

EMBRYONIC STEM CELL-DERIVED POPULATIONS RETAIN THEIR TUMORIGENIC POTENTIAL

By

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ABSTRACT OF THESIS

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Multiple sclerosis (MS) is a neurodegenerative disorder, which results in the death of oligodendrocytes, the myelinating cells of the central nervous system (CNS). Although drug therapies can abate the progression of MS, no treatment has reported significant repair of lost myelin. One possible solution would be to use an embryonic stem cell therapy to derive oligodendrocytes. However, ESC studies have stressed the need for have a homogenous cell population, because ESCs produce teratomas, benign embryonic tumors consisting of all three tissue layers. In this thesis, mouse ESCs were differentiated to oligodendrocytes using a six-step protocol. At each stage of the differentiation protocol, we asked whether teratogenic ESCs persisted within the differentiated cell population. If ESCs persisted, the cell population would have the ability to generate embryoid bodies (EBs). It was found that at each stage of differentiation, we were able to generate EB-like bodies. However, the frequency of EB formation decreased as differentiation progressed indicating a progressive reduction in the number of ESCs. The differentiated cell populations were characterized by immunocytochemistry and RT-PCR and compared and contrasted to starting ESC populations. We conclude that undifferentiated ESCs remain within the heterogeneous population of cells. Therefore, a possible cell therapy must have all ESCs removed from the final differentiated cell population.

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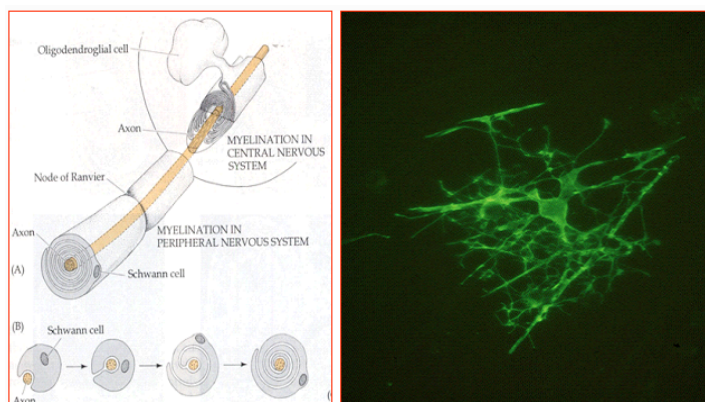
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1. INTRODUCTION

Neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, Parkinson's, and multiple sclerosis (MS) all involve forms of cell death. Apoptosis is programmed cell death initiated from within the cell; whereas, necrosis is cell death due to insults including toxins and trauma. MS is a particular neurodegenerative disorder, which results in the death of oligodendrocytes (OLs), the myelinating cells of the central nervous system (CNS). Myelin is a lipid-rich membrane that insulates and increases the speed of electrical impulses along an axon (Figure 1). The loss of myelin destabilizes axons and their ability to conduct impulses. MS is the most common neurodegenerative disorder in young adults, and it occurs mostly in the western, early adulthood population (Frohman, et al., 2006; Hauser and Oksenberg, 2008). Early symptoms of the disease include loss of dexterity and weakness, and as the disease progresses cognitive defects and spasms. There are many factors that influence the progression of MS including genetic and environmental components. Combined, these factors contribute to the autoimmune response, which induces inflammation in the CNS. MS is diagnosed through MRI scan, where loss of myelin is visualized through plaque formations, an area of increased inflammation and demyelination.

Figure 1: Myelin-forming oligodendrocytes. (a) Schematic of CNS myelin. (b) a single oligodendrocyte from rodent brain in co-culture with neurons, stained for myelin basic protein. One soma (asterisk) extends multiple arms that T-intersect (arrow) and form compacted myelin sheaths on neuronal axons.



MS has a familial genetic component, and studies have indicated the human leukocyte antigen (HLA) gene cluster on chromosome six is a candidate region. In a genome wide analysis conducted by the International Multiple Sclerosis Genetics Consortium in 2007, 930 families with MS were studied against 500, 000 single nucleotide polymorphism arrays. The study noted a correlation with disease and the gene *HLA-DRB1* on chromosome 6p21.3 as well as other non-HLA genes, such as *IL2RA* and *IL7R* (IMSGC, 2007). Since MS is associated with immune response genes, treatment options have incorporated global immunosuppression using anti-inflammatory drugs and targeting immune response pathways (Vogel and Hemmer, 2002; Bauman and Kasper, 2004). For example, MS treatments have targeted T-cell mediated inflammation. Studies conducted by D'Souza et al. (1996) and Dowling et al. (1996) have indicated the Fas ligand death signaling pathway, thus providing therapeutic targets to prevent cell death. Current drug therapies include immunomodulatory agents (Marrie and Rudick, 2006) such as interferon β -1b (Betaseron), interferon β -1a (Avonex and Rebif), and glatiramer acetate (Copaxone). Galetta et al. (2001) examined the efficacy of these drugs in treatment and disease activity. In each trial, it was observed that there was a reduction in lesion size as well as the number of new lesions that formed. In addition, a three year clinical trial studying the treatment of Betaseron observed that early treatment of initial MS symptoms prevent severe disability (Kappos et al., 2008). Although drug therapies can abate the progression of MS, no treatment has reported significant repair of lost myelin (Prineas et al, 1993; Bechmann, 2005).

New approaches in treating MS have examined the biology of oligodendrocytes and their interactions with axons. These studies have shown that transplants of myelin-

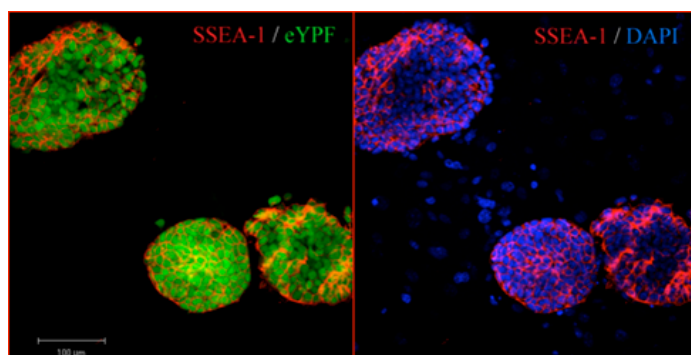
forming cells can actually rebuild lost myelin and repair axons (Dubois-Dalcq and Armstrong, 1990; Groves et al., 1993; Kuhn et al., 1995; Cao et al., 2005; Keirstead. et al., 2005; Perez-Bouza et al., 2005; Chen et al., 2007). Therefore, an emerging strategy for MS treatment is to combine immunosuppression with cell transplants to generate oligodendrocytes for remyelination. The concept of cell-based therapy is based on the hematopoietic stem cell (HSC) model. Bone marrow transplants have demonstrated that multipotent HSCs can reconstitute all blood cell lineages as well as maintain the HSC population (Weissman et al., 2001).

Stem cells are defined as self-renewing cells that can, under appropriate conditions, generate specific differentiated cells types (Rossant, 2008). Multipotent stem cells, such as HSCs, have the ability to self-renew and only produce cell types from their particular lineage. Multipotent neural stem cells have been located in the subgranular zone in the dentate gyrus of the hippocampus, the subventricular zone of the lateral ventricle, and the brain parenchyma of the adult brain (Schluz, 2008). Using a mouse model depicting MS, Pulchino et al. (2003) injected neural stem cells both intravenously and into the cerebral ventricles. In both instances, significant numbers of donor neural stem cells reconstituted brain tissue as well as generated new myelin.

In human studies, Parkinson's Disease (PD) treatments have implemented fetal brain grafts for dopamine neuron replacement (Lidvall and Kokaia, 2006). The PD studies indicate that the immune rejection of fetal neural transplants, as the source for neural stem cells, may be a downfall to this approach; thus, alternatives have been proposed. For example, pluripotent embryonic stem cells (ESCs) can generate all cell types of the embryo (Evans and Kaufman, 1981; Martin, 1981). Mouse ESCs differentiated into

oligodendrocytes were able to produce myelin when transplanted into the spinal cords of myelin deficient rats (Brüstel et al. 1999), therefore providing an example of cell-derived therapy. Recently, ESC-derived oligodendrocytes completely rescued the myelin-mutant Shiverer mouse (Kiel et al, 2007; Windrem et al., 2008).

Figure 2: Mouse embryonic stem cells. The ESC line 7ac5 (eYFP⁺) growing on a monolayer of mouse embryo fibroblasts and stained with the ESC marker antibody SSEA-1.



These studies suggest it may soon be possible to provide a

cell-based therapy approach in treating MS. However, studies to date have also have stressed the need for a homogenous population of oligodendrocytes. ESCs (Figure 2) have the ability to differentiate into many cell types. They also possess the ability to form teratomas and malignant carcinomas (Carson et al., 2006; Addis, et al, 2008; Lau et al, 2008, Amariglio et al. 2009). Mouse ESCs were discovered upon studies of embryonic carcinoma cells (Evans et al., 1981). This raises the question of tumorigenicity for ES-derived cell therapeutics. Embryonic tumors are normally found in the gonads, but they can form in other locations within the developing fetus. There are two categories of this type of tumor: teratomas, which are benign, and teratocarcinomas, which are malignant. The histology of these tumors can consist of a disorganized array of somatic and extraembryonic tissues. Advanced tumors can have fully formed adult structures such as teeth and hair. The malignancy of these tumors is caused by a subset of cells, the embryonic carcinoma cells. This subpopulation also has the ability to self-renew and differentiate into various tissue types of ectoderm, mesoderm, and endoderm.

Many studies have examined the tumorigenicity of ESCs. When a population of ESCs is injected into immuno-compromised mice, teratomas develop. Studies conducted by Erdü et al. (2003) and Riess et al. (2007) examined the ability of transplanted undifferentiated ESCs to repair motor function after induced brain trauma using animal models. It was found that although motor function improved, tumors formed. However, not all ES-derived transplant studies generate tumors. Dihné et al. (2006) used partially differentiated ESCs to form neuronally committed precursor cells, and this population of cells was injected into trauma-induced lesions in mice. The study varied the duration in which the pre-differentiated neural cells were cultured in differentiation medium. The pre-differentiated cells, which had the longest duration in differentiation medium, produced no tumors in the transplanted area, while the cells cultured for a shorter duration were tumorigenic.

These studies lead to the question, will ES derived cell populations form teratomas during ES cell based therapies? The studies described in this thesis address this question for ESC-derived myelin forming cells. Following a modified differentiation procedure outlined by Billion et al. (2002), mouse ESCs were differentiated to oligodendrocytes using a six-step protocol (Figure 3). At each stage of the differentiation protocol, we asked whether teratogenic ESCs persisted within the differentiated cell population. If ESCs persisted, the cell population would have the ability to generate embryoid bodies (EBs). It was found that at each stage of differentiation, we were able to generate EB-like bodies. However, the frequency of EB formation decreased as differentiation progressed indicating a progressive reduction in the number of ESCs. The differentiated cell populations were characterized by immunocytochemistry and RT-PCR and compared

and contrasted to starting ESCs population. We conclude that undifferentiated ESCs remain within the heterogeneous population of cells. Therefore, a possible cell therapy must have all ESCs removed from the final differentiated cell population.

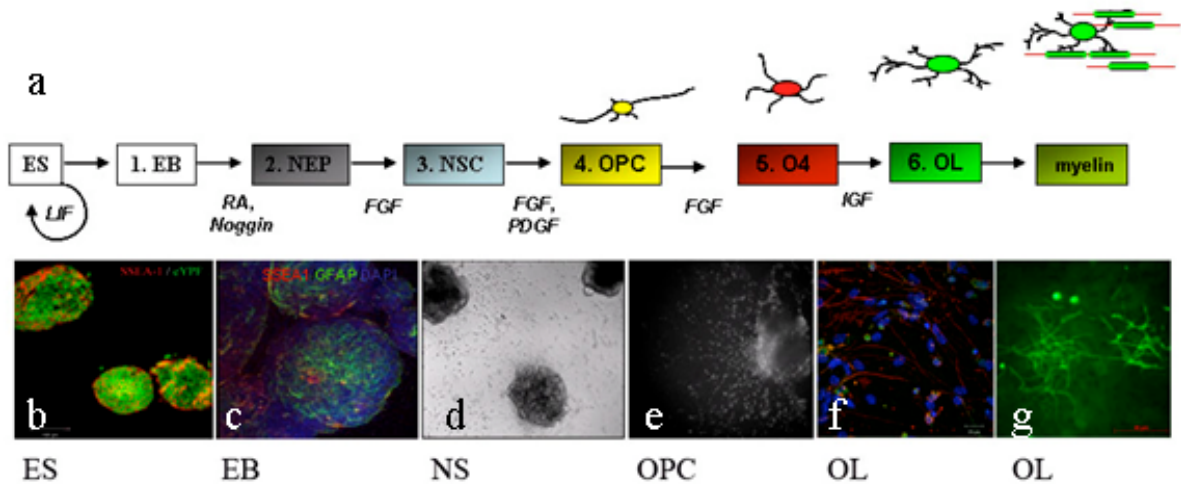


Figure 3: Oligodendrocytes from pluripotent stem cells in vitro. (a) ESCs can sequentially generate EB (embryoid bodies) NS (neural stem) cells and OL progenitors (OPCs) via factors that promote neural induction (FGF induced BMP antagonists) then neural tube ventralization (Shh). OPC maturation to O4 progenitors and postmitotic OLs is driven by PDGF and inhibited by FGF. (b-f) Morphology and marker expression in ESC-derived cultures *in vitro*. (b) mESC (SSEA1); (c) EB (GFAP); (d) NSC (phase; axon outgrowth); (e) ES-derived OPCs (Olig2:GFP⁺); (f) ES-derived OLs (O4⁺, Olig2:GFP⁺, DAPI); arrows mark myelin sheaths; (g) spinal cord-derived OLs and myelin (MPB⁺).

2. Materials and Methods

2.1. Reagents

Recombinant growth factors were obtained from R&D, Inc. (Minneapolis, MN). Primary antibodies were obtained from Chemicon (Temecula, CA) and Developmental Studies Hybridoma Bank (Iowa City, IA). Secondary fluorescent conjugate antibodies were obtained from Molecular Probes (Eugene, OR). G-Olig2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cell culturing media and supplements was obtained from Gibco Invitrogen (Carlsbad, CA). Cells were grown in a humid-filled incubator at 37°C with 5% carbon dioxide. The basal media was Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Invitrogen).

2.2. Cell Culture

a. ESC Culture G-Olig2 mouse ESCs (Xian et al., 2003; Xian et al., 2005) were cultured on irradiated mouse embryonic fibroblasts (MEF) monolayer in ES Media, which contains DMEM supplemented with 15% fetal bovine serum (FBS), 0.1 mM β -mercaptoethanol, and 1% nonessential amino acids (Invitrogen). This was supplemented with 1.25 ng/mL (125 units) leukemia inhibitor factor (LIF, Chemicon), and this media was replaced daily. When ES colonies grew to a size where they approached contact with neighboring colonies, they were passaged with 0.25% trypsin (Invitrogen) to dislodge the cells. ES media was added to stop the reaction, and the cells were collected by centrifugation (1,000 rpm, 10 minutes). The media was then removed, and the cells were resuspended with fresh media. Depending on the density of cells, cells were diluted to 1:2 or 1:3.

b. MEF MEFs were provided by Mary Kiel from the McKinnon lab. Pregnant mice at 13.5 post coitus were anesthetized, and the embryos were removed from the uterus and then washed three times in 1X Phosphate Buffered Saline (PBS). The organs and heads were removed, and the remainder was diced into small pieces. To break down the tissue, 1 mL 0.25% trypsin was added, and the tissue incubated at 37°C for 10 minutes. To stop the reaction, 4 mL of DMEM with 15% FCS was added. The tissue clumps were then manually dissociated by pipetting up and down several times, and the mix was placed into a centrifuge tube to settle for 5 minutes. The supernatant fluid was removed, the cells were resuspended with MEF media [DMEM supplemented with 10% FBS, 1% nonessential amino acids, and 1% Penicillin/Streptomycin (Invitrogen)] and were incubated at 37°C in culture plates. Generally, these cells reach confluency after 3-4 days, and were passaged 3 times as per the ES cell passaging method, in order to expand the population. Cells were collected and cryoprotected at 5×10^6 cells/mL in Freeze Down Media [DMEM, 20% volume FBS, and 10% dimethyl sulfoxide (Fisher Scientific, Bridgewater, N.J.)]. To prepare mitotic arrested MEFs for feeder layers in ESCs cultures, the frozen MEFs were irradiated at 4,000 rads (γ -irradiation source, Rutgers Environmental) then returned to liquid nitrogen storage until use.

c. ES differentiation G-Olig2 cells were cultured following a sequential ligand addition (six steps) protocol for oligodendrocyte differentiation (Figure 3). To form embryonic bodies (EBs), ESCs were cultured as suspension cultures in bacterial dishes with ES Media without LIF for 96 hours. On Day 4, neural stem (NS) cell induction began. To

enhance EBs adhesion, EBs were transferred to tissue culture plates which had been coated in 100 $\mu\text{g/mL}$ poly-ornithine (Sigma) in boric acid pH=8.4. The ES media was supplemented with 50 ng/mL Noggin (R&D) and 1 μM Retinoic Acid (Sigma) to induce neural sphere (NS) formation. To promote neural progenitor cell (NPC) formation, the cells (Day 8) were cultured with 50:50 mixture of DMEM-F12, 2% B27 plus Neural Basal Media, 1% N2 supplement (all from Invitrogen) and supplemented with 1 μM FGF2 (R&D). At Day 11, this was replaced with 50:50 DMEM: NBM supplemented with FGF plus 30 ng/mL sonic hedgehog (SHH, R&D) to promote oligodendrocyte progenitor cell (OPC) formation. On Day 14, to promote maturation to the O4 stage (Figure 3), the cells were cultured with the 50:50 DMEM-F12 and NBM media supplemented with 10 ng/mL PDGF-AA and 30 μM T3. Lastly to optimize oligodendrocyte formation, the cells were cultured with R1236 media, a media that contains 1% Penicillin/Streptomycin, 100 $\mu\text{g/mL}$ sodium pyruvate (Invitrogen), 20 nM selenium (Sigma), 50 ng/mL insulin (Sigma), 100 $\mu\text{g/mL}$ bovine serum albumin (Sigma), 60 $\mu\text{g/mL}$ N-acetyl cysteine (Sigma), and 10 μM forskolin.

2.3. EB Adherence and Fixation

To compare conditions for optimal EB adherence, cover slips were placed in 24 well-plate and were treated with either 0.1% gelatin, with antibody SSEA-1 (stage specific embryonic antigen-1), or untreated. An EB aliquot was placed in each well under the various conditions, and the plate incubated at 37°C for 2 hours. The efficiency was determined by counting the number of EBs and EB-like bodies that adhered to the cover slip and the total number of EBs and EB-like bodies per well.

EBs and EB-like bodies were fixed on chamber slides or 12 mm cover slips with 2% paraformaldehyde (Fisher, room temperature, 20 minutes). The samples were rinsed three times with PBS. Chamber slides were stored with approximately 1 mL PBS, covered in parafilm, and kept at 4°C. Cover slips were stored similarly with 0.5 mL PBS and the plate was wrapped with parafilm.

2.4. EB-like Assay

Cells derived from ES to OL (stages 1-6) were collected as a single cell suspension by trypsinization with 0.25% trypsin, centrifuge (1, 000 rpm, 10 min), and resuspended in with ES media without LIF. These cells were plated in bacterial dishes at varying cell concentrations starting at 5×10^4 and increasing up to 5×10^6 cells/plate. EB-like bodies were counted at 48 and 96 hours after plating. EB-like bodies were transferred to new bacterial dishes each day of culturing, in order to allow their continued growth and prevent differentiation. For this assay, a cell mass was considered an EB if it filled the requirement of a hollow, spherical body that did not adhere to the surface of the plate.

2.5. Transcript Analysis

a. RNA isolation RNA was extracted from samples using Trizol reagent (Invitrogen) following the manufacturer's protocol. For cells that were adherent, 1 mL Trizol reagent per 35 mm or 100 mm dishes was added directly to the plate. For EBs and EB-like bodies, the non-adherent cells were centrifuged for 5 minutes in order to collect bodies suspended in media. Cells were counted and then re-centrifuged to collect cells. The supernatant was removed, and the surface of the pellet was rinsed gently with 1X PBS so

not to disturb the pellet. As per the protocol, 1 mL of Trizol was used to resuspend 5×10^6 up to 1×10^7 cell for 5 minutes to break up the EBs, then 0.2 mL chloroform was added. Samples were centrifuged (12,000 rpm, 15 minutes, 4°C), the aqueous phase was removed, and 1 mL of 2-propanol was added. After a 10 minute incubation (room temperature), the samples were centrifuged (12,000 rpm, 10 minutes, 4°C), the supernatant was removed, then the pellet was rinsed with 70% ethanol and centrifuged (7,500 rpm, 5 minutes, 4°C). The supernatant was removed, the pellet was dried and was dissolved with 50 μL distilled water.

b. RNA purification In order to remove any contaminating genomic DNA from RNA extracts, RNA samples were purified with RNeasy Kit (Qiagen, Valencia, CA) following the manufacturer's protocol using DNase I reagent (Qiagen). RTL Buffer was added to 3-4 μg of total RNA samples to inhibit the RNases and keep the RNA intact. Ethanol was added to the samples to bind the RNA onto a silica-based membrane column, which was then centrifuged (10,000 rpm, 15 seconds, room temperature). Next, the RNA samples were washed with RW1 buffer and centrifuged. A mixture of DNase I and RDD buffer in 1:8 dilution was added to degrade any contaminating genomic DNA, and each sample was incubated (room temperature, 15 minutes). The samples were washed with RW1 buffer and centrifuged. Next, the samples were washed with RPE buffer and were centrifuged. This wash step was repeated, and the samples were centrifuged for 2 minutes at 14,000 rpm to remove all liquids. Last, 50 μL ddH₂O was added to each sample to elute the RNA.

c. ODs: OD readings were taken with the Ultrospec 2000 (Pharmacia, Piscataway, N.J.) to determine the amount of total RNA in each sample. The apparatus was blanked with 100 μ L distilled water. Each sample underwent a serial dilution, and a reading was taken of each dilution. Serial dilutions were prepared with 2 μ L of sample with 100 μ L distilled water (1:50 dilution). An additional 100 μ L distilled water was added to the sample (1:100 dilution). Next, 100 μ L was removed and 100 μ L distilled water was added (1:200 dilution), and this was repeated for the final dilution (1:400 dilution). For samples with an initial Abs₂₆₀ value less than 0.010, the volume of RNA sample used was increased up to 10 μ L of sample to 100 μ L distilled water (1:10 dilution). RNA concentration was determined by multiplying the 1:100 dilution OD value by 40 and then dividing this value by 10 to achieve μ g/ μ L, based on the formula that an RNA solution at 40 μ g/mL yields an absorbance of 1.00 (Maniatis et al., 1982).

d. cDNA Preparation First-strand cDNA was prepared from RNA samples starting with 1 μ g RNA and 0.5 μ g oligo (dT)₁₂₋₁₈ (Invitrogen), following the manufacturer's protocol. Samples were denatured at 65°C (5 minutes), then placed on ice to allow the oligo (dT) to anneal to the poly(A) tail of messenger RNAs. A mixture of First-Strand Buffer, 10 mM DTT, 0.5 mM dNTP, and 200 Units M-MLV reverse transcriptase was mixed with the samples for a total volume of 20 μ L. The samples were incubated for 1 hour at 37°C, to produce a first strand DNA copy of the RNA sample, and then the samples were stored at -20°C. This reaction produces a 1:1 conversion of RNA to cDNA.

Gene target	Primer Name	Oligonucleotide sequence	Genbank Access
GAPDH	hGAPDH 447f GAPDH643r	5'-AGACAAAAGGGTCATCATCTC-3' 5'-CTGTGGTCATGAGTCCTTCCA-3'	NM_002046
TUB β 3	TUB β 3.199F TUB β 3.542R	5'-AGCGGATCAGCGTCTACTACA-3' 5'-ATACTCCTCACGCACCTTGCT-3'	BC031357
Nestin	mNestin 916f mNestin 1096r	5'-AGCAGGAGAAGCAGGGTCTA-3' 5'-TCAGGAAAGCCAAGAGAAGC-3'	BC060693
SOX2	hSOX2.910f hSOX2.1116r	5'-GCACATGAACGGCTGGAGCAACG-3' 5'-TGCTGCGAGTAGGACATGCTG TAGG-3'	NM_003106
AFP	h&m.alfaFTPR.1611F h&m.alfaFTPR.1786R	5'-GTCCCTCCTGCATTCTCTGA-3' 5'-TCCAACAGGCCTGAGAAATC-3'	BC066206
ActA	h&mActa.953f h&mActa.1124R	5'-AGGGGGCACCACATATGTACC-3' 5'-GATCCACATCTGCTGGAAGG-3'	NM_001613.2
Olig2	h.mOlig2.427f h.mOlig2.689r	5'-CACCAAGAAGGACAAGAAGCA -3' 5'-GTAGATCTCGCTCACCAAGTCG -3'	AB028697
Oct3/4	mOct3.423f mOct3.576r	5'-GAGGAGTCCCAGGACATG AA -3' 5'-AGATGGTGGTCTGGCTGA A-3'	BC068268
Vimentin	h&mVim.1001F h&mVim.1216R	5'-TCCAAGTTTGCTGACCTCTCTG-3' 5'-GCGGCCAATAGTGTCTTGGT-3'	NP_035831
PDGFR α	hPDGFR α .212f hPDGFR α .474r	5'-CTTTCATTACCCTCTATCCTTCC-3' 5'-TAAATGTGCCTGCCTTC-3'	NM_006206.3

Table 1. Primer Pairs. The primer pair tested for gene expression within cell populations. Target gene is listed with primer name, following the forward (f) and reverse (r) oligonucleotide sequences prepared by IDT. The size of the expected band is listed with the Genbank Access for each gene.

e. PCR Oligodeoxynucleotides primers (Table 1) for polymerase chain reactions (PCR) were obtained through the UMDNJ-RWJMS DNA Core Facility and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). For PCR reactions, 100 ng cDNA was mixed with PCR Buffer (Invitrogen), 2.0 mM MgCl₂ (Invitrogen), 0.2mM dNTP, 0.2

μ M forward primer, 0.2 μ M reverse primer, and 1.25 U Taq DNA polymerase (Invitrogen). The samples were cycled through the following program: 94°C for 3 minutes, 55°C for 2 minutes, 72°C for 3 minutes using an Eppendorf Mastercycler Gradient (Eppendorf, Westbury, NY). This program was repeated thirty times to amplify the cDNA sample, and then heated to 72°C for 7 minutes and finally cooled to 4°C. Primer pairs included GAPDH (cytoskeletal), tubulin β 3 (ectoderm), α -feto protein (endoderm marker), nestin (ectoderm), neural filament (ectoderm), activin a (mesoderm), Sox2 (embryonic stem cells), and Oct3 (embryonic stem cells).

f. Gel Electrophoresis To visualize the PCR products, 10-20 μ L(20-40%) of the PCR product was mixed with 2 μ L 6X loading buffer (Manatis et al., 1982). The samples were size-separated by electrophoresis at ~80 V on a 1.2% agarose gel in Tris-Acetate buffer with 0.5 μ g/mL ethidium bromide. Results were visualized using an ultra violet light source, and photographs were taken using a Polaroid camera.

2.6. Histochemistry

Prior to adding antibodies, the samples were blocked with PBS containing 2% normal goat serum (Sigma) and incubated at room temperature for 30 minutes. For extracellular antigens, the slides or cover slips were rinsed three times with PBS; for intercellular antigens, the cells were permeabilized by rinsing the slides or cover slips three times with PBS containing 0.1% Triton-X100 (PBT). The primary antibodies were added to the cover slip or chamber slide sample at appropriate dilutions with PBS or PBT, and they were incubated for at least 100 minutes at room temperature. The

following primary antibody dilutions were used: SSEA-1 was prepared at 1:50 dilution; vimentin was prepared at a 1:25 dilution; PAX6 (paired box gene 6) was prepared at 1:25 dilution; GFAP (glial fibrillary acid protein) was prepared at 1:100 dilution. After incubation with the primary antibody, the cover slips or chamber slides were rinsed with PBS three times. The appropriate secondary antibodies (1:200 dilution) were added for at least 1 hour at room temperature. Finally, the cover slips or chamber slides were rinsed three times with PBS, then mounted on a glass slide with a small volume of Vectashield mounting media containing the nuclear dye DAPI (Vector Laboratories, Inc., Burlingame, CA). The slides were then visualized on epifluorescent microscope, and photographs were collected on a Zeiss LSM 510 META confocal microscope. Photographs were assembled using Adobe Photoshop version 8.0.

2.7. Animal Studies

To examine the ability of embryonic stem cells to form tumors *in vivo*, a teratoma assay was performed. Male Rag^{-/-} (immune deficient) mice were used as the host. Mice were anesthetized with an intraperitoneal injection (300 μ L) of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine (Sigma). Following a procedure conducted by Anderson et al. (1996) and Prokhorova et al. (2008), the right kidney was exposed through a lateral incision, and 1×10^6 cells were injected under the kidney capsule. The incision was closed, and the mouse was monitored for recovery from surgery and subsequent tumor formation.

3. RESULTS

3.1. Maximizing the efficiency of forming embryoid bodies from ESCs

Embryoid bodies (EBs) are formed by the aggregation of embryonic stem cells under non-adherent conditions in media without LIF, which promotes differentiation. To maximize the efficiency of forming EBs, G-Olig2 ESCs were collected, resuspended in media, and counted for the EB formation assay. As shown in Figure 4a, we adopted a nonmenclature for ES-derived EBs as EB0. EBs derived from subsequent steps in the ESC-to-oligodendrocyte pathway (Figure 3) are designated by their starting population (EB1, EB2, etc.).

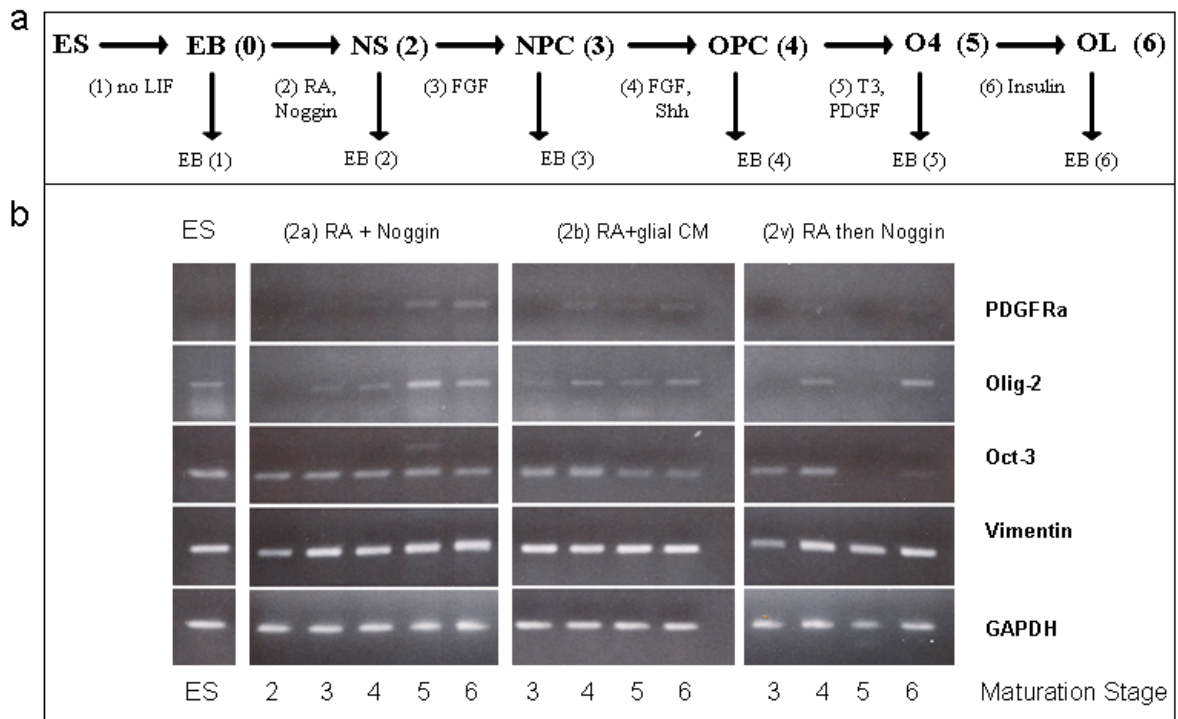
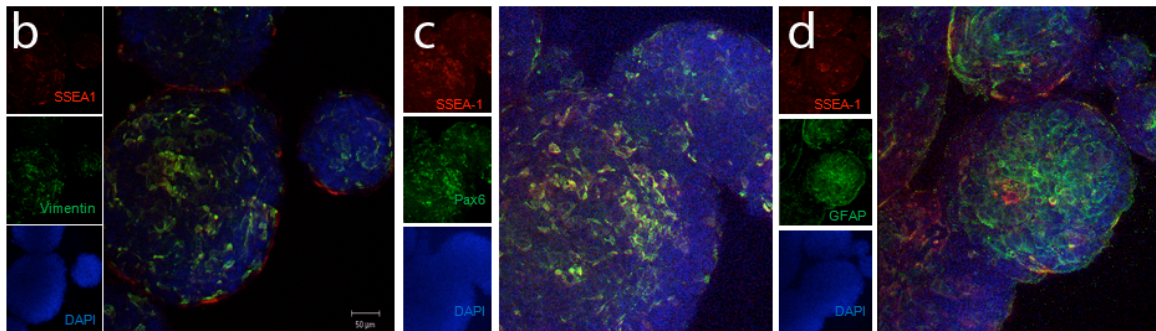
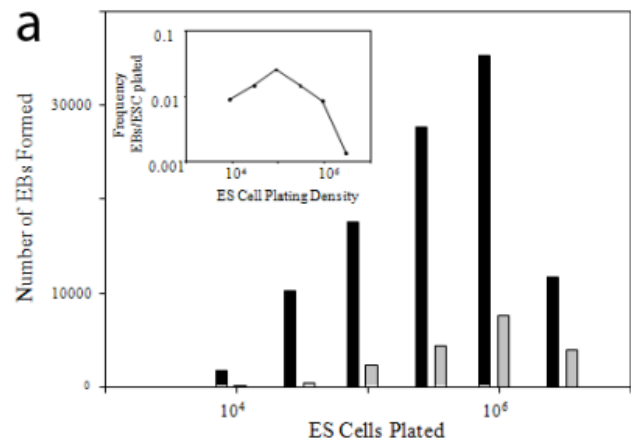


Figure 4: ESC -to- OLs in vitro. (a) Scheme for sequential enrichment of stem-derived OLs (Steps 1-6), and EB- potential at each stage defining nomenclature assigned to each EB population. (b) RT-PCR analysis of lineage marker expression in mESC-derived cell types. Lane numbers correspond to culture stage in panel A, and the culture conditions at step 2 are detailed above each group in panel B; glial CM, conditioned media from primary rat brain glial cultures.

Our first question was to determine if cell density affect the number of EBs that form. G-Olig2 cells were plated at densities starting from 9×10^3 cells/plate and increasing to 3×10^6 cells/plate. Cells were cultured with ES media with 15% FBS. Since the goal was to optimize the total number of EBs per ESCs plated, ESCs were cultured in petri plates rather than by hanging droplets, a method which allows for a single EB. EBs were transferred to a new petri dish after 24 hours in order to remove contaminating MEF cells and to prevent the EBs from attaching to the remaining MEF cells. An aliquot was removed from these plates at time intervals 48 hours and 96 hours, and EBs were counted under a 10X objective in phase contrast. As shown in Figure 5a, the number of EBs formed varied depending on the number of cells plated. The frequency of EB formation in these assays varied from $(14.4 \pm 3$ to $50.1 \pm 18) \times 10^{-3}$ (Table 2). The maximum number

Figure 5. EB0 formation and histochemical analysis of ESC-derived EBs. (a) ESC derived EB0 formation at T=48hrs (gray) and T=96hrs (black). The insert indicates frequency at T=96hr. ESC (SSEA1) and germ layer specific markers. EB0 were stained with SSEA-1 (b-d), Vimentin (b), GFAP (c), and Pax6 (d). It is noted that there is high expression of the differential markers and a weaker expression of SSEA-1.



of EBs formed at 96hrs was at a density of 1×10^6 ESCs plated. Therefore in subsequent experiments, G-Olig2 ESCs were plated at 1×10^6 cells/plate to achieve the maximum number of EBs. This would optimize the ability of ESCs to form EBs, and therefore increase our ability to identify cells with EB forming ability in other ES-derived differentiated cultures.

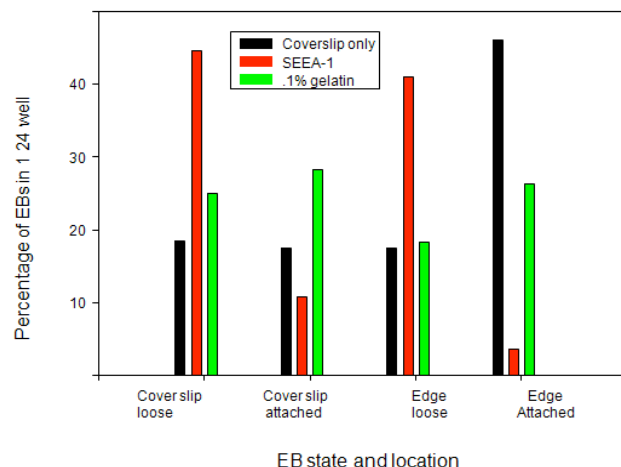
Table 2. Frequency of Embryoid Body Formation.

<u>Cell Population</u>	<u>EB Frequency ($\times 10^{-3}$)</u>
EB0*	14.4 ± 3 50.1 ± 18
EB1	5.6 ± 2
EB2	0.06 ± 0.01
EB3	5.3 ± 3
EB4	0.5 ± 0.2
EB5	3.071 ± 2
EB6	0.7 ± 0.2

The frequency for EB and EB-like formation was calculated by the number of EB/EB-like formed versus the number of cells plated. At EB0, two readings were taken, thus two frequencies were recorded.

In these ESC culture studies, the total number of EBs decrease from T=48hr to T=96hr at each plating density tested (Figure 5a). There are several possible reasons for a decrease in the number of EBs between the two time intervals. One possibility is the aggregation of smaller EBs, and consistent with this, we observed that EBs at T=96hr were considerably larger than at T=48hr. The size of the EBs cannot solely be due to cell division. We also observed a greater decrease in EB numbers at the higher cell plating condition, which is also consistent with aggregation (Figure 5a). A second possibility is that EBs counted at T=48hrs subsequently adhered to the bacterial dish, and these were not counted at T=96hrs. At the present time, we cannot exclude either potential explanation.

Figure 6. Adherence of EBs to various conditions. To optimize the number of EBs for subsequent fixing and staining, EBs were cultured on sterile 12 mm glass cover slips treated with 0.1 % gelatin, a 1:20 dilution of SSEA-1 antibody, or untreated on a 24 well plate for 2 hours at 37°C. The total number of EBs were counted and categories denoted the location of the EB. There was a greater percentage of EBs that attached to the cover slip with gelatin, and this method was used for all EBs.



To determine if the aggregate EB0 formed were indeed EBs, we used both histochemistry and RT-PCR analysis. EB samples on glass coverslips were fixed in paraformaldehyde according to the Methods Section. In order to optimize the staining of EBs on coverslips we first examined conditions for their adherence (Figure 6). We plated EBs in a 24 well dish containing cover slips which had been coated with either 0.1% gelatin, SSEA-1 antibody, or left untreated. In the untreated condition a majority of EBs adhered to the tissue culture plastic and only 15% of EBS were attached to the glass coverslip. In SSEA-1 treated conditions, almost all EBs were not attached. In contrast, 60% of EBs in the gelatin coated condition were attached, and many EBs adhered to the coverslips (Figure 5). For all subsequent staining assays using cover slips, the EBs were attached with 0.1% gelatin. This improved our recovery of EB-like bodies for histochemical analysis.

ESCs and EB0 were stained with primary antibody SSEA-1, a pluripotent stem cell marker. As illustrated in the Figure 5b-d, SSEA-1 expression was detected in the 48hr EB0 sample. In addition, 48hr EB0 were immunoreactive with vimentin, a cytoskeletal marker for mesoderm and parietal endoderm (Figure 5b). EBs were also positive for PAX6, a transcription factor expressed in ectoderm (Figure 5c), and GFAP, a cytoskeletal

filament protein expressed in ectoderm (Figure 5d). This analysis indicates that the aggregate bodies include differentiated cells from all three germ layers; therefore, these aggregates represent true EBs.

3.2. ESC differentiation

G-Olig2 cells were cultured under the Billon et al. (2002) protocol for oligodendrocyte differentiation. Figure 3 diagrams the progression of derived cell types in the oligodendrocyte differentiation pathway, which denotes six derived cell populations: EB, NS, NPC, OPC, O4, and OL. To monitor the maturation of OL lineage cells in this process, we used RT-PCR analysis of gene expression as performed by Mary Kiel from the McKinnon lab (Figure 4b). In these studies, the ESC marker Oct3 decreased and the OPC and OL markers Olig2 and PDGFR α increased from stages 2 through 6, as compared to the control GAPDH. At each stage of differentiation, a sample of the derived cells populations were brought to a single cell suspension, and we used our EB assay to determine whether the cell population could produce EBs. If EBs formed, this would indicate ESCs still persisted in the derived cell population.

Starting with ESCs, the resulting EBs were termed as EB0. The EB0 were dissociated into single cells and were allowed to aggregate. As indicated in Figure 7a, the dissociated EB0 were indeed able to re-aggregate into EB-like bodies (EB1), suggesting that pluripotent cells persisted within the EB0 bodies. As with EB0 formation, we observed more EBs formed at 48hrs than at 96hrs. Unlike EB0 formation, the frequency of EB1 was 5.6×10^{-3} (Table 2), or up to 10 fold less than EBs formed when we started with ESCs. This is consistent with our SSEA1 staining, which indicated that

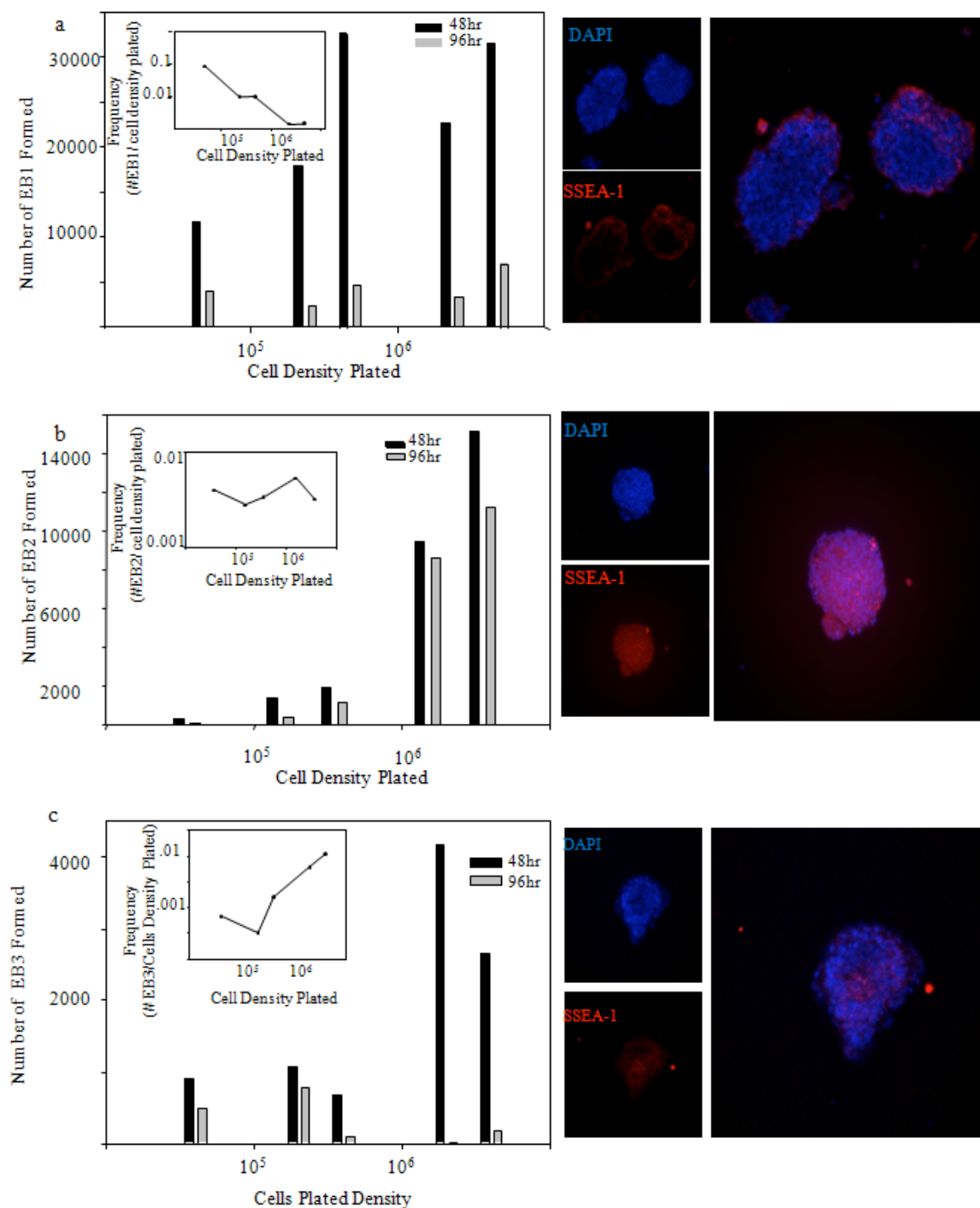


Figure 7. EB Formation and SSEA-1 expression. a-f. The ability for derived cell populations to form EB-like bodies were counted at T=48hrs and T=96hrs and stained with SSEA-1. The inserts represent the frequency of EB-like body formation. All EB-like bodies were stained with SSEA-1. The EB-like bodies were examined at 20X magnification.

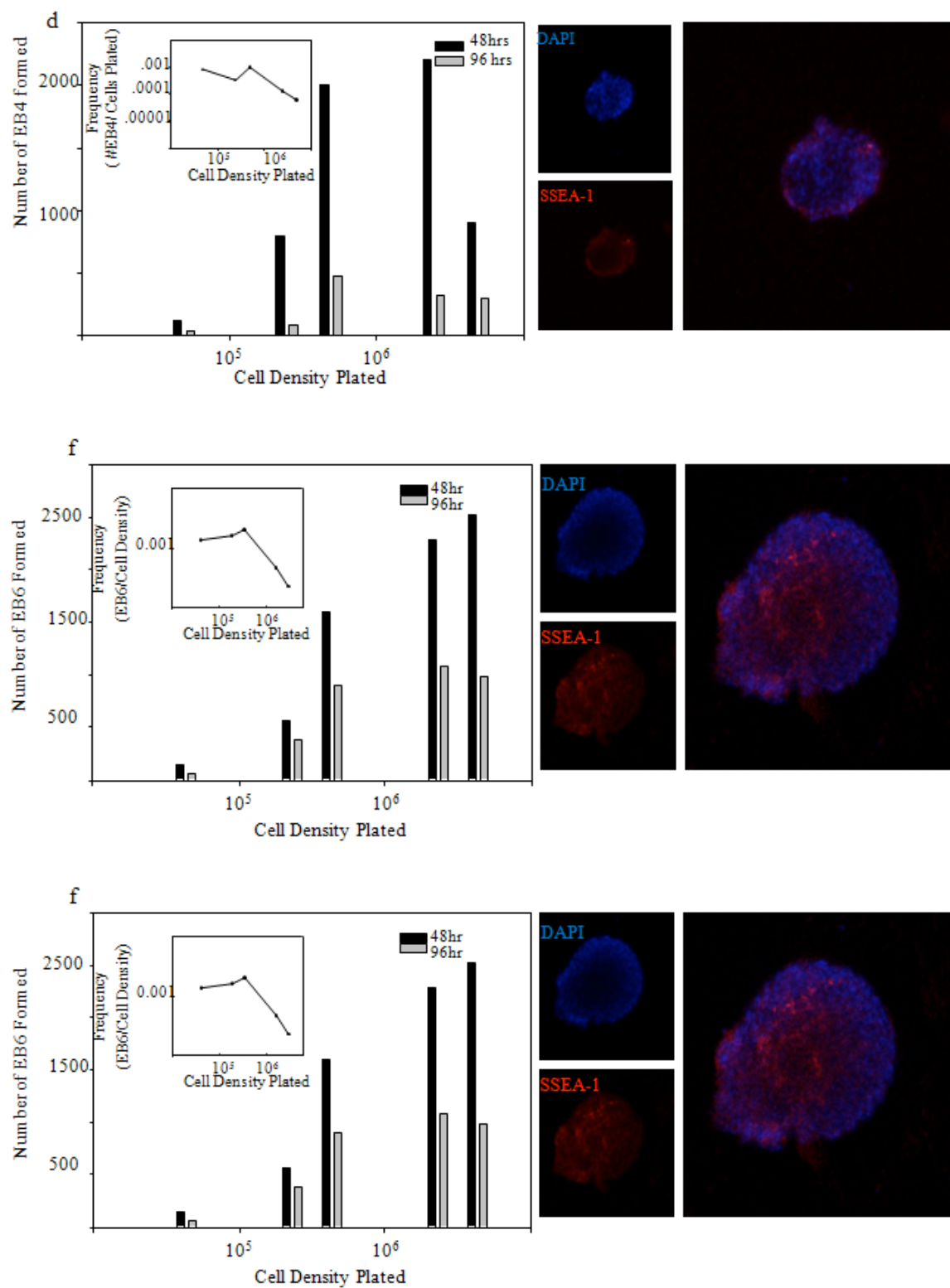


Figure 7 (cont'd).

some ESCs are present in EB0. Since there are fewer cells that have the potential to form EBs, there are less undifferentiated cells than in the EB0 cultures. However, EB0 cultures still retain the potential to form EB-like bodies.

In the oligodendrocyte differentiation pathway, NS cells are formed upon adding Noggin and RA. NS cells cultured on a monolayer were next dissociated into single cells and were allowed to aggregate. If EBs formed from this population, this would suggest that ESCs still persisted in the NS derived cell population. As indicated in Figure 7b, the dissociated NS cells were indeed able to aggregate into EB-like bodies (EB2), suggesting that pluripotent cells persisted in the NS cell culture. The greatest number of EB2 form at a cell population of about 2×10^6 cells/plate. EB2 formed at a frequency $0.06 \times 10^{-3} \pm 0.013$ (Table 2), or approximately 90 fold less than the frequency of EB1 formation. There was less certainty here on frequency, as there are several potential sources of error. One possibility is the counting the number of EB2s, and a second possibility is obtaining an aliquot with a composite of the total population. However, this decrease in the ability to form EBs indicates that as the ESC population became progressively more differentiated, there were fewer undifferentiated ESCs in the derived cell population.

The following stage in oligodendrocyte differentiation is NPCs formation, where NPCs formation is enhanced upon removing FBS and providing 50:50 DMEM-F12 and NBM media supplemented with FGF. NPCs cultures were dissociated into single cells and were allowed to aggregate. Again, if EBs form from this population, this would indicate ESCs remained in the NPC population. EB-like bodies that form from dissociated cells from NPC populations are defined as EB3. As indicated in Figure 7c, the dissociated NPCs were able to form EB3, with the greatest number of EB3 formed

at T=96hr in a density of 2×10^6 cells/plate. Similar to EB0, there are less EB-like bodies at T=96hrs than T=48hrs. We calculated that EB3s formed at a frequency $5.3 \times 10^{-3} \pm 3$ (Table 2), which is 90 fold greater than EB2 formation and is a frequency closer to EB1 formation. Again there are several sources for possible error, including counting the number of EB3s, and obtaining a representative composite of the total population.

Another possibility could be that these ESCs retain an ability to form EBs similar to the EB1 stage, and the EB2 stage frequency should be similar to EB1 and EB3 formation.

The next stage in oligodendrocyte differentiation is OPC formation, which is amplified by the addition of SHH to instruct gliogenesis (Chen et al., 2007). OPC cultures were dissociated into single cells and were allowed to aggregate. EB-like bodies from dissociated OPC populations were defined as EB4. As shown in Figure 7d, EB4 do form at T=48hr and T=96hr, where the greatest number of EBs form at a cell density of 5×10^5 cells/plate at T=96hr. Similar to EB0, there were more EB-like bodies forming at T=48hrs than T=96hrs. EB4 formed at a frequency $0.46 \times 10^{-3} \pm 0.18$ (Table 2), which is about 10 fold less than EB3 formation. Again this was consistent with the general result of fewer cells in this derived cell population that have the ability to form EB-like bodies.

Continuing along the oligodendrocyte differentiation pathway, the 50:50 DMEM-F12 and NBM was supplemented with PDGF and T3 to promote O4 maturation. To test whether EBs form from this derived cell population, O4 cells were dissociated and were allowed to aggregate. EB-like bodies that form from dissociated cells from O4 stage cells were defined as EB5. Figure 7e illustrates the formation of EB5 at T=48hr and T=96hr, thus showing the similar aggregation pattern to EB0. At T=96hrs, EB-like bodies form at a density of 2×10^6 cells/plate. EB5 formed at a frequency $3.01 \times 10^{-3} \pm 1.8$

(Table 2), which is roughly equivalent to EB4. This suggests that these cultures retained a significant number of undifferentiated ESCs, thus had the ability to form EBs.

Finally, O4 cells were induced to differentiate into oligodendrocytes. OL cultures were then dissociated into single cells and allowed to aggregate. EB-like bodies that form from dissociated OL stage cells were defined as EB6. As indicated in Figure 7f, EB6 indeed form. Similar to EB0 and the other EB-like bodies, more EB-like bodies formed at T=48hrs versus T=96hrs. Also the greatest number of EBs form at a cell density of 2×10^6 cells/plate. EB6 formed at a frequency of $0.69 \times 10^{-3} \pm 0.18$ (Table 2), which is about 4 fold less than EB5. This again suggests that OL cultures had fewer cells that could generate EB-like bodies, and therefore, fewer ESCs within the heterogeneous cell population. However, within the derived cell population there were cells that still had the ability to form EB-like bodies.

Results from these EB-like forming assays, these results indicate that at all stages of differentiation these cultures have the potential to produce EB-like bodies. This reflects the heterogeneous nature of these cell populations. It is also important to note the decreased potential for EB-like bodies in later derived cell populations as compared to early differentiation stages. Overall, cells need to be plated at least 2×10^6 cell/plate in order to form EB-like bodies in derived cell populations, and the EB frequency dropped from 14×10^{-3} (ESCs) to 7×10^{-4} , or 20-fold.

3.3. EB formation from ESC-derived cultures

To examine the characteristics of the EB-like bodies, histochemistry and RT-PCR analysis were performed. EB-like bodies derived from stages 1 through 6 were stained with SSEA-1 to examine whether pluripotent cells existed within the derived cell population. Figure 7a-f illustrates that SSEA-1 is expressed in these bodies, thus showing that there are pluripotent cells persisting through the differentiation process. A sample of 48hr EB2, derived from the NS cell population, was further examined using antibodies to vimentin, GFAP, and PAX6. As with EB0, these EBs were immune reactive for all markers examined (data not shown).

EB-like bodies were also examined for transcript expression using cDNA analysis using Reverse Transcriptase followed by Polymerase Chain Reaction (RT-PCR, Figure 8). RNA was extracted from samples using Trizol reagent and then purified with Promega RNeasy Kit with DNase I RNase Free to rid genomic DNA. Several primer pairs were used in this analysis (Table 1) including primers that specifically amplify GAPDH (cytoskeletal), tubulin β 3 (ectoderm), α -fetal protein (endoderm marker), nestin (ectoderm), activin a (mesoderm), the oligodendrocyte lineage transcription factor (Olig2), Sox2 (stem cell), and Oct3/4 (stem cell). The left panel of Figure 8 examines the lineage gene expression from ES to oligodendrocytes. The ESC markers Sox2 and Oct3/4 were strongly expressed in early stages (ESC and EB0), while in more differentiated cell populations (NS-OL), differentiation-specific markers were present. This recapitulates the expression pattern from Figure 4b. All expression patterns were normalized to the GAPDH expression.

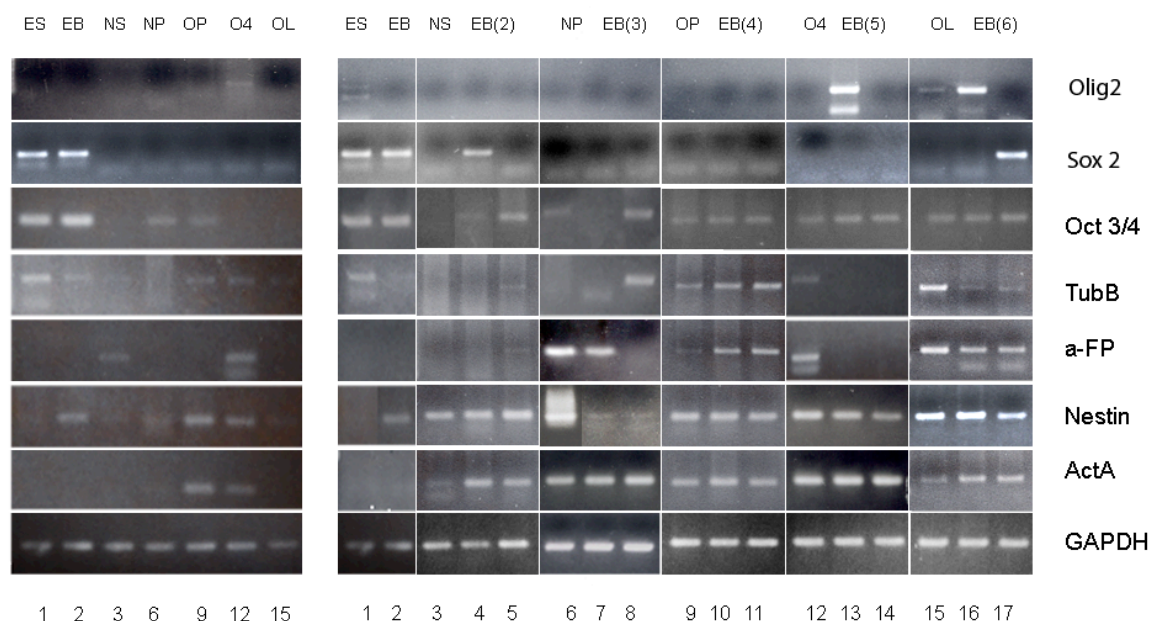


Figure 8. RT-PCR analysis. Amplification of cDNA from cultures of various stages of OL differentiation (ES through OL, left panel), and embryoid bodies derived from each of these stages (right panel). Each sample was amplified with the PCR primer pairs as indicated on the right. Lanes 1-3,6,9,12,15: ES-to-OL cultures. Lanes 4,5: 48hr, 96hr EB2. Lanes 4,5: 48hr, 96hr EB2. Lanes 7,8: 48hr, 96hr EB3. Lanes 10,11: 48hr, 96hr EB4. Lanes 13,14: 48hr, 96hr EB5. Lanes 16,17: 48hr, 96hr EB6. GAPDH, housekeeping control to verify the integrity of the cDNA.

The right panel of Figure 8 demonstrates the expression of these markers in EB-like bodies derived from the differentiated populations. Though there is no expression of Sox2 and Oct3/4 in the NS stage, Sox2 re-appears in EB2 T=48hrs and again in EB6 T=96hrs. Similarly, Oct3/4 also re-appears in the EB2 T=96hrs. In addition, Oct3/4 is also known to be expressed in OPC populations (Figure 4b) as well as the derived EBs from these stages. There are several possibilities for the re-appearance of these early undifferentiated markers. Since each RNA extraction was derived from a separate cell culture, it is possible that stem cells remained in each individual culture at these differentiation stages. Thus our cell culture conditions could have allowed this ESC population to expand during the EB formation. As for Oct3/4 expression within the OPC to OL stages and in their respective EBs, it is thought that once there is expression in the

starting population, the cells expressing Oct3/4 will continue to express Oct3/4 in the derived EB population.

Tubulin β 3 (Tub β 3) and nestin are neuronal differentiation markers, and both are progressively expressed in more differentiated populations. The right panel of Figure 8 indicates Tub β 3 is strongly expressed in OPC cultures and its derived EB3. Similarly, OL staged cells exhibit this same pattern, yet expression decreased in the derived population. O4 cultures include cells which do express Tub β 3, but the EB5s do not express Tub β 3. Perhaps these culture conditions do not allow for expression of Tub β 3 within the EB5s, since even in the EB6 populations, the Tub β 3 expression decreases, whereas nestin is expressed starting in EB0 population, and it is continually expressed throughout the stages and the derived EBs. It appears that once this gene is activated towards a neural lineage, cells within these cultures continue to express this gene.

Other cell type markers were also examined. Activin A, a marker for mesoderm induction was continually expressed in cell stages NS to OL including the derived EBs (Figure 8, right panel). Like Nestin, it appears that once the gene is active the cell populations continue to express the gene. It is important to note that although the cells were differentiated towards a neural lineage, there were mesoderm type cells in the populations. Similarly, Alpha-feto protein (AFP) expression, an endoderm marker, indicated there were endoderm type cells present in these cultures. Expression of AFP appeared to oscillate. There was strong expression in NPC population and the signal remained in the EB3 T=48hr. However, there was no expression in the EB3 T=96hr. There was weak expression with the OPC population, but then remained strong within the EB4 populations. In the O4 stage cells, there was strong expression of AFP, but at later

stages there was a varied expression pattern. Lastly, the OL staged cells and EB6 had strong AFP expression, indicating that the composition of cells within the cultures varied greatly. In Figure 8, the left panel demonstrates that there was AFP expression only in NS and O4 stage cells, whereas in the right panel, there is a greater range of expression. This indicates that the differentiated populations had varying numbers of cells expressing this endoderm marker.

In total, these results demonstrate that the process of ESC differentiation as detailed in Figures 3 and 4a does not ensure that all ESCs in the starting population enter differentiation. In addition, these results indicate that these EB-like bodies, are truly EBs since the differentiated populations contained gene expression of all three tissue types. The persistence of EB-capable ESCs in these cultures is an important observation because it signals the absolute need to cell sort the final OL population before these cells are safe for cell transplant and myelin therapy.

4. DISCUSSION

ESC-derived oligodendrocytes can provide a possible cell-based therapy for demyelinating diseases. However, we must first devise a protocol for the efficient differentiation of OL-lineage cells from ESC populations *in vitro*. In this thesis I used an established protocol for ESC-to-OL differentiation *in vitro*. The cell populations were examined through histochemical and RT-PCR analysis, and the results indicated that the ESC population does progress to neuronal lineages. The derived cell population also displayed endoderm and mesoderm expression patterns as well. This shows that other cell types persisted in culture, thus a potentially harmful factor in a cell based therapy. Thus the EB assay displayed that ESCs remained in the heterogeneous cell population. Throughout the differentiation protocol, populations of derived cells were examined for their ability to form embryoid bodies, an *in vitro* assay for teratomas. We found that these populations of differentiated cells could form EBs. This suggests that ESCs remained within the ESC-derived population. The frequency of EBs formation decreased as the cell populations became more differentiated, indicating that the populations retained less ESCs as differentiation continued. However, the possibility of forming teratomas *in vivo* remains a major concern for ESC based therapy.

The discovery of pluripotential ESCs arose from research studying teratomas and teratocarcinomas. The embryonal carcinoma (EC) cells are thought to be the malignant counterpart to ESCs in that they possess the potential to differentiate to all three germ layers. In addition, EC cells can contribute to the developing embryo to produce chimeras; however, they do not contribute to the germ-line cells due to their genetic

aberrations. There is debate whether the EC cells retain their malignant characteristics once formed in the chimera (Papaioannou and Rossant, 1983; Oosterhis and Looijenga, 2005). Unlike ESCs, EC cells can differentiate into extraembryonic cell types found in the placenta and yolk sac. Early teratoma and teratocarcinoma studies used transplanted blastocytes underneath the kidney capsules of mice and rat. Under histological examination, tumors were formed and contained differentiated tissues as well as undifferentiated stem cells. In addition, the stem cell derived tumors did not undergo malignant transformation, where their morphology and genotype were unchanged (Damjanov, 2005). These studies lead to the establishment of ES cell line *in vitro*. Evans and Kaufman (1981) obtained mouse blastocysts and removed the inner cell mass to culture *in vitro*. The ESCs had the potential to form embryoid bodies and differentiate into other cell types as well as self renew. Also, the cell line was injected into mice and teratocarcinomas formed. In addition, these cells were able to contribute to the developing embryo to form chimeras. To separate these cells from the EC cell line, karyotyping revealed that the ESCs retained their normal karyotype; thus, an ES cell line was prepared *in vitro*. ESCs are closely related to teratocarcinomas, and it is important to note their tumorigenic potential. Werbowetski-Ogilvie et al. (2009) examined the neoplastic progression of the variant cell lines derived from the human stem cell line, H9. Through FACS analysis, morphology examination, tumor formation, and aCGH analysis, the variant H9 cell lines had increased tumorigenic ability.

Differentiation protocols have outlined procedures to derive a favored cell type; however, studies are still required to maximize the efficiency of this process. The Billon

et al. (2002) protocol had outlined a five-step procedure, where growth factors are given at specific time points, allowing for oligodendrocyte differentiation in approximately two and half weeks. Currently, we have devised a six-step oligodendrocyte differentiation protocol based on Billon et al. (2002). Unlike the Billon protocol, we added Noggin (Smith et al. 1993; Izael et al., 2006) in our first stage to promote an environment similar to development of neural ectoderm, and currently, our protocol allows us to produce oligodendrocytes within a two-week time frame. The main goal of any differentiation protocol should be to optimize the percentage of desired tissue type as well as reduce the time frame to produce the product, and to prepare a homogeneous population. There are several components that still require examination to maximize the efficiency of forming ESC-derived oligodendrocytes. The first step in these procedures involves EB formation, and providing the optimum number of EBs allows for a sufficient population of cells that can be used for further differentiation. We found that 1×10^6 ESCs achieved the highest frequency of EBs formed; thus, this density was used to perform subsequent differentiation studies.

A study conducted by Dihné et al. (2006) generated a mouse EGFP⁺ ESC line and generated immature post-mitotic neurons using conditions similar to the Billon et al. (2002) procedure. They cultured mESCs using the same conditions and altered the duration of exposure time to the factors by increasing the number of days the cultures remained in a given condition, the longest duration being eighteen days. In their *in vivo* studies, mice were given lesion in their brains with stereotaxic injection of 1 μ L of 60 nmol of quinolinic acid three days prior to transplantation. ESC-derived cell populations

were injected into the trauma area at a density of 100, 000 cells/ μ L. It was noted that the neural precursor cells that remained in the *in vitro* conditions the longest produced no tumors, whereas traditionally cultured cells produced tumors within one month after transplantation. This study indicates that a prolonged culturing time may be more suitable in order to avoid teratoma formation. This is similar to our current study, where the frequency of EB-like body formation decreased as cell populations became more differentiated.

EB-like masses formed at each stage of differentiation; however, the frequency of masses formed decreased as cell populations became more differentiated. Nonetheless, the cell populations still had the ability to form EB-like bodies. The expression of ActA, AFP, nestin and Tub β 3 in these EB-like bodies indicates the presence of all three tissue germ layers (neural ectoderm, mesoderm, and endoderm) , thus showing that these EB-like bodies are true EBs. Moreover, this distinguishes these bodies as EBS and not neural spheres, since they express germ layer markers other than neural. In addition, these populations of cells also expressed ESC markers such as Oct3/4 and Sox2. Gene expression analysis indicated that Oct3/4 is expressed in all stages of differentiation and in EB-like bodies (Figure 9). Although Oct3/4 has been known to be expressed in OL staged cells, histochemistry results indicated that the pluripotential ESC cell marker SSEA-1 is also expressed in these EB-like bodies (Figure 7a-e).

Multipotent stem cell treatments have been implemented in Parkinson's Disease (PD) patients. A study conducted by Lindvall et al. (1990) obtained tissue from eight week old

aborted fetuses, which contain the unique population of immature dopamine neurons. Mesencephalic tissue was then transplanted in the anterior, middle, and posterior part of the left putamen of PD patients, who underwent immunosuppression in order to prevent rejection of the tissue. Within two months after the procedure, the patient had increased mobility and less rigidity in movement; thus the study indicated a potential therapy for the loss of dopamine neuron PD patients. However in a double-blind patient trial, thirty-four patients with advanced PD were studied against a placebo group. One-third of the patients received transplant tissue from one donor, the second third of the patients received tissue from four donors, and the final third of the patient group was the placebo group. The patients with the transplant initially had improvement in motor functions; yet, after six months period when immunosuppression drugs were stopped, there was an increase in PD symptoms, consistent with graft rejection. The PD patients were studied for two years and there was no significant improvement in PD symptoms (Freed et al., 2001; Olanow et al., 2003). One downfall in using allografts (non-self) of multipotent neural stem cells is immune rejection. Thus, ESC therapy could only be a viable alternative if the ESC lines used were closely matched to the histocompatibility loci of the graft recipients. Alternatively, it may be possible to derive autologous (patient-specific) ESC lines using the newly emerging field of induced pluripotent stem (iPS) cell reverse engineering.

Despite the possible complications with ESC derived tissues, there can still be a future for a cell-based therapy. A study by Windrem et al. (2007) examined the remyelination and rescue of the hypomyelinated Shiverer mouse. The mice had been

mated with Rag2^{-/-} mice to produce an immune-compromised mouse population, which was then injected with human glial progenitor cells at birth. The mice achieved a substantial recovery of normal neurological phenotype, and upon dissection, the mice exhibited high-efficiency of remyelination, as compared to the untreated mice, which died within five months after birth. Kiel et al. (2008) conducted a cell replacement therapy by injecting G-Olig2 ESCs into 3.5 day blastocysts homozygous for the Shiverer (MBP-null) mutation. The blastocysts were then implanted into pseudopregnant recipients and offspring were genotyped. The extent of chimerism was analyzed through Q-PCR amplification of the wild-type region of the myelin basic protein (*Mbp*) intron 1. Their results indicated that chimeras expressed a range of G-Olig2 derived (wild-type MBP) cells, whereas control mice did not express *Mbp*. In addition, G-Olig2-derived MBP expression was examined using RT-PCR and histochemistry, and their results demonstrated that G-Olig2 ESCs were able to generate OLs and form myelin within the chimeric animals. Through motor function analysis, the chimera mice displayed improved function depending on the percent of chimerism with significant improvements in animals with greater than 7% wild-type (ES-derived) cells. In addition, the age of death of animals correlated with G-Olig2 chimerism, and one animal was completely rescued by the ESC therapy (Kiel et al., 2008).

Such pre-clinical studies using mouse model systems have indicated the potential benefits of an ESC based therapy, and possible solutions should be considered to make safer ESCs. One possibility is the use of genetically engineered ESCs. Billon et al. (2002) also devised a system where they could select against ESCs via a *hygromycin*-

thymidine-kinase fusion gene in the *Oct4* locus upon exposure to Gancyclovir. This would remove surviving ESCs in the differentiated population, since Oct4 is expressed in ESCs and not differentiated cells. They then selected for neuroepithelial cells via an introduced *β geo* gene in the *Sox2* locus. When cultures were exposed to G418, any non-neural cells in which the *Sox2* locus is not expressed would be removed. This study provides a means to remove of unwanted ESCs in derived cultures and also promotes a specific lineage. ESC-derived oligodendrocytes can provide a possible cell-based therapy for demyelinating diseases, and genetically engineered ESCs might be a potential solution. It will be of interest to examine whether EB-like bodies form in OL cultures derived from such genetically engineered ESCs. If such engineered cells do not produce EBs, this would overcome the tumorigenic hurdle and bring ESC-derived cells closer to clinical use.

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