inducing neurogenesis	+ *in vitro* after growth factor starvation experiment - in co-culture/aggregate experiment
MAG	Oligodendrocyte marker		- in monolayer experiment
Vimentin	Astrocyte intermediate filament protein*		+ *in vitro*

2.4 Injections of AHNPs into Chick Embryo Brains

AHNPs were prepared for injection by trypsinizing them in 0.25% trypsin/0.02%EDTA (CellGro) until they detached from the bottom of the culture dish and separated into single cells while incubated at 37^{o}C; this process took approximately four minutes with mild tapping of the cell dish after two minutes. Media with serum was added to inactivate the trypsin and the cells in solution were counted using a hemacytometer. Following centrifugation at 800 rpms for five minutes, cells were resuspended in the necessary volume of Medium 199 (Gibco) to achieve a cell concentration of $1x10^4$ cells/5μL/embryo, equating to $2x10^6$ cells/mL. Approximately 25 μL of 1% fast green (in distilled water) was added to 600 μL of cell suspension in order to aid in visualizing the cell suspension during injection experiments.

Incubated eggs were inspected at embryonic day 5 (E5) and holes were cut into the air pocket, exposing the top membrane that covered the yolk and embryo. After wetting the membranous covering with Medium 199, the membrane was removed. Using the PV380 Picopump pressurized microinjector (World Precision Instruments), approximately five microliters of cell solution were injected equally into the optic tectum and hindbrain of the E5 chick embryo, as shown in Figure 6. Five drops of 50 mg/mL ampicillin (a broad-spectrum antibiotic) in water were added over the injection site and the hole in the egg was sealed with tape. Embryos were incubated at 37.5^{o}C to the embryonic day necessary for brain removal and dissection (usually E12-E15).

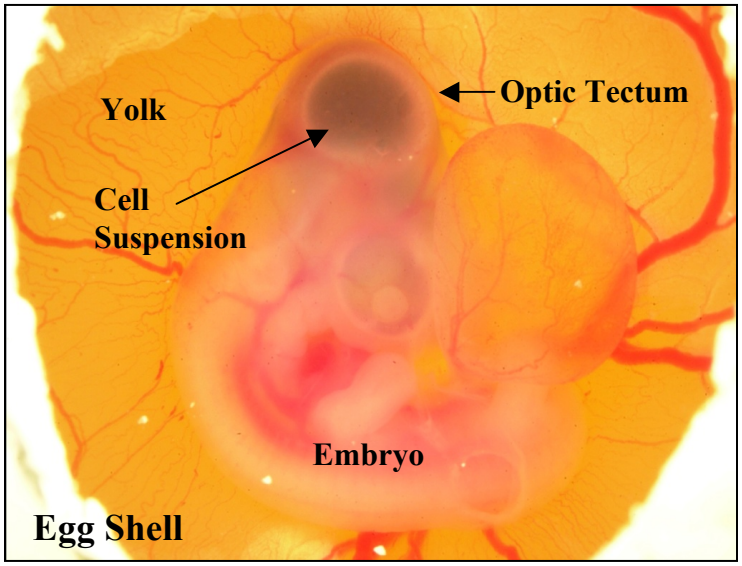

Figure 6. **Cell suspension injected into the optic tectum of E5 chick embryo brain.**

2.5 Observation of AHNPs in chick embryo brains

2.5.1 Preparation of chick embryo brains

Embryos which had been injected with AHNP cell suspensions at E5 were sacrificed between E12 and E15, or occasionally at E17, in order to allow for nearly complete brain development. The whole brain was removed, as was the pia mater, the innermost meningial layer surround the brain. Brains were removed and dissected in calcium- and magnesium-free Tyrode's saline solution (CMF, Fischer Scientific). After fixation in 2% paraformaldehyde in phosphate buffered saline (PBS) overnight, brains were dissociated into the three brain parts: forebrain, optic tectum (midbrain), and hindbrain. These brain parts were then embedded in agarose blocks (3.5% agar and 8% sucrose in PBS, all materials from Fischer Scientific) and vibratomed into 200 micron thick sections. Sections were screened using a dissection microscope equipped

for epifluorescence to determine the presence of green fluorescing cells, as pictured in Figure 7.

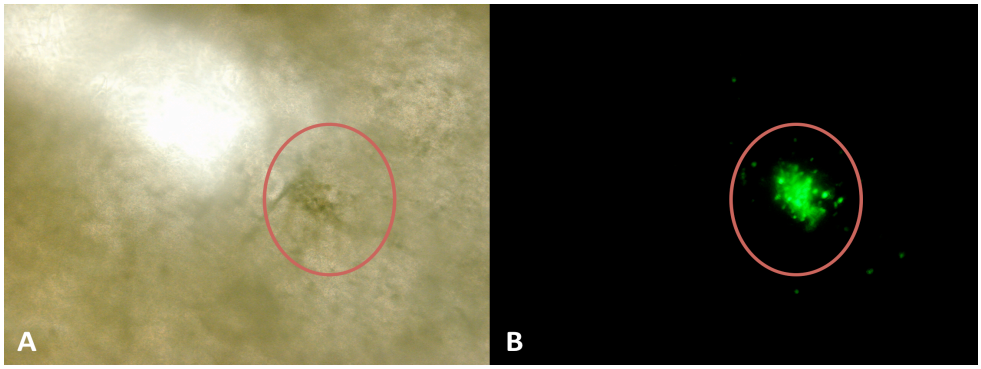

Figure 7. 200 micron-thick vibratome section of E17 OT chick brain with AHNP/GFP+ cells. Encircled in red in both panels are the AHNPs. A) Phase image of vibratome section; B) GFP fluorescence.

2.5.2 Tissue staining of 200 micron thick vibratome sections

Two methods were explored in attempts to immunostain the 200 micron thick vibratome sections, though neither proved successful. The first method from Luque et al. (2001), used a fixative solution (Dent's solution) made up of four parts methanol and one part DMSO (dimethysulfoxide). Tissue sections were rinsed in PBS and then fixed in the Dent's solution for two hours. Sections were then rinsed with methanol twice for approximately five minutes before a third rinse in fresh methanol for one hour at -80^{o}C. Sections were brought to room temperature before rinsing twice with PBS for five minutes each. PBS was then replaced with primary antibodies (as previously described) for an overnight incubation. The next day, the primary antibody solution was removed, the sections rinsed five times with PBS and then incubated overnight in secondary antibodies (as previous described). On the third day,

the secondary antibody solutions were removed, sections were rinsed again for five times with PBS and tissues were then transferred into new wells for overnight exposure to 70% glycerol/30% PBS solution for clearing of the samples on a rotator plate. Sections were mounted in glycerol/PBS on the fourth day.

The other method explored involved the use of saponin to create minute pores in the tissue and allow for antibodies to bind; this method had been suggested by a faculty member who had successful results employing this method. Sections embedded in agarose were transferred into a six-well culture plate and rinsed twice with PBS before incubation overnight with primary antibody and 0.05% saponin (diluted in PBS). The following day, five rinses of PBS and 0.05% saponin were completed for an hour each before overnight secondary antibody incubation; again the antibody incubation was performed with secondary antibody solution and 0.05% saponin. On the third day, five more rinses of PBS and 0.05% saponin were done for an hour each and then the sections were put in 70% glycerol/30% PBS solution overnight. Sections were mounted after a 30 minute rinse in PBS on the fourth day of the procedure.

2.5.3 Cryostat sectioning

AHNPs that formed co-culture aggregates with chick brain cells were fixed in 2% paraformaldehyde (in PBS) overnight before carefully transferring them from culture dishes to aluminum foil chambers where they were embedded in Tissue Freezing Medium overnight in the -80^{o}C freezer. The cryoblocks were then cryostat

sections and cut into 16 micron thick sections (object and chamber temperatures were set in range of -23 to -25^{o}C using the Leica CM3050 S Cryostat housed in Wolf Hall.

2.6 Protein detection via Western blot analysis

Western blotting is a technique that denatures proteins and separates them first by molecular weight, then allowing for specific proteins to be detected via antibodies. Proteins are denatured in sodium dodecyl sulfate (SDS) and gives them an overall negative charge, allowing for separation in polyacrylamide gel electrophoresis (PAGE). Separated proteins can then be transferred to a polyvinylidene fluoride membrane and stained using antibodies directed against the protein under investigation (Texeira 2009).

2.6.1 L1 and Pax-6 expression detected by Western blot

AHNP cell extracts were prepared by lysing cells in RIPA (Radio Immuno Precipitation Assay) lysis buffer with a Complete Mini Protease Inhibitor (PI) Cocktail tablet (Roche). Protein concentrations were determined using bicinchoninic acid (BCA) assay kit (Pierce). Serial dilutions of 0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 μgrams/mL of bovine serum albumin (BSA) in RIPA/PI were prepared and absorbances were measured on a spectrophotometer at 562nm λ in order to construct a standard curve. Absorbance of cell lysates were measured and compared to the standard curve to determine total protein concentration.

After determining total protein concentration, the cell lysates were prepared with LDS (lithium dodecyl sulfate) and proteins were denatured in a 70^{o}C water bath for ten minutes. Equal amounts of protein were loaded into each lane (for L1 and Pax-6 experiment, protein concentration was 10.7 micrograms of total protein). NuPage MOPS SDS PAGE Running Buffer (Invitrogen) diluted 1:20 with distilled water was used in running the gel.

Prepared protein samples and MagicMark standards (Invitrogen) were loaded into the lanes of a NuPage 4-12% Bis-Tris gel (Invitrogen) and the gel was set to run for two hours at 120 volts. Following the electrophoresis step, a PVDF membrane (Invitrogen) was wetted in methanol. The membrane and gel were aligned and placed in NuPage Transfer Buffer (Invitrogen) and 10% methanol in distilled water and allowed to transfer overnight in the cold room (4^{o}C) while running at 30 volts.

The following day, the membrane was placed in blocking solution made up of 0.01% Tween-20 (Fischer Scientific) and 5% non-fat powdered milk in PBS for two hours at room temperature while on a rocker. The L1 and Pax-6 membrane was then incubated with a 1:1000 dilution of the primary antibody for Pax-6. This incubated endured one hour at room temperature on the rocker. The membrane was rinsed three times for 15 minutes each with the rinsing buffer solution of PBS and 0.01% Tween-20 (no milk was added in the rinsing buffer). The membrane was then placed in a solution made up of blocking solution and 1:5000 dilution of goat anti-mouse peroxidase labeled secondary antibody for two hours at room temperature on a

rocker. The membrane was then rinsed three times for 15 minutes each in the rinsing buffer. Enhanced chemiluminescence (ECL) blotting substrate (Pierce*)* was prepared by mixing equal parts of enhancer solution and peroxide solution. The membrane was immersed in the ECL for one minute and then developed onto autoradiograph film (Denville) using the SRX101A Konica Minolta Developer located on the third floor of Wolf Hall.

After developing, the membrane was stripped using Restore Plus Western Blot Stripping Buffer (Thomas Scientific) and the membrane was re-probed with anti-β-tubulin antibody to normalize loading protein and L1CAM. Again, blots were developed onto autoradiograph film.

2.7 Co-culturing AHNPs and chick embryo brain cells

2.7.1 Monolayer of brain cells and AHNPs

In order to simulate the developing brain *in vitro*, AHNPs were co-cultured with a monolayer of chick embryo brain cells (E7). Chick embryos were sacrificed and brains were totally dissected while in CMF. The brain was minced using forceps and a transfer pipette in order to triturate the cells. After centrifugation of minced pieces at 800 rpm for three minutes at 4^{o}C, CMF was aspirated and brain fragments and cells were resuspended in one milliliter of 0.25% trypsin and incubated for twenty minutes in 37^{o}C; following this incubation, the cells were put on ice for five minutes. Then, two milliliters of soybean trypsin-inhibitor (SBTI) – DNAase I (0.003g DNAase I, Sigma; 0.03g SBTI, Sigma, in 100 mL Medium 199) were added

to the test tube and the cells were centrifuged again at 800 rpm for three minutes at 4^{o}C. The supernatant was aspirated and cells were resuspended in two milliliters of SBTI and triturated with a Pasteur pipet approximately fifteen times.

At this point, two milliliters of fresh SBTI was added, the cell tube was inverted and then placed on ice for five minutes to allow cells to vertically undergo differential sedimentation. The uppermost two milliliters of SBTI-DNAase I were removed and placed in another test tube. This process was repeated after triturating the cells again and then adding two milliliters of fresh SBTI-DNAase I for differential sedimentation. After at least four milliliters of cells were isolated, a cell count was performed with the hemacytometer. Brain cells were seeded on 0.2 mg/mL poly-L-ornithine treated 12 mm diameter coverslips in a 24 well plate for immunostaining; cell counts were approximately 1x10^{6} for the co-culture experiments. Cells were plated in Medium 199 (Gibco), supplemented with 10% FBS, pen/strep (Gibco), and 2mM L-glutamine (CellGro).

After brain cells had formed a monolayer on the coverslip (after approximately 14 days), AHNPs were trypsinized and seeded at 1x10^{4} cells per well onto the monolayer. Cells were then incubated in a humidified environment at 37^{o}C with 5 % CO_2.

2.7.2 PolyHEMA protocol

PolyHEMA (poly-2-hydroxyethyl methacrylate) forms a hydrogel in water and when culture dishes are coated with this substance, cells do not adhere and

grow in suspension. AHNPs and chick embryo brain cells were cultured on polyHEMA coated plates in order to form aggregates of cells and to determine how the cells interacted.

The polyHEMA solution was made from 2.4 grams of poly-HEME (Sigma cat. #P-3932) added to 20 mL of 190 proof ethyl alcohol. This solution was put in the water bath set at 65^{o}C for eight hours and then let cool. After it cooled, 35mm tissue culture multi-well plates were triple coated with polyHEMA solution. This involved adding 3 mL of polyHEMA solution to the plates while they were rotating on a rotator and drying.

Chick embryo brain cells were isolated following the same procedure as outlined previously for making a monolayer of cells. After counting chick embryo brain cells, the cells were seeded at 1x10^{6} cells per well onto the triple-coated polyHEMA plates. AHNPs were trypsinized and seeded at 1x10^{4} cells per well two days after the chick cells had been added.

2.8 Fixed cell and co-culture immunocytochemistry

Immunostaining procedures for fixed cells on coverslips and co-culture sections followed the same protocol. For preparation of antibodies and blocking buffer solutions, see section 2.3 Antibodies and Reagents. Post-fixation, sections and cells were rinsed with PBS and then incubated with primary antibody for one hour and kept in the dark for the duration of the antibody incubation. Controls were incubated in blocking buffer solution during this incubation. After the hour was complete, the

primary antibody solution was removed and the cells and sections were triple rinsed in PBS for five minutes each. After this rinse, all sections and cells were incubated in the dark for one hour in secondary antibody solution. Following the secondary antibody solution, cells and sections were rinsed with PBS for five minutes, incubated for five minutes with 10 μg/ml bisbenzamide (nuclear staining), rinsed twice more with PBS for five minutes and finally mounted or coverslipped with glycerol in PPD solution (70% glycerol solution, 0.05% p-phenylenediamine in roughly 30% water).

Chapter 3

RESULTS

3.1 Characterization of protein expression via immunocytochemistry

In order to characterize the AHNPs, immunocytochemistry, as outlined in Materials and Methods section, was performed in order to detect proteins expressed by the AHNPs that were suggested in the published data of Walton et al. (2006); this therefore led to multiple immunostaining experiments. Of particular interest, AHNPs were reported to express GFAP *in vitro*. After immunostaining, AHNPs were found to have this astrocyte marker (Figure 8).

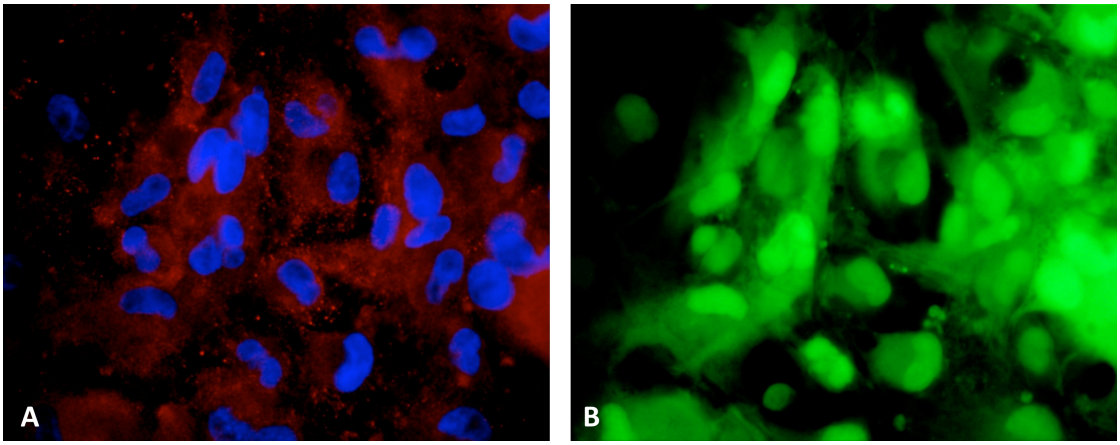

Figure 8. GFAP immunostaining of fixed *in vitro* AHNPs on coverslips. A. Merged image of GFAP (in red) and nuclear staining (bisbenzimide, blue). B. GFP fluorescence of the AHNP/GFP cells. Both photos were taken with a 40x objective.

Further characterization experiments via immunostaining were performed. Of particular interest was vimentin, an intermediate filament marker known to be expressed in astrocytes. This was investigated in order to assess differentiation in future experiments where it would be expected that vimentin expression would change. Walton et al. (2006) examined a similar protein, nestin, but due to the lack of a nestin antibody, I chose to investigate the AHNP expression of vimentin. AHNPs were positively immunostained for vimentin expression (Figure 9).

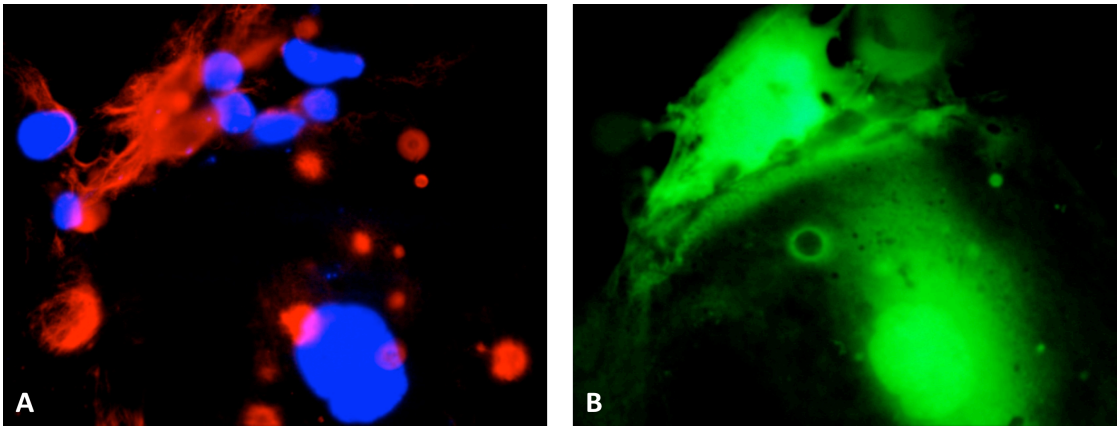

Figure 9. **Vimentin immunostaining of AHNPs *in vitro*.** A. Merged image of vimentin staining (red) and nuclear staining (blue). B. GFP fluorescence of the AHNP/GFP cells. AHNPs were fixed on coverslips and immunostained. Both photos were taken with a 40x objective.

Lastly, immunostaining of L1CAM and Pax-6 were performed. The AHNPs that expressed GFP only needed to be evaluated for any *in vitro* levels of L1 expression as well as Pax-6 (a known L1 transcription factor). Pax-6 expression was meant to aid my determination of a mechanism to upregulate L1 expression in the AHNPs. Pax-6 immunostaining showed positive results but not all of the Pax-6

staining colocalized to the nucleus as would be expected of a typical transcription factor (Figure 10).

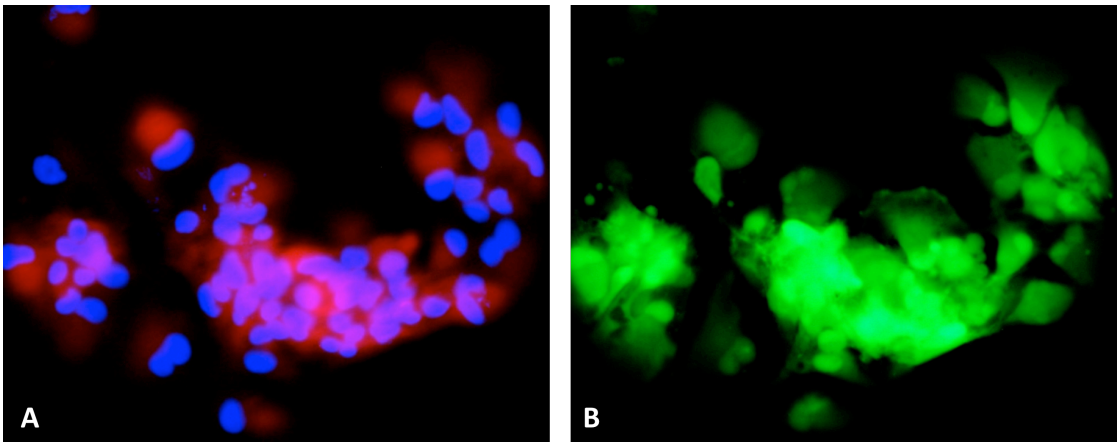

Figure 10. AHNPs were positive for L1CAM transcription factor, Pax-6. **A**. Merged image of Pax-6 (red) and nuclear (blue) staining. B. GFP fluorescence of the AHNP/GFP cells. AHNPs were fixed on coverslips and immunostained. Photos were taken with a 20x objective. Some of the Pax-6 staining is colocalized to the nucleus but not all.

Due to these results, AHNPs were assessed for normal L1 expression *in vitro* and were found to be positive for L1CAM, using two different antibodies as described in the Materials and Methods section (and as indicated in Figure 11). *In vitro* analysis of L1 expression was meant to establish that L1 expression prior to vector infection experiments (which modulated the amount of L1ecto expressed in AHNPs) would help extrapolate if a difference in excessive L1 would further increase cell differentiation or interactions *in vivo*.

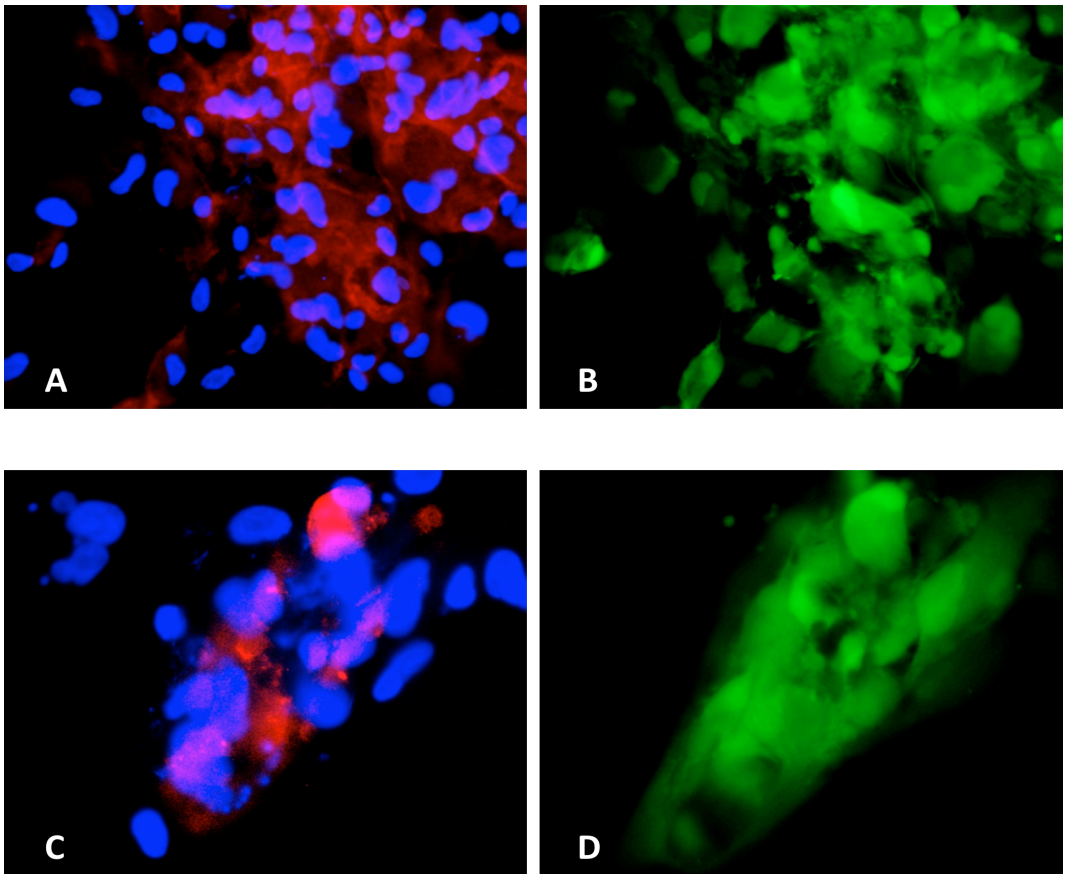

Figure 11. AHNPs were positive for L1CAM using two different antibodies. A. Merged image of L1 (red) and nuclear (blue) staining. B. GFP fluorescence of the AHNP/GFP cells in panel A. A and B are AHNPs that were immunostained using UJ127 antibody and photographed with a 20x objective. C. Merged image of L1 (red) and nuclear (blue) staining. D. GFP fluorescence of the AHNP/GFP cells in panel C. C and D are AHNPs that were immunostained using NCAM-L1 C-20 antibody and photographed with a 40x objective.

3.2 Western blotting analysis confirm AHNP expression of L1 and Pax-6

Western blotting analysis was pursued in order to reaffirm AHNP expression of L1 and Pax-6, particularly after the unexpected cytoplasmic staining of Pax-6 transcription factor. AHNP cell lysates were prepared and Western blot analysis was performed as described in the Materials and Methods sections. The blot

was first examined for L1 expression using the UJ127 antibody, which binds to the extracellular portion (ectodomain) of the L1 molecule as well as for Pax-6 expression. Figure 12, panel A, shows the Western blot and both positive and negative controls run simultaneously.

The same Western blot was then stripped and re-probed in order to evaluate loading controls using β-tubulin and to re-evaluate L1 expression (in the lane that was previously used for Pax-6) for the presence of cleaved L1 using NCAM-L1 C-20, which binds to the cytoplasmic epitope of L1 and would have further showed a proteolyzed 32-kDa fragment in the lane. The absence of the fragment indicated that NCAM-L1 C-20 bound to the cytoplasmic epitope intracellularly and that full-length L1CAM was present in the AHNPs (Figure 12, panel B).

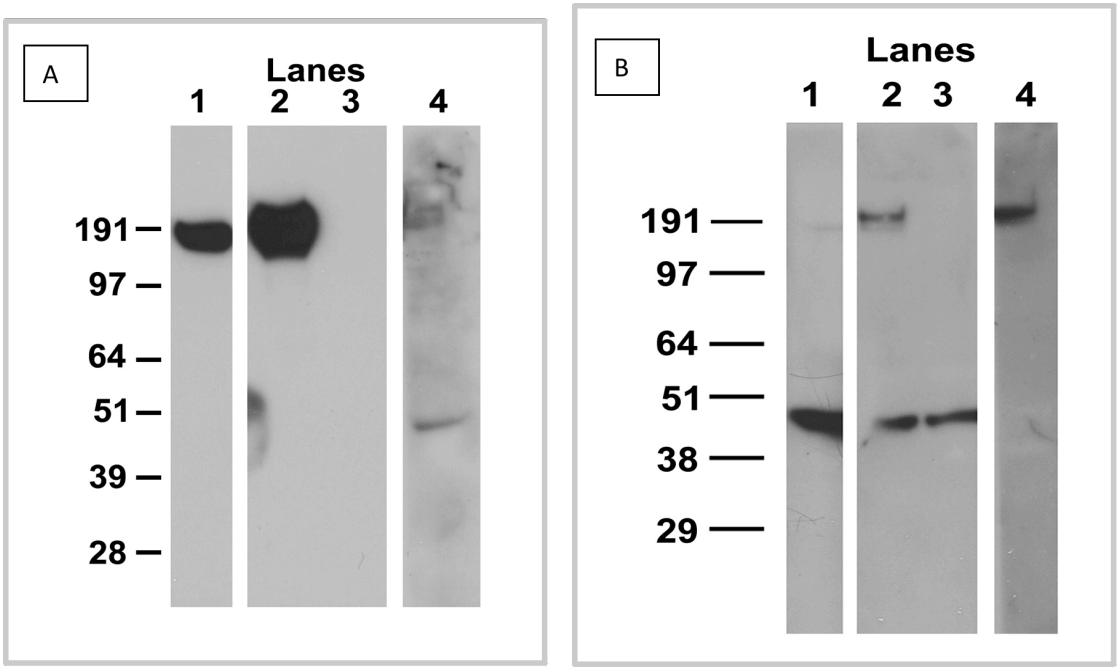

Figure 12. L1CAM and Pax-6 expression as evaluated by Western blotting analysis. A. Lanes were used as follows: 1 – UJ127 antibody detection of L1CAM in AHNPs, 2 – QT6 (quail fibroblast cells) transfected with human full-length L1, serving as a positive control, 3 – normal QT6 cells as a negative control for L1, 4 – Pax-6 antibody detection in AHNPs.
B. Lanes are the same cell lysates as those lanes in panel A, but reprobed for: 1 – β-tubulin loading control, 2 – β-tubulin loading control, 3 – β-tubulin loading control, 4 – AHNPs reprobed for L1using NCAM-L1 C-20 antibody.

3.3 Co-culture of AHNPs and chick embryo brain cells

Following *in vitro* analysis of AHNPs, the differentiation capabilities of the cells were assessed using an *in vitro* technique that simulated cell-cell interactions mirroring those of AHNPs in the normal brain, in hopes that these conditions would then induce AHNP differentiation. Therefore, AHNPs were cultured simultaneously (co-cultured) with embryonic chick brain cells that had been isolated as described in

the Materials and Methods sections. These cell to cell interactions were studied using two different techniques (and are also described in their respective sections of Materials and Methods): (1) adherent monolayers of chick brain cells cultured with AHNP/GFP cells and (2) non-adherent chick brain cells grown on polyHEMA-coated plates that created chick brain cell aggregates (as opposed to the monolayers) cultured with AHNP/GFP and AHNP/L1LE cells.

3.3.1 Monolayers of AHNPs and E7 chick brain cells prove to be difficult

Forming monolayers of E7 chick brain cells on coverslips was performed as outlined in the Materials and Methods section. Following the initial plating of embryonic chick brain cells, AHNPs were added once the chick brain cells appeared to be a confluent monolayer. This typically occurred roughly 16 days after the initial plating as a result of large chick brain cell aggregates that slowly melted to interact with the tissue culture dish (Figure 13). AHNPs were then added and the notation for culturing these cells tracked the days upon which AHNPs had been in co-culture with the chick brain cells. AHNPs were cultured in a different medium than had been previously used in order to allow the embryonic chick brain cells to continue to grow. Medium 199 + 10% FBS was used instead of the DMEM:F12 plus growth factor supplements that the AHNPs were cultured *in vitro* with; therefore, this may have been a source of potential complications during the co-culture experiments.

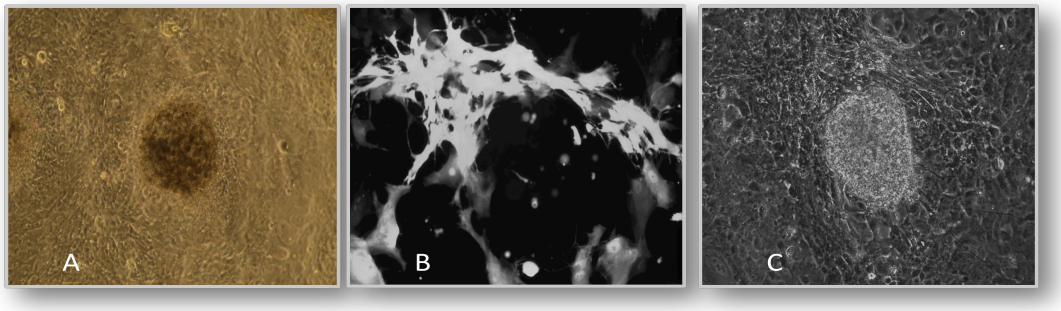

Figure 13. Embyronic chick brain cells and AHNPs co-cultured in monolayers. A. Phase contrast, live image of chick brain and AHNP cell co-cultures, 2 days after AHNPs were added to the co-culture, E7 + 16 + 2. B. Live image, 20x objective fluorescence of AHNP/GFP cells in the same co-culture. C. Live image, 20x objective phase contrast of the AHNP/GFP cells.

In order to begin evaluating the co-cultures of signs of differentiation, the AHNPs and chick brain monolayers were first immunostained for vimentin. Figure 14 shows very low levels of vimentin co-localization with the AHNPs, suggesting a loss of an astrotypic feature and therefore differentiation; however, the AHNPs were still minimally interacting with the embryonic chick brain cells and had burrowed their way underneath the monolayer of chick brain cells. These results began to suggest that the AHNPs would require lots of time in order to assess differentiation and interaction between cells.

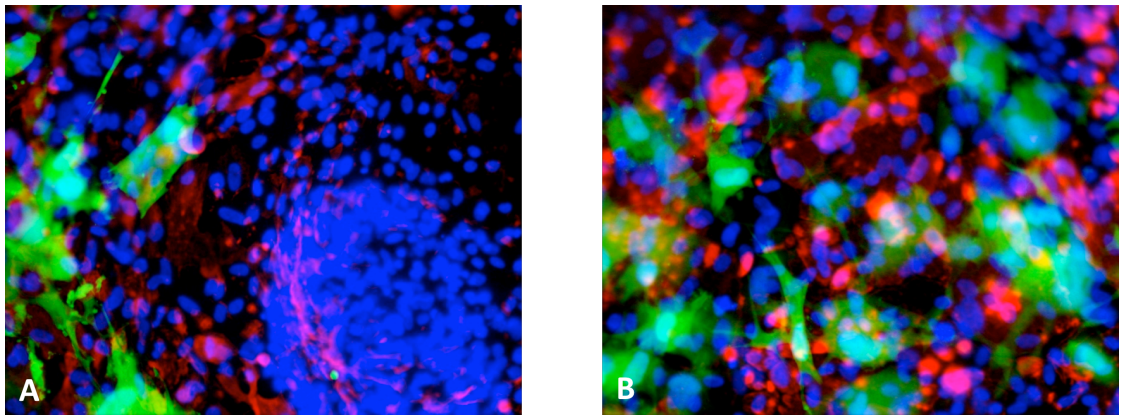

Figure 14. AHNP monolayer co-culture with E7 embryonic chick cells shows lack of interaction between cell types. A. Merged staining image of E7 + 10 days post-introduction of AHNPs in co-culture for vimentin (red), AHNP/GFP (green) and nuclei (blue). Chick brain cells are very prevalent on right half of this image and show very little interaction with the AHNPs. B. Merged staining image of E7 + 21 days post-introduction of AHNPs in co-culture for vimentin (red), AHNP/GFP (green) and nuclei (blue). This shows much more cell displacement but cells were not interacting and were in different planes of focus when images were assessed. Both pictures are of fixed and immunostained cells on coverslips and taken with a 20x objective.

Furthermore, in order to assess if any differentiation had occurred, immunostaining was done for oligodendrocytes, the one neural cell type that the AHNPs showed no previous cell marker for *in vitro* (Figure 15); chick brain cells did test positive for oligodendrocyte markers but the AHNPs did not. Finally, the monolayers were immunostained for L1 expression in order to evaluate if the AHNPs grown in a different media while in the presence of chick brain cells altered the L1 expression. AHNPs still expressed L1 in monolayer co-cultures (Figure 16).

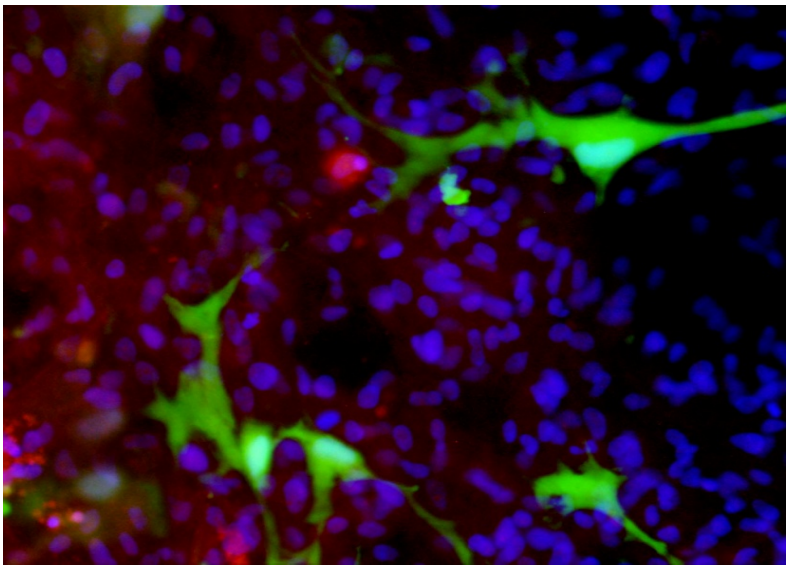

Figure 15. Co-culture monolayers do not show positive differentiation of AHNPs into oligodendrocytes. MAG (red staining) is seen in chick cells. The merged AHNP/GFP (green) cells with the nuclear (blue) staining show no colocalization of MAG and green AHNPs. Cells were fixed and immunostained on coverslips and photos taken with a 20x objective.

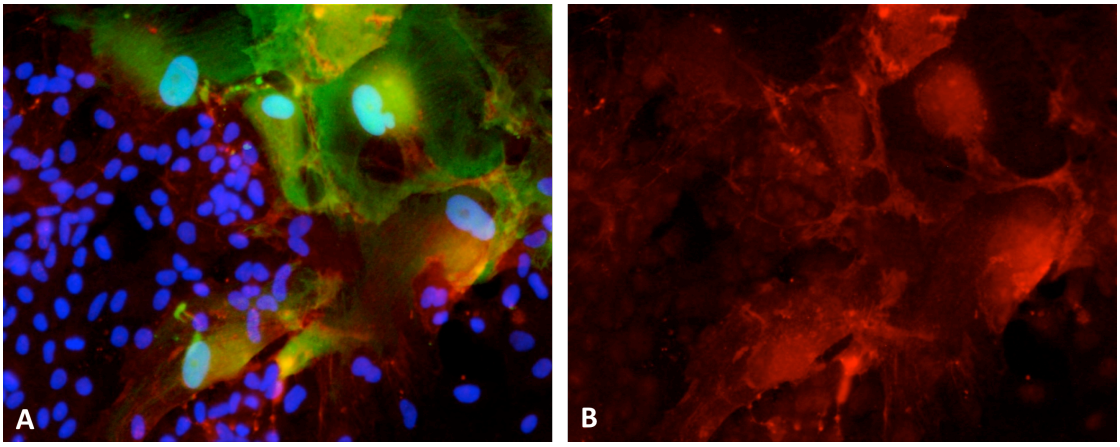

Figure 16. AHNPs still express L1 *in vitro* in monolayer experiments. A. L1CAM (red) still expressed (staining seen used UJ127 antibody) and colocalized to AHNP/GFP (green) cells. Nuclear (blue) staining was merged in with this photo. B. L1CAM (red) immunostaining only.Cells were fixed and immunostained on coverslips; photos taken with a 20x objective.

3.3.2 Chick brain cell aggregates and AHNPs show more cell-cell interactions

After investigating methods to overcome the lack of AHNP and chick brain cell interaction, the polyHEMA protocol (as outlined in the Materials and Methods section) was developed in order to keep co-cultured chick brain cells and AHNPs from adhering to the tissue culture dish and aggregate together in suspension. Harvesting embryonic chick brain cells and seeding AHNPs was performed as outlined in the Materials and Methods sections and cultures were then embedded in Tissue Freezing Media, cryostat sectioned, and immunostained. The sections were evaluated for similar cell and differentiation markers that had been previously studied up to this point. The largest difference in the co-culture method had been the introduction of full-length L1LE added to the AHNPs in order to assess any cellular differences. The infection of AHNPs with L1LE vector is outlined in the Materials and Methods section as well.

Upon introducing chick brain cells to the polyHEMA coated tissue dishes, chick brain cells formed aggregates *in vitro*; aggregates remained intact upon the addition of AHNP/GFP and AHNP/L1LE cells (Figure 17).

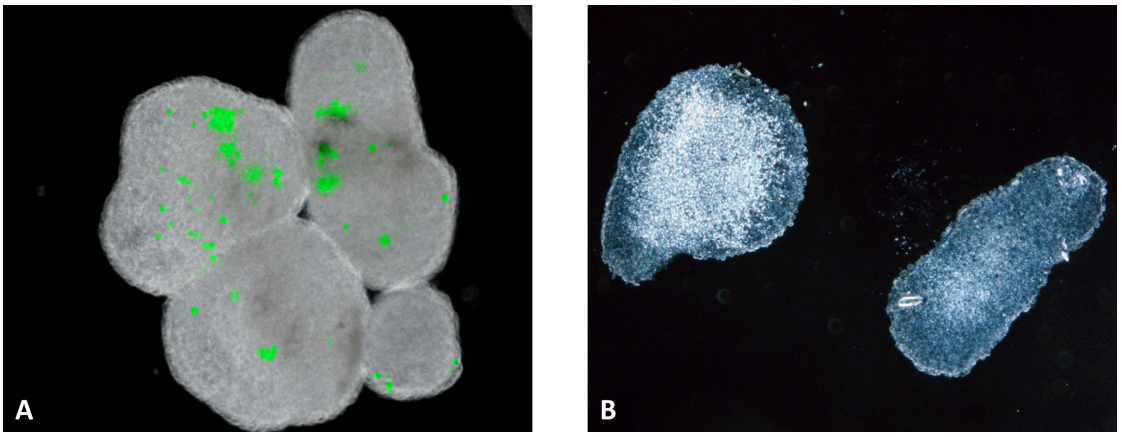

Figure 17. Chick brain cell aggregates with AHNPs. A. Co-culture image, modified via Adobe PhotoShop to overlay the phase contrast co-culture image of the aggregate and show the GFP+ AHNP image. Modifications were made by adjusting the settings using specific filters for color and blending the images by adjusting the percentage of opacity. Both pictures merged in panel A were taken using a 4x objective and were live imaging. B. Live imaging, phase contrast of aggregates in culture, a mixture of E15 chick brain embryo brain cells and AHNPS taken with a 4x objective.

Before beginning the experiments with the AHNP/L1LE cells, it need to be affirmed that the AHNP/L1LE cells being used for the various experimentations adequately expressed L1 and that the cells had not changed as a result of the transfection. Immunostaining of the AHNP/L1LE cells was performed. This method was chosen as opposed to a Western blot analysis which would have lysed the cells and taken much longer to harvest more cells for future experiments. AHNP/L1LE cells did positively immunostain for L1 expression (Figure 18). Again, vimentin expression was evaluated for first in order to detect any changes between AHNP/GFP cells and AHNP/L1LE cells (Figure 19). Vimentin immunostaining of both cell types reaffirmed that the cells being cultured at this point in time were non-differentiated in

comparison to previous culturing experiments and that the addition of L1 had not induced the AHNP/L1LE cells to begin differentiation.

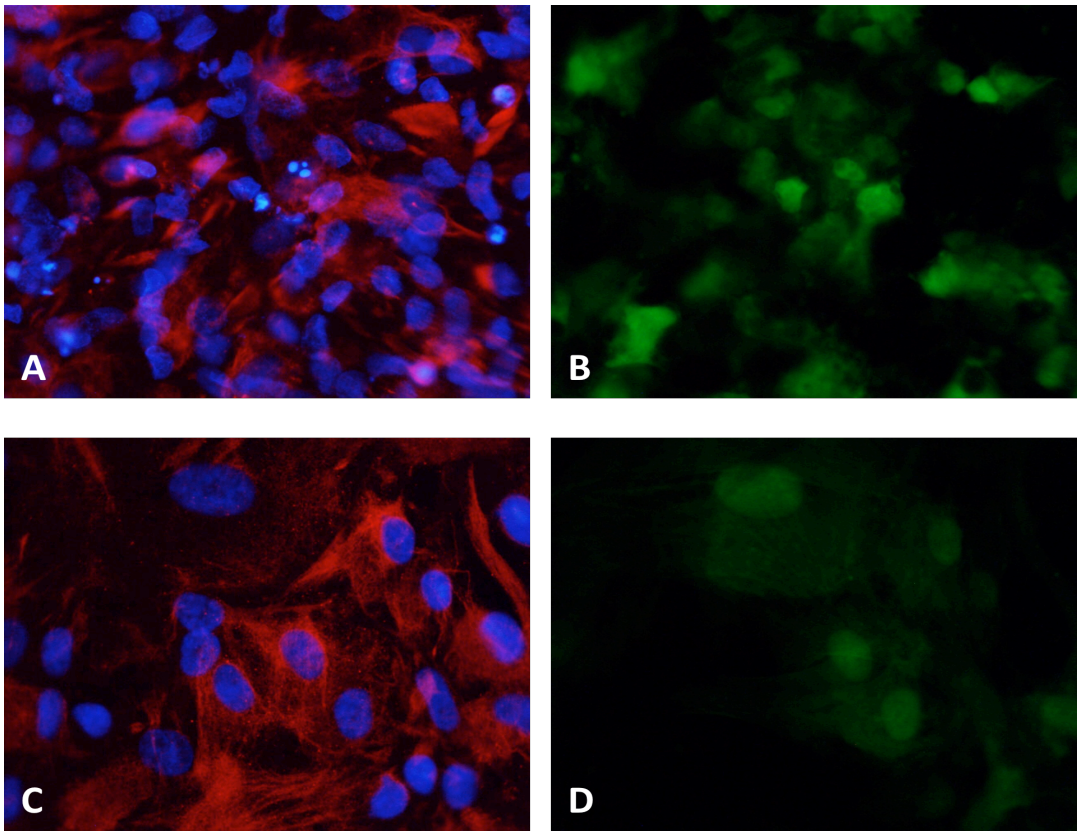

Figure 18. AHNP/L1LE cells express L1. UJ127 antibody was used to detect L1 (red) expression. AHNP/L1LE cells were also GFP+ and are therefore detected as green fluorescent cells. Panels A and B show the merged immunostaining and nuclear (blue) staining and the green fluorescent cells respectively. Panels C and D show another example of L1 staining in the AHNP/L1LE cells and are also the merged immunostaining and nuclear (blue) staining and fluorescence image respectively. Cells were fixed and immunostained on coverslips; photos were taken using a 20x objective (A and B) and a 40x objective with immersion oil (C and D).

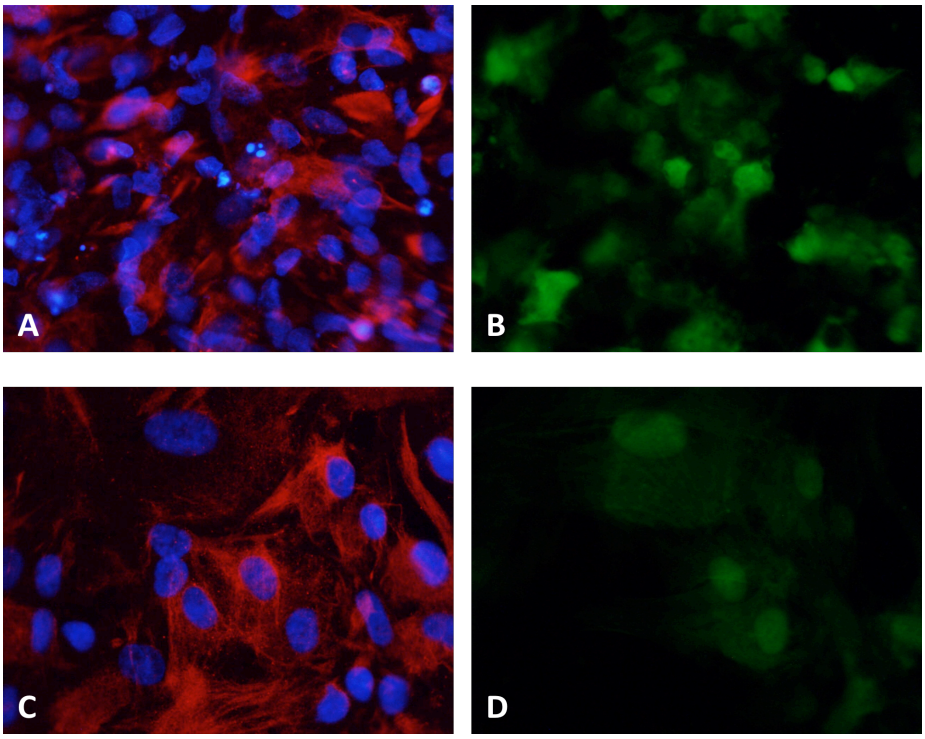

Figure 19. AHNP/GFP and AHNP/L1LE cells both retained their vimentin expression after successive passages and transfection experiments. A. Merged image of vimentin (red) and nuclear (blue) staining in the AHNP/GFP cells while below, in panel B the AHNP s (green) fluoresce green as a result of the GFP expression. C. Merged image of vimentin (red) and nuclear (blue) staining of AHNP/L1LE cells next to panel A for comparison and below in panel D the AHNP/L1LE cells fluoresce green due to GFP expression vector transfection. Cells were fixed and immunostained on coverslips; photos taken with a 40x objective.

After co-culturing both AHNP/GFP and AHNP/L1LE cells in aggregates, co-cultures were embedded, sectioned, and immunostained sections for various differentiation markers. It was of particular interest to observe co-cultures expression for glutamine synthetase (GS) and β-tubulin (via TUJ1 antibody) as indicators of differentiation. GS staining in published data (Walton et al., 2006) suggested that the AHNPs would express GS *in vitro*. Under the current study, this observation could

not be confirmed since co-culture studies of AHNP/GFP cells did not show any positive GS immunostaining (Figure 20). No analogous results were obtained for the AHNP/L1LE aggregates due to poor sectioning; none of the cryostat sections that were retrieved showed any indication of AHNP/L1LE cells being present (there was no green fluorescence in any of the aggregate sections). It cannot be definitely ascertained that the AHNPs differentiated from these results because GS staining appears to have been colocalized to the nucleus, and glutamine synthetase is typically observed as a cytoplasmic enzyme. This suggests problems with either the GS antibody or immunostaining protocol.

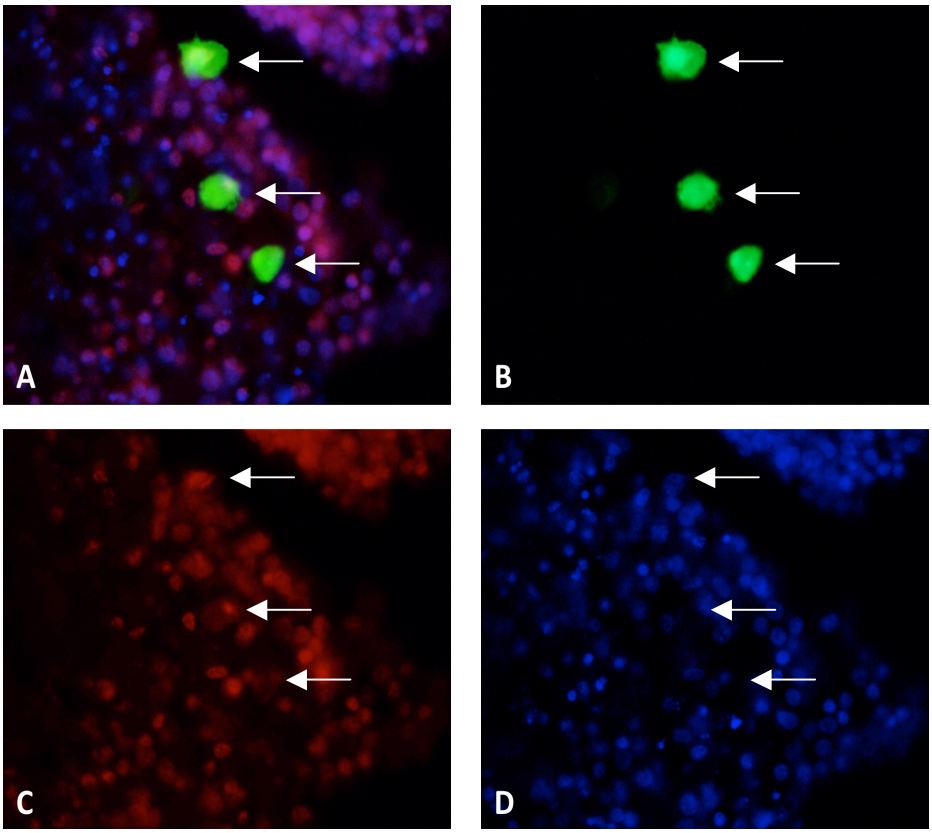

Figure 20. Immunostained section of AHNP/GFP cells in aggregate show negative GS expression. A. Merged image of panels B, C, D. B. GFP expression of AHNPs in aggregate. C. GS (red) staining. D. Nuclear (blue) staining. AHNP/GFP cells did not colocalize the GS staining in the cytoplasm, but instead it appears to be nuclear. Arrows are used to identify the AHNP/GFP cells in all panels of the image. Sections were cut 16 microns thick and had been fixed prior to embedding; photos taken with a 40x objective.

Additionally, immunostaining for neuronal differentiation showed no expression of β-tubulin via TUJ1 antibody staining. Neither the AHNP/GFP cells nor the AHNP/L1LE cells showed positive β-tubulin expression (Figures 21 and 22).

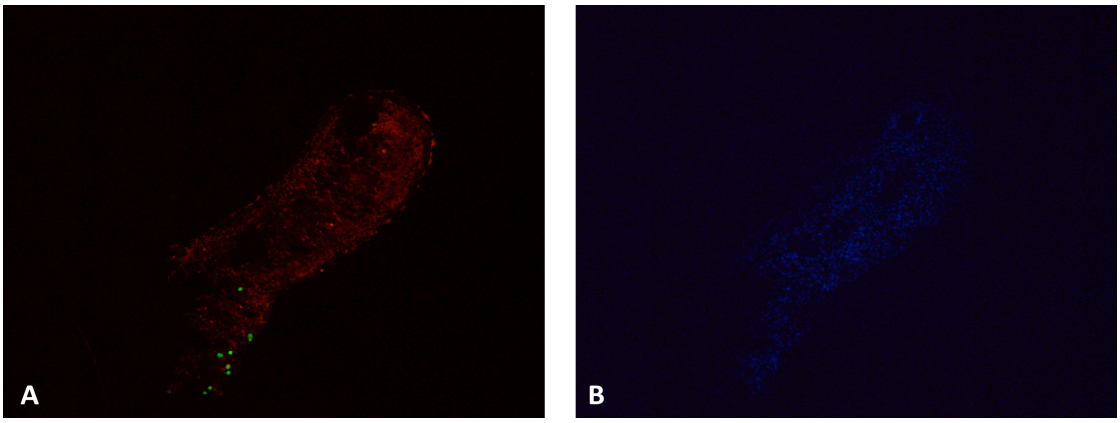

Figure 21. **AHNP/GFP co-cultures show no β-tubulin expression.** A. Merged image of TUJ1 staining (red) and AHNP/GFP (green) cells. B. Nuclear staining (blue) of the same section. Sections were cut 16 microns thick and had been fixed prior to embedding; photos taken with a 4x objective. Better images were not found when revisiting these sections for it appeared that the GFP positive cells had been bleached of their fluorescence.

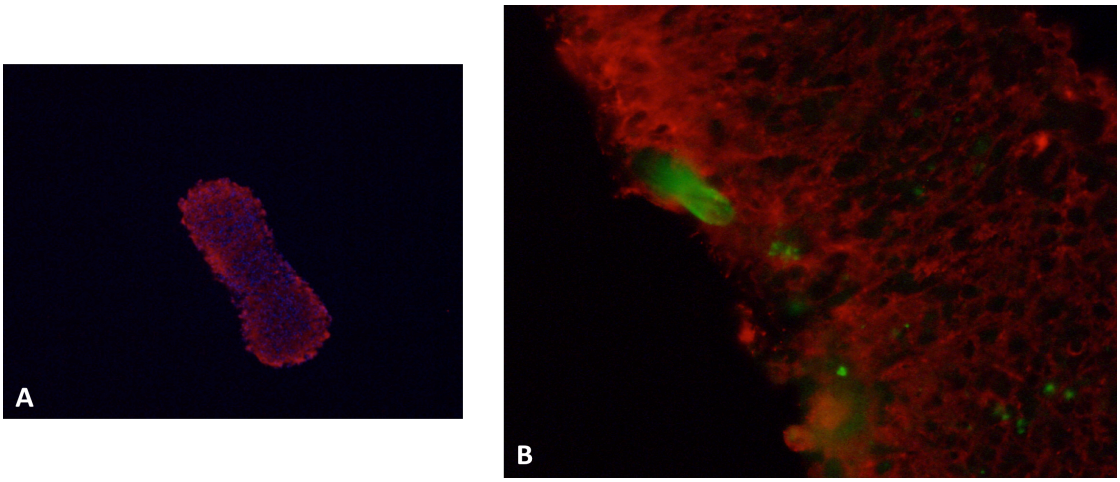

Figure 22. **AHNP/L1LE co-cultures show similar lack of β-tubulin expression.** A. Merged image of TUJ1 staining (red) and nuclear (blue) staining as photographed using a 4x objective. Green fluorescent cells were visible but not at the magnification presented. B. Zoomed in on the left side of panel A (using a 20x objective), shows AHNP/L1LE (green) but no colocalization with the TUJ1 staining (red). Sections were cut 16 microns thick and had had been fixed prior to embedding.

Finally, in an attempt to compare the co-culture aggregate method with that of the monolayer method, immunostaining for the presence of oligodendrocytes was done. The immunostaining was performed with a different antibody (O4 instead of MAG), but analysis of the co-culture of AHNP/GFP cells in an aggregate showed a positive result (Figure 23). O4 is expressed earlier than MAG, so monolayers might have been O4+ as well.

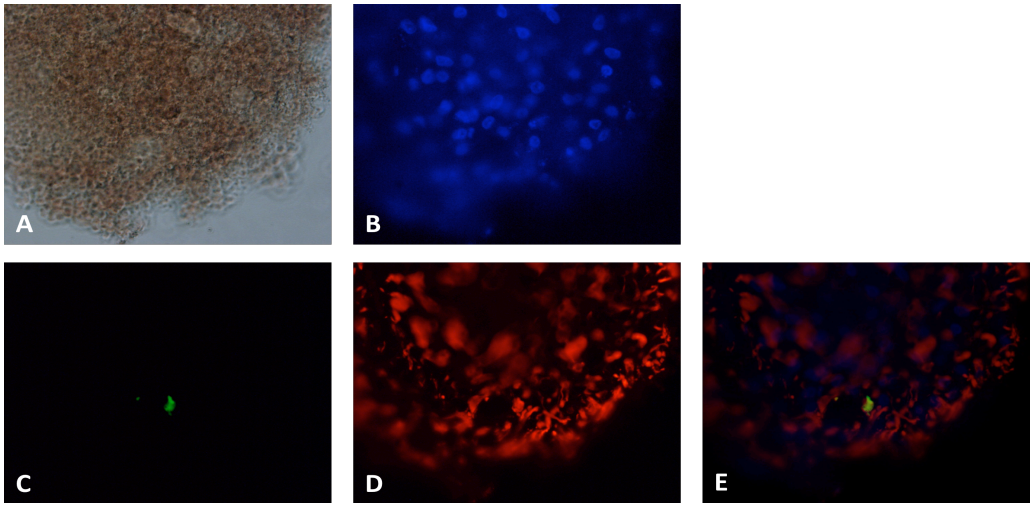

Figure 23. AHNP/GFP co-culture shows O4+ staining. A. Aggregate image in phase contrast. B. Nuclear staining (blue). C. AHNPs (green) fluorescing. D. O4 immunostaining (in red). E. Merged image of panels B, C, and D; colocalization of all three stainings indicated by the yellow color of the AHNP in the center of the picture. Photos were taken with a 40x objective and immersion oil. Sections were cut 16 microns thick and had been fixed prior to embedding.

3.3.3 Growth factor starvation experiments induce positive expression of neuronal markers in AHNPs *in vitro*

In order to assess the integrity of the GS and β-tubulin (TUJ1) staining done in the co-cultures, immunostaining of the AHNPs was done *in vitro* and without

growth factor supplements in order to induce neurogenesis (mirroring similar procedures performed by Walton et al., 2006). After growth factor starving AHNP/GFP and AHNP/L1LE cells, glutamine synthetase staining again appeared colocalized to the nucleus and images at a higher magnification suggested that the cells were autofluorescing (Figure 24).

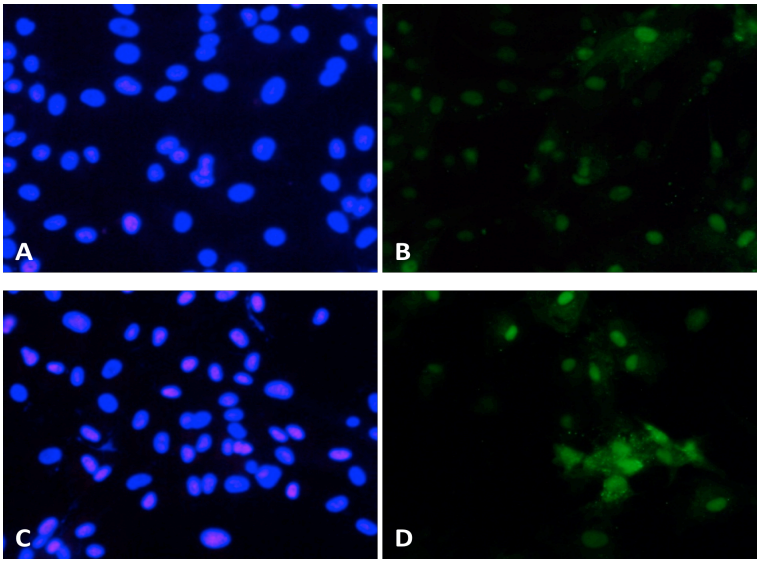

Figure 24. Growth factor starvation of AHNP/GFP and AHNP/L1LE cells fail to show any signs of GS immunostaining. A. Merge of nuclear (blue) and GS (red) staining of AHNP/GFP cells. B. AHNP/GFP cells fluorescing green. C. Merge of nuclear (blue) and GS (red) staining of AHNP/L1LE cells. D. AHNP/L1LE cells fluorescing green. Colocalization of GS and nuclear staining suggests poor GS staining and likely autofluorescence. Cells were fixed and immunostained on coverslips. Photos were taken with a 20x objective.

Unlike GS immunostaining, TUJ1 immunostaining resulted in positive β-tubulin expression in both AHNP cell lines that had been growth factor starved (Figure 25). This begins to suggest that the AHNPs were in fact undergoing neurogenesis,

however, further experiments would be required to ensure the accuracy of these results.

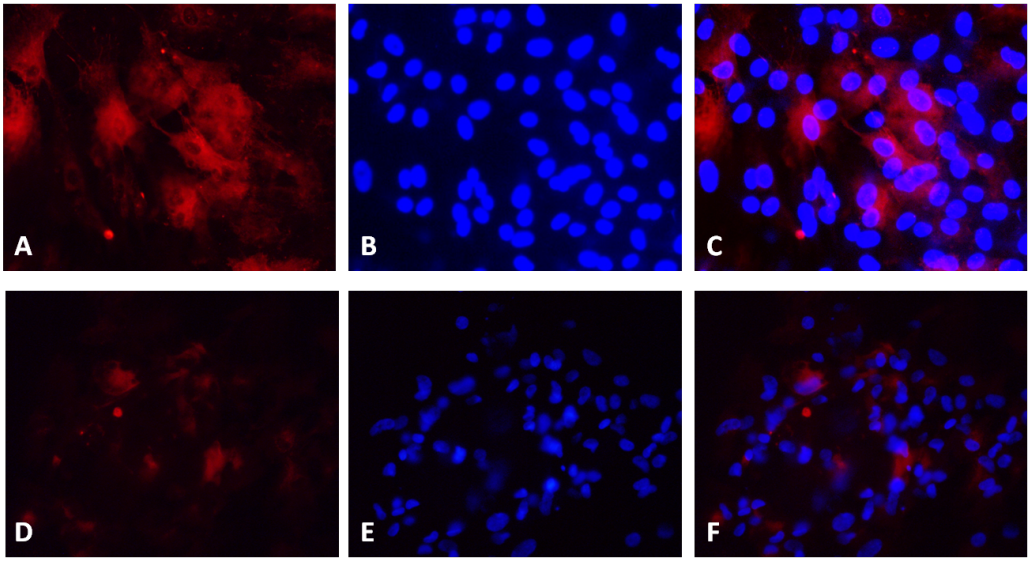

Figure 25. Growth factor starvation of AHNP/GFP and AHNP/L1LE cells show positive β-tubulin expression. A. AHNP/GFP TUJ1 (red) staining. B. AHNP/GFP nuclear (blue) staining. C. AHNP/GFP merged image of TUJ1expression and nuclear staining. D. AHNP/L1LE TUJ1 (red) staining. E. AHNP/L1LE nuclear (blue) staining. F. AHNP/L1LE merged image of TUJ1 expression and nuclear staining. Images show TUJ1 staining in the cytoplasm of the cells. Cells were fixed and immunostained on coverslips. Photos were taken with a 20x objective.

Chapter 4

DISCUSSION

4.1 Immunocytochemical characterization of AHNPs *in vitro* reveals the expression of L1 and other proteins of interest

One of the main goals of this study was to characterize the AHNPs *in vitro* in order to understand if the cells being studied expressed particular known markers (as published by Walton et al., 2006) and to ensure that the cells had maintained their viability since being stored and thawed for use after being given to the Galileo lab. In order to establish a point of comparison, vimentin expression, an intermediate filament known to be present in immature astrocytes, was examined (Bramanti, et al., 2010). The AHNPs were vimentin positive and showed vimentin expression throughout multiple experiments, particularly the co-culture experiments with the AHNPs and E7 chick embryonic brain cells. AHNPs were previously described as astrocyte-like cells and therefore the expression of vimentin was not unexpected. It had been hoped that the loss of vimentin expression would appear as a result of co-culturing with chick brain cells. This novel vimentin immunostaining further confirms that the AHNPs possess progenitor cell qualities.

The AHNPs were found to express glial fibrillary acidic protein (GFAP) *in vitro* as was expected in comparison to previously published studies (Walton et al.,

2006). No previous research had examined the presence of L1CAM in the progenitor cells and the current study demonstrated the presence of full-length L1 *in vitro* and of the transcription factor, Pax-6, although it was not localized to the nucleus. Western blot analysis further confirmed the immunocytochemsitry results for the full-length L1 protein and Pax-6 expression.

Additionally, in order to encourage differentiation of the AHNPs, the AHNPs were cultured and growth factor starved. Unlike the work done by Walton et al. (2006), the AHNPs were not given additional chemical supplements that forced neurogenesis. Without the growth factor supplements, the AHNPs were simulating the typical *in vivo* stimulus for neuron differentiation. The occurrence of differentiation was confirmed by *in vitro* growth factor starved TUJ1 AHNP immunostaining indicating the presence of β-III-tubulin, a neuronal marker. Further evaluation of this technique could allow for the development of *in vitro* growth of neurons for neurodegenerative diseases (Lie et al., 2004). Unfortunately, AHNP cultures were limited as cell cultures would experience passage doublings of up to 20 before cells began to senesce, an unexpected result in contrast to the 60 passage doublings of Walton et al. (2006). Therefore, it was not possible to immunostain the cells for TUJ1 and glutamine synthetase *in vitro* without growth factor starving them in order to ascertain if growth factor starvation had in fact signaled for progenitor differentiation.

4.2 Co-culture studies provide alternative means of AHNP study to the chick embryo model

AHNPs survived in the chick embryo brain, which will allow for future *in vivo* studies. If the protocol for immunostaining 200-micron thick vibratome sections could be perfected or if vibratome sections were cryostat sectioned in order to retrieve smaller sections, then this would allow for binding of antibodies to AHNPs and developing chick brain cells. This approach would allow for evaluation of the degree of interaction between AHNPs and the chick embryo brain.

The development of co-culture studies via polyHEMA and monolayers allowed for the *in vitro* evaluation of AHNPs and their dynamics with chick embryo brain cells *ex vivo*. Co-culture studies via the polyHEMA method proved to be more time efficient than the monolayer method. Many diverse interactions between AHNPs and chick brain cells were seen. It was interesting to note that due to the polyHEMA coating of the culture dishes, AHNPs could have formed AHNP-only aggregates of cells, though no green-only clusters were observed; both AHNP types appeared to interact with the chick brain cells in the aggregates. Normal *in vitro* culturing of AHNPs was very contact-dependent so it does appear that physical contact between AHNPs could have happened.

When screening the 16 micron thick cryostat sections of aggregates, green fluorescent AHNPs were not always found. A solution to this problem would be to readjust the number of seeded cells per experiment in order to ensure that AHNPs are sufficiently concentrated in aggregates. The finding of novel O4 positive staining

was something that Walton et al. (2006) was unable to find in the AHNPs that were engrafted into the mice model. This further justifies continuing to pursue this method of cell growth and analysis. In order to detect multiple stem cell types, better antibodies and a diverse array of secondary antibodies could be used that would allow for images displaying immunostaining (and ideally phenotypes) of multiple neural cell types.

Additionally, the monolayer experiments could have provided data as to the motility of AHNPs. Changes in L1 expression over the course of the monolayer experiments was not expected, but had differentiation occurred where AHNPs were to become more glial like, changes in L1 could have indicated glioma genesis and possible increases in invasiveness and motility (Yang et al., 2011). Monolayer experiments should be repeated in the future for AHNP/GFP and AHNP/L1LE cells and observed via time-lapse microscopy in order to assess cell to cell interactions and track cell movement.

4.3 Conclusions

Despite the limited research that has been completed on adult human neural progenitor cells, it appears that these cells display the potential plasticity that could enable future scientists to develop protocols that expand human progenitor cells into other neural cells (Walton et al., 2006). The current study should be expanded to more fully assess differentiation. Eventually, motility and invasiveness of AHNPs in the brain can be reviewed and compared; further distinctions between AHNP/GFP and

AHNP/L1LE can be made based on the presence and interactions of L1. It would be very interesting to see if the AHNP/L1LE cells prefer differentiation toward glial cells that over-express L1or if they continue to obtain exogenous signals that induce neuronal differentiation. Additional experiments to track L1 changes would have included determining ADAM-10 presence and degree of expression between AHNP/GFP and AHNP/L1LE cells and Western blot analysis of AHNP culture media over the course of aggregate experiments and *in vitro* immunostaining for the presence cleaved L1.

Interactions of AHNPs with other cells in culture would be very important to study in order to understand signaling mechanisms and communication, particularly via integrins and the expression of glial markers that may provide insight into gliomagenesis (Canoll and Goldman, 2008). Additionally, further studies of the AHNPs L1 upregulation and any connection to gliomas would complement previous work done in the Galileo lab that showed that the presence of L1 upregulating glioma metastasis and invasion (Yang et al., 2011).

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