

Engineering Embryonic Stem Cells for Myelin Cell Therapy

by

Dorota Sadowski

A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements for the degree of

Master of Science

Graduate program in Physiology and Integrative Biology

written under the direction of

Randall McKinnon, Ph.D.

and approved by

New Brunswick, New Jersey

May, 2009

ABSTRACT OF THE THESIS

Engineering embryonic stem cells for myelin cell therapy

by

Dorota Sadowski

Thesis Director:

Randall D. McKinnon

Cell replenishment using stem cell-derived transplants holds great promise for therapy in human disease. However, clinical therapeutics in the central nervous system (CNS) requires targeted migration for lesion repair. For CNS grafts, homing the graft population to the appropriate site for tissue repair is non trivial since surgical access to wound is not risk free. Our objective is to modify stem cells to generate lineage specified CNS glia that ‘home’ into lesions, focusing on myelin repair for multiple sclerosis and spinal cord injury. Myelin protects neuronal axons, and in pre-clinical models ES-derived OL progenitor cell (OPC) grafts can regenerate myelin forming oligodendrocytes (OLs) and rescue the phenotype of myelin-defective mice. Our studies focus on engineering ES cells to express PDGFR α , the trans-membrane receptor for Platelet-Derived Growth Factor, which directs OPC migration during brain development. Prior work identified a ‘rheostat’ function for PDGFR α , with low levels of PDGF activating PI3K to initiate migration and high levels activating PLC γ to inhibit migration. This predicts that PLC γ -uncoupled receptors would direct ES-derived OPCs toward PDGF infused brain lesions.

We addressed the rheostat model by engineering mouse ES cells to express wild-type and signal transduction mutant versions of PDGFR α . First we generated a medium cocktail named '2i5S' that allows the the long-term expansion of mouse ES cell lines, which is optimal for our engineering experiments. Next we introduced PDGFR α transgenes into mES cells *in vitro*. A very low number of stable ES transformants were obtained after transfection, and this directed us towards using a set of vectors (TRE.PDGFR α) with tetracycline inducible promoter for control of transgene expression. Lastly, we used myelin mutant shiverer mice to evaluate the potential of ES-derived OPCs to generate myelin *in vivo*. Our results showed substantial improvements in both motor function and longevity in animals with 7% of ES^{wt} derived cells present in the pup. Therefore this suggests that 7% ES-derived stem cell engraftment is the minimal level that is required for functional cell replacement therapy.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the members of my committee: Randall McKinnon, Yufang Shi and Zui Pan for their generous donation of time and guidance in the pursuit of my master's degree.

I would also like to specifically thank the following individuals: Randall McKinnon for his mentorship, guidance, and inspiration throughout my graduate studies; Mary Kiel MS for her friendship, inspiration, guidance, and generous donation of time; Cui- Ping, Marisa Apicella for their friendship, support and collaboration on the study.

Finally, I wish to thank my family and friends for patience, encouragement, undying support, and love. Without these people, I can definitely state that none of this would be possible. Thank you.

TABLE OF CONTENTS

| | |
|--|-----|
| ABSTRACT | ii |
| ACKNOWLEDGEMENTS | iv |
| LIST OF TABLES | vi |
| LIST OF FIGURES | vii |
| 1. INTRODUCTION | |
| 1.1 Background..... | 1 |
| 2. MATERIALS AND METHODS | |
| 2.1 Materials..... | 8 |
| 2.2 Cell Culture..... | 8 |
| 2.3 Embryonic Stem cell differentiation in vitro..... | 10 |
| 2.4 Antibodies..... | 11 |
| 2.5 Immunocytochemistry..... | 12 |
| 2.6 Recombinant DNA..... | 13 |
| 2.7 Embryonic Stem Cell transfection..... | 15 |
| 2.8 Total RNA extraction..... | 16 |
| 2.9 RT-PCR (Reverse Transcription-Polymerase Chain Reaction)..... | 17 |
| 3. RESULTS | |
| 3.1 Optimizing mouse ES cell culture conditions..... | 19 |
| 3.2 Optimizing mouse ES to OL differentiation in vitro..... | 22 |
| 3.3 DNA transfection of mES cell in vitro..... | 26 |
| 3.4 Inducible (Tet-ON) gene expression in mES cells in vitro..... | 29 |
| 3.5 ES cell therapy: rescue of Shiverer mice with mES cells in vivo..... | 31 |

| | |
|--------------------|----|
| 4. DISCUSSION..... | 33 |
| 5. REFERENCES..... | 39 |

LIST OF TABLES

| Table | Title | Page |
|-------|--|------|
| 1 | Table 1. List of recombinant plasmid vectors used..... | 14 |
| 2 | PCR Primers for RT-PCR..... | 18 |
| 3 | ES (7ac5) stable transformants (G418 ^r)..... | 27 |

LIST OF FIGURES

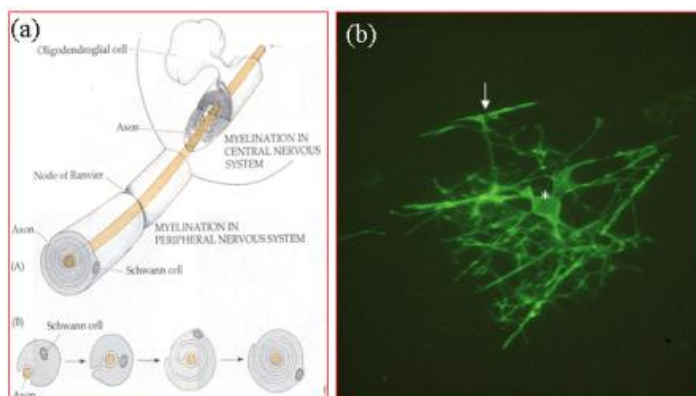
| Figure | Title | Page |
|--------|--|------|
| 1 | Myelin forming oligodendrocyte..... | 1 |
| 2 | Mouse embryonic stem (ES) cells..... | 3 |
| 3 | OLs from pluripotent stem cell in vitro..... | 4 |
| 4 | The “RTK Rheostat”..... | 6 |
| 5 | Template for mES cell culture conditions..... | 19 |
| 6 | 2i5S media for ES cell self-renewal..... | 20 |
| 7 | ES cell self-renewal in minimal sera conditions..... | 21 |
| 8 | ES cell to OL Differentiation..... | 23 |
| 9 | Histochemical analysis of mES-derived cell..... | 24 |
| 10 | mouse ES cell-to-OPC/OL differentiation in vitro..... | 24 |
| 11 | Transient eGFP expression in G-Olig2 ES cells..... | 26 |
| 12 | Stable transgene expression in 7ac5 ES cells..... | 29 |
| 13 | Luciferase (Luc): tetracycline inducible (Tet-ON) expression.. | 29 |
| 14 | TRE- PDGFR α : Cre-mediated recombination..... | 30 |
| 15 | ES cell rescue of Shiverer mutant mice..... | 31 |
| 16 | Survival of MBP ^{shi} : G ^{OLig2} ^{GFP} chimeras..... | 32 |

1. INTRODUCTION

1.1 Background

The brain contains three major classes of glia cells; microglia, astrocytes and oligodendrocytes. Oligodendrocytes (OL) produce and maintain myelin sheaths on neuronal axons (Fig.1) to facilitate proper conduction of action potential and to support the survival of these axons (Chen et al., 2007). OLs have the highest metabolic rate of any cell in the brain, and it has been estimated that they can create up to three times their weight in myelin membrane per day and eventually support membrane up to 100-times the weight of the cell (Connor and Menzies, 1996).

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.



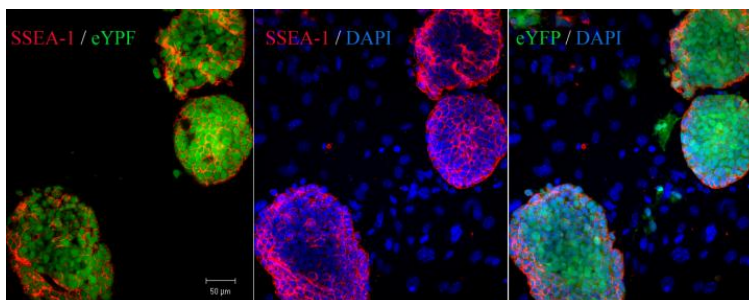
OLs originate from multipotent neuroepithelial stem cells in the central nervous system (CNS), which first generate proliferating OL precursors cells (OPCs). During embryogenesis, OPCs originate at specific loci of the neural tube and migrate out to populate all regions of the CNS. Recent studies in mice have shown that at embryonic day 12 the first OPCs start to appear in the ventral domain and then migrate into the lateral and dorsal part of the developing spinal cord. The OPCs found in the developing

thalamus, hypothalamus and cerebellum are generated by the diencephalon region of the forebrain (Battiste et al., 2007; Cai et al., 2005; Vallstedt et al., 2005).

Spinal cord injuries (SCI), cause damage to white matter which is composed of myelinated fiber tracts also known as axons that carry signals to and from the brain. It also causes damage to the gray matter causing segmental losses of motor neurons and interneurons (Filbin, 2003). The myelin sheath is very important in proper neurological functioning in the central nervous system (Bauman and Kasper, 2004; Blight, 2002). One of the neurological disorders called multiple sclerosis (MS) is caused by disrupted myelin, which is being attacked by patient's own immune system. In the MS patients, the disrupted myelin affects the ability of nerve cells in the brain and spinal cord to communicate with each other. Thus for both SCI and MS, there is an urgent need to find an approach to induce regeneration of dysmyelinated axons, since spontaneous remyelination after the trauma is insufficient to promote significant or stable recovery (Frohman et al., 2006).

There is evidence that suggests that failure to repair myelin after SCI is due in part to inhibitory molecules secreted by myelin. The normal role of these inhibitors is thought to prevent axons from extending new branches and making inappropriate connections. Another reason thought to inhibit axon regeneration after trauma is due to inflammatory response that promotes glial scarring and sends out chemical signals that block axonal growth. There is an urgent need for new approaches for repair and regeneration. One of the emerging strategies is to transplant myelin forming cells to promote myelin repair of intact axons which have lost their myelin sheaths.

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



Embryonic stem (ES) cells represent an unlimited source of pluripotent cells (Fig. 2), generated from the inner cell mass of a mouse blastocyst (Bradley et al., 1984; Evans and Kaufman, 1981; Martin, 1981). These cells can potentially generate all three germ layers in culture and, if injected into a blastocyst, they incorporate and participate in organogenesis and have potential to produce a chimeric animal. ES cell lines have been routinely derived from mice since 1981. In vitro studies demonstrated the ability of these cells to propagate indefinitely in cultures with “feeder layers” of mitotically inactivated mouse embryonic fibroblasts (MEFs) in the presence of serum and Leukemia Inhibitory Factor (LIF) (Evans and Kaufman, 1981; Smith et al., 1988). They are also able to differentiate into many different cell types. For example, following treatment with retinoic acid and specific growth factors (Fig. 3), ES cells can be differentiated into neural lineage including OPCs and mature oligodendrocytes (Billon et al., 2002; Brustle et al., 1999; Glaser et al., 2005). Recent breakthroughs in induced pluripotent stem (iPS) cell technology has attracted much attention, due to their potential to advance human stem cells research. iPS cell have the potential to generate patient-specific ES cells for therapy, while no longer invoking ethical issues. Patient-specific (autologous) iPS cells may improve the quality of therapeutic approaches for cell transplantation by eliminating one of the biggest concerns, immune rejection.

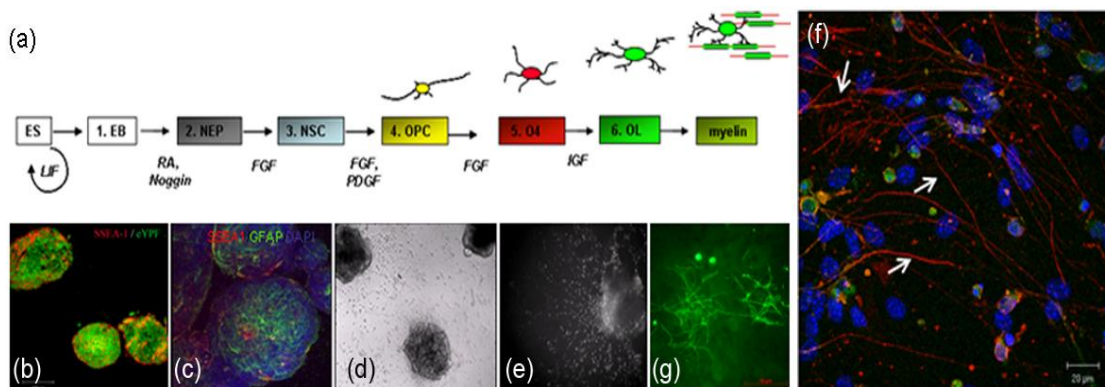


Figure 3: OLs from pluripotent stem cells in vitro. (a) ES cells can sequentially generate EB (embryoid bodies) NS (neural stem) cells and OL progenitors (OPCs) via factors that promote neural induction (FGF induced BMP antagonists) then neural tube ventralization (Shh). OPC maturation to O4 progenitors and postmitotic OLs is driven by PDGF and inhibited by FGF. (b-f) Morphology and marker expression in ES cell-derived cultures *in vitro*. (b) mES cell (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⁺).

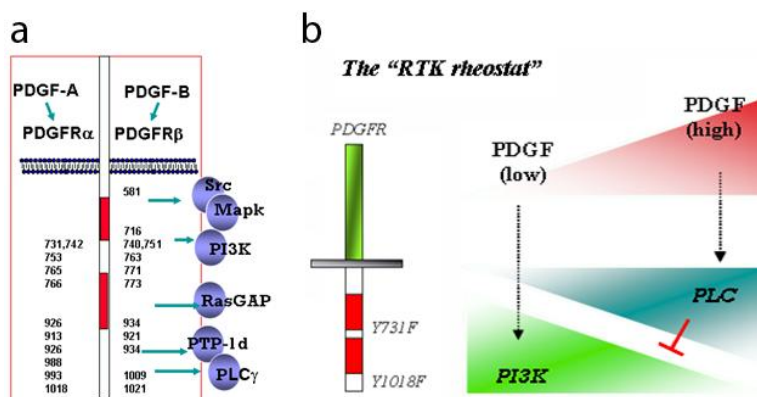
There are other significant issues that could limit the efficacy of therapeutic cell transplantation. For example, transplant approaches for the brain or spinal cord repair also need to concentrate on instructing ES cell differentiation with specific signals prior to grafting, and on directing ES-derived cells to reach their appropriate targets *in vivo*. Our studies have focused on generating OPCs from mouse ES cell lines (Fig. 3). It is crucial to have the ability to instruct lineage differentiation prior to grafting to facilitate any repair of demyelinated areas in MS or SCI patients. One of the first protocols to use mESCs to derive OLs was established by Brustle's group in 1999. *In vitro* experiments involved an initial differentiation step to neural progenitors in the presence of mitogens such as FGF2 and EGF (Brustle et al., 1999). After expansion of the cultures, further differentiation was first instructed by the ventralizing factor Shh, then OPCs were amplified in the presence of FGF2 and PDGF to yield bipotential glial progenitors. These cultures were then used for transplant purposes in animal models of demyelinating diseases (Brustle et al., 1999; Lowell et al., 2006; Ying et al., 2003).

The migration pattern of OPCs is not a random event. The progenitors follow the precise routes of migration characteristic of each CNS region. This suggests they respond to specific molecules or combinations of molecular cues and several extracellular soluble signals are already known. One of the cues that influence OPCs migration is netrin-1, a laminin-related glycoprotein expressed by the ventral midline of the developing CNS. It was shown that netrin binds to its transmembrane receptor Unc40 and Unc5 and mediates dorsal-ventral neuronal migration and axon guidance (Fruttiger et al., 1999). Studies in the rodent optic nerve also demonstrated that netrin acts as a repulsive cue in the migration of the OPCs. Several studies suggested that netrin can either attract or repel OPCs. How OPCs switch their response is a question that needs to be answered.

A second key regulator of OPC migration is platelet-derived growth factor (PDGF). The PDGF trans-membrane receptor PDGFR α was found to be specifically expressed in rodent and human OPCs, and PDGF promotes OPC migration and proliferation during development (Armstrong et al., 1990; McKinnon et al., 2005; Noble et al., 1988). Early experiments using PDGF-knock-out mice showed a severe dysmyelination phenotype in all parts of the brain, most evident in the cerebellum, but also in the optic nerves of the animal (Fruttiger et al., 1999). Previous studies also revealed PDGFR α expressing OPCs in normal white matter of CNS, but importantly not within areas of inflammatory demyelination (Franklin and Blakemore, 1997). These OPCs appear to have a migration defect in the adult brain. These results also suggest that by modifying grafted OPCs to express PDGFR α we can direct the migration of OPCs and grafted cells may become a potential source of remyelinating population of cells in vitro.

Figure 4. The “RTK-Rheostat” (a) RTK signaling; schematic of PDGFR α and PDGFR β s. Ligand induced receptor dimer activates the kinase domain (red) which auto-phosphorylates specific tyrosines. Numbers correspond to intracellular phosphotyrosines which recruit and activate second messenger pathways. Y731P activates PI3K, and Y1018P activates PLC γ . When

mutated to phenylalanine they are neither phosphorylated nor activate the associated signaling pathway. (b) The RTK Rheostat Schematic of PDGFR α -receptor signaling uncoupled mutants; Y to F substitution of tyrosine 731 uncouples the receptor from PI3K, while Y1018F uncouples PLC γ . Red box: tyrosine kinase domain. Right: the Rheostat model suggests that PI3K-driven motility is activated when OPCs are in low ligand levels, and suppressed via PLC-gamma when they enter high domains of PDGF.



The key is to control OPCs migration and to discover the precise mechanism of how these cues act. Recent microchemotaxis assays together with aggregation assays studies in vitro revealed that in the presence of active Fyn and downstream cyclin dependent kinase 5 (Cdk5), there was a significant increase of PDGF stimulated OPCs migration. Biochemical studies and loss of function experiments demonstrated that Cdk5 acts as mediator of a sequential cascade linking Fyn kinase to WAVE2 after PDGF stimulation (Miyamoto et al., 2008). This data provides new insights into OL development, however it also raises questions that need to be addressed to fully understand OPC migration.

Recent studies in the McKinnon lab have revealed how PDGFR α directs OPC migration in a PDGF gradient (Fig. 4). At low ligand levels, PDGFR α receptors activate PI3-kinase to stimulate OPC migration. At high ligand levels, PDGF α activates PLC γ which inhibits PI3K signaling and OPC migration (McKinnon et al., 2005). Thus PLC γ signaling would be predicted to block OPC migration into an area of high PDGF concentration. Conversely, OPCs which express PDGFR α that have been modified so

they cannot activate PLC γ should migrate into an area of high PDGF, such as a PDGF-infused lesion in MS or SCI patients.

In this thesis our objective was to direct the migration of oligodendrocyte progenitors by modifying ES cells to generate OPCs that ‘home’ into lesions. We first modified culture conditions for maintaining undifferentiated mouse ES cell lines *in vitro*. We generated a medium cocktail, combining inhibitors of the Wnt effector GSK3 plus MAP kinase effector MEK, which can support long-term expansion of mouse ES cell lines. The media now called ‘2i5S’ contains a reduced amount of FBS and no LIF molecule, therefore eliminating daily refeeding, as well as reduced chances of culture contamination and lastly it reduced the cost.

Previous studies suggested that PDGF directs migration by a rheostat that activates P13K and PLC γ pathways at different ligand levels. To address the rheostat model we engineered mouse ES cells to express wild type and signal transduction mutant versions of PDGFR α . Using eGFP plasmid as our control and then bi-cistronic vectors of pMO.PDGFR α -ires-Neo with plasmid DNA introduced by transfection into our ES cell lines resulted in very low frequency of stable transformants. To better control transgene expression we employed a second set of vectors (TRE.PDGFR α) with a tetracycline inducible promoter. Introduction of TRE.PDGFR α constructs into Ainv15 cells is still in progress. Lastly, we used shiverer mice to evaluate the potential of graft ES-derived OPCs cell population to repair myelin. The results showed substantial improvement in both motor function and longevity in animals with 7% of ES^{wt} derived cells present in the chimeric pups. Therefore, this suggests that 7% ES-derived stem cell engraftment is the minimal amount of cells that are required for functional cell replacement therapy.

2. MATERIALS AND METHODS

2.1 *Materials.*

Cell lines used in this study include mouse ES lines 7ac5, G-Olig2 and Ainv15, all obtained from American Type Culture Collection (ATCC, Rockville MD). NIH3T6 cells were obtained from the McKinnon lab, and primary mouse embryo fibroblasts (MEFs) were generated de novo from mouse embryos by Mary Kiel in the McKinnon lab, as outlined below. For immune histochemistry, primary antibodies were obtained from specific vendors as indicated, and secondary antibodies conjugated to specific fluorescent fluorophores (Alexa 350/UV; Alexa 488/green; Alexa 510/red) were obtained from Molecular Probes (Eugene, OR). Recombinant growth factors were obtained from Research & Development Inc. (R&D Inc., Minneapolis MN). Cell culture materials were obtained from Bethesda Research Laboratories/Grand Island Biological Co. (BRL/Gibco, Gaithersburg MD). Oligodeoxynucleotide primers were purchased from Integrated DNA Technologies (Coralville Iowa) through the RWJMS DNA Core Facility.

2.2 *Cell Culture*

Mouse Embryonic fibroblasts (MEF) were obtained from day 13.5 post coital embryos of mice that are neomycin (G418) resistant [C57Bl/6J-Tg (pPGKneobpA) 3Ems/J, Jax Labs, Bar Harbor ME). Cells were expanded in complete Dulbecco's Modified Eagles Medium (DMEM, Gibco) with 1mM sodium pyruvate, penicillin/streptomycin (0.1mM), 1% non-essential amino acids, and 10% (vol:vol) Fetal Bovine Serum (FBS, Atlanta Biologicals, Atlanta GA) in humidified air with 5% CO₂ at

37° C. When the cells reached confluency, they were dislodged from the culture flasks using 0.25% trypsin solution (Gibco), then either replated in complete culture media (1:3 dilution of original volume) or collected by centrifugation (1000 rpm, room temperature, 10 min) then resuspended in 1 mL of freeze-down media (90% FBS, 10% DMSO) and cryopreserved in liquid nitrogen in a Nunc cryopreservation vial. To mitotically arrest MEF cells for use as stem cell feeder layers, frozen cells were gamma-irradiated at 4000 rads and returned to liquid nitrogen storage for future use.

In this study we used two mouse embryonic ES stem cell lines, 7ac5 (SCRC-1033) and G-Olig2 (SCRC-1037), both obtained from American Type Culture Collection (ATCC, Rockville MD). 7ac5 cells were developed by co-transfecting ES cells with linearized pCX-EYFP and circular pPGKPuro (Hadjantonakis et al., 1998) and puromycin to select Yellow Fluorescent Protein (YFP)-expressing transfectants. G-Olig2 cells were developed by targeted insertion of a GFP-Neomycin phosphotransferase (GFP-Neo) cassette into the Olig2 locus of RW4 ES cells (Xian et al., 2003). Transfected cells were selected in 250 ug/ml G-418 (Xian et al., 2003). The GFP construct is activated when these cells differentiate into OPCs, which allows the visualization and separation of live cells (Fig. 3).

The Mouse ES cells were cultured on gamma-irradiated MEF cells in ES media, which consists of DMEM supplemented with 15% FBS, 1mM sodium pyruvate, Pen/Strep (0.1mM), 0.1 mM beta mercapthoethanol (B-MeOH), and 1% nonessential amino acids (Invitrogen). This was supplemented with 1.25 ug/ml (125 units) of Leukemia Inhibitory Factor (LIF, R&D Inc.), and the media was changed daily. When ES colonies

approached high density (Fig. 2), the cultures were trypsinized then replated at a lower density (passaged), usually every other day.

As outlined in Results, we also examined conditions for the expansion of mES cells in media lacking LIF and with reduced serum. Our analysis was based on recent work of Austin Smith (Buehr et al., 2008) using inhibitors of the MAP kinase effects MEK (PD325901) and of the Wnt effector GSK3B (CHR9402). As detailed in Results, we identified a media formulation now termed 2i5s which promotes ESC self renewal. Importantly, 2i5S does not require daily replenishment as we can maintain ES colony growth on a refeed schedule of 3 times/week. This media contains DMEM, 5% FBS, 1mM Na pyruvate, 0.1mM Pen/Strep, 0.1 mM B-MeOH, 1% non-essential amino acids, 3 mM CHIR94021 and 0.1 mM PD325901 (both from Stengent, San Diego, CA).

2.3 Embryonic Stem cell differentiation in vitro.

Experiments were carried out using 7ac5 and G-olig-2 cell lines in parallel to test different media conditions that would result in the most efficient induction of oligodendrocyte progenitor cells (OPCs). The experiments were divided into six stages of differentiation, each one representing different combination of growth factors and varied time points between each specific stage.

Step 1: Actively growing cells were trypsinized and transferred onto 10 cm bacterial dishes in ES media plus 15% serum without LIF. Withdrawal of LIF induced cells to aggregate and form embryoid bodies (EB) within 4 days of incubation under these conditions. **Step 2:** On day 4, floating EBs were transferred onto 10 cm poly-ornithine coated dishes and cultured in the presence of 10 uM of Retinoic Acid (RA, Sigma) and

Noggin (50 ng/ml, R&D Inc.) to promote neural differentiation. **Step 3:** After 4 more days, the media was replaced by 50/50 mixture of DMEM-F12 plus N2 supplement, and Neurobasal medium plus B27 supplement (Gibco) including Fibroblast growth factor FGF2 (20ng/ml). The N2 supplement includes insulin (25ug/ml), transferrin (100 ug/ml), progesterone (6 ng/ml), putrescine (16 ug/ml), sodium selenite (30 nM) and BSA (50 ug/ml). **Step 4:** After 3 days of incubation in these conditions, 300 ng/ml of Sonic Hedgehog (Shh, R&D Inc.) was added to the cell cultures to promote OPC formation. The cell cultures were grown in these conditions for the next 4 days. **Step 5:** After OPCs formation FGF2 was removed and cells were then grown in the same media containing Platelet Derived Growth factor (PDGF, 10 ng/ml) and triodo-thyronine (T3, 40 ng/ml, Sigma) to amplify the OPC population. **Step 6:** After four days with PDGF and T3 the cell cultures were transferred to R1236 media with high insulin (10 ug/ml) to facilitate the final maturation into oligodendrocytes. R1236 media contains penicillin/Streptomycin, Sodium Pyruvate (1 mM), Transferrin (100 ug/ml), selenium (30 nM), insulin (50 ng/ml), bovine serum albumin (100 ug/ml), N-acetyl cystein (50 ug/ml), and forskolin (10 uM), obtained from Sigma (St. Louis, MO).

2.4 Antibodies

Primary antibodies were used at the indicated dilutions in PBS at room temperature (1-2 hrs) or at 4° C (overnight incubations). Antibodies recognizing OL lineage extracellular markers were obtained as tissue culture supernatants from their respective hybridoma cell lines. These included mouse monoclonal anti-ganglioside antibody A2B5 (IgM;1:10), anti-galactocerebroside antibody O1 (IgM, 1:30) and anti-sulfatide antibody

O4 (mouse IgM, 1:10). Polyclonal rabbit antisera against the intracellular OL antigen myelin basic protein (IgG 1:20) was from Chemicon Inc. Mouse anti Neural Cell Adhesion Molecule (NCAM; IgM, 1:10) and polyclonal rabbit anti-Neuro-Glia 2 (NG2) chondroitin sulfate proteoglycan (IgG, 1:100) were obtained from Chemicon. The ES cell markers, goat anti-SSEA1 (IgG, 1:30) was obtained from Developmental Studies Hybridoma Bank (U. Iowa, IA).

2.5 Immunocytochemistry

For immunochemistry, cells were cultured on sterile 13 mm glass cover-slips, or in 4 well permanox chamber slides (Lab Tek). The media was removed from adherent mES cells followed by washing with 1x Phosphate Buffered Saline (PBS) (Gibco). Cell cultures were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and then stained with specific differentiation markers to determine the identity of cell types in these cultures. Before adding the antibodies, the cells were incubated with PBS containing 2% normal goat serum (NGS, Sigma) for 45 min to block non-specific binding sites. When using antibodies that identify intracellular antigens, the cells were first permeabilized by incubating in PBS containing 0.1% Triton X-100 (PBT). The cells were exposed to primary antibodies for 60 min at room temperature, then rinsed extensively with PBS and exposed to isotype-specific secondary antibodies conjugated to fluorescent tags for 45 min at room temperature at a 1:200 dilution (Molecular Probes, Eugene OR). After extensive washes, the coverslips and chamber slides were then mounted under glass coverlips in media containing the nuclear fluorescence dye DAPI (Vector Labs, Burlington CA). Cells were examined by epifluorescence (Zeiss Axiovert

100TV) and photographed on a Zeiss LSM510 confocal microscope, (Zeiss Inc, Germany) and images were assembled using Adobe Photoshop 6.0

2.6 Recombinant DNA.

Recombinant plasmids used in the studies described, and their source, are listed in Table 1. A complementary DNA (cDNA) encoding human PDGF α -Receptor (PDGFR α) was obtained by Dr. McKinnon from Dr. Stew Aaronson (NIH Bethesda), and specific mutations were introduced by oligodeoxynucleotide site-directed mutagenesis by Chris Edwards in the McKinnon lab (McKinnon et al., 2005). These mutations were confirmed by di-deoxy DNA sequencing (C. Edwards, unpublished). For cDNA expression studies, several distinct expression vectors were employed (see Figure 11). All cloning steps were performed by Dr. McKinnon. First, the cDNAs were subcloned into the bicistronic vector pMo.iresNeo placing the inserts under transcriptional regulation of the Moloney murine leukemia virus long terminal repeat (LTR) cis-regulatory sequence. Second, the cDNAs were subcloned into the Creator expression vector system (Clontech, Palo Alto CA). This is a convenient system for subcloning into a variety of expression vector platforms (Fig. 14).

For the Creator system, the PDGFR α cDNA was subcloned into the transition vector pDNR-dual placing the coding sequences between two tandem 'loxP' (locus of x-over) sites. This cloning was performed in two steps. First a 2 kilobase (kb) fragment encoding wild-type PDGFR α amino terminal sequences (N'PDGFR α) was inserted into pDNR dual between the BamH1 and EcoRI sites. Second, a 1.6 kb fragment encoding wild-type PDGFR α carboxy terminal sequences (C'PDGFR α) was amplified by polymerase chain

reaction (PCR) using linker adaptors primers (Table 1). The 3' primer was constructed so that the translation termination (stop) codon was replaced with a Proline, and the cDNA open reading frame would be fused in-frame with a 6x-histidine epitope tag encoded by pDNR-dual. The PCR product was then cut with appropriate restriction enzymes for insertion into the N'PDGFRa vector, generating pDNR-PDGFRa (Figure 14a). This second cloning step was repeated for each site-specific mutant cDNA construct (Y731F; Y762F; Y1018F), and each pDNR-PDGFRa vector was then subjected to DNA sequence analysis to ensure the junction sequences were of the correct nucleotide sequence, and that the appropriate wild-type or point-mutant version of PDGFRa was present in each construct.

Table 1. List of recombinant plasmid vectors used.

| Plasmid | Promoter-Expression | Source |
|---------------------|---------------------------------------|--|
| pSV2.Neo | SV40-Neomycin phosphotransferase | T. Matsui, NCI/NIH McKinnon lab unpublished unpublished unpublished |
| pSV2-PDGFRa | SV40--PDGFRa | |
| pSV-PDGFRa (Y731F) | Chris Edwards, McKinnon lab | |
| pSV-PDGFRa (Y762F) | | |
| pSV-PDGFRa (Y1018F) | | |
| pMo-PDGFRa | MoMuLV-PDGFRa | McKinnon lab |
| pCMV-eGFP.N1 | CMV-Green Fluorescence Protein | Clontech Inc. |
| pBS185 | CMV-Cre recombinase | Clontech Inc. |
| pDNR-dual | | Clontech Inc. |
| pLP-3'eGFP | CMV-eGFP | Clontech Inc. |
| pLP-PDGFRa-3'eGFP | CMV-PDGFRa-eGFP | R.McKinnon |
| pLPS-eGFP | CMV-eGFP | Clontech Inc. |
| pLPS-PDGFRa-GFP | CMV-PDGFRa-eGFP | R.McKinnon |
| pTet-ON | recombinant Tet-transactivator (rtTA) | Clontech Inc. |
| pTRE2 | Tetracycline response element (TRE) | Clontech Inc. |
| pTRE-PDGFRa | TRE-PDGFRa | R.McKinnon |
| pTRE-luc | Tet-Luciferase | Clontech Inc. |
| pTRE-DsRed | TRE-Red Fluorescence Protein | Clontech Inc. |

The second phase of these constructions was insertion of PDGFRa into Clontech 'acceptor' vectors pLPS-3'GFP, pLP-ires2-GFP, and pLP-TRE2 (Fig 14b) using Cre-mediated recombination (Clontech). Individual pDNR-PDGFRa constructs were incubated with Cre recombinase and one acceptor vector, allowing Cre to recombine the vectors at the loxP sites, and the resulting DNA was used to transform E.coli DH5alpha. After this transformation step, recombined plasmids were selected on agar plates containing ampicillin and chloramphenicol. Using these selection conditions only plasmids containing the PDGFRa insert recombined into the acceptor vector can be obtained, as this construct places the CmR gene under control of a bacterial promoter. The resulting colonies were screened for each PDGFRa insert and acceptor vector combination, and in each case over 90% of colonies contained the appropriate recombined DNA fragments. All recombinant vectors were verified by direct DNA sequence analysis of the junction regions between vector and inserts (RWJMS DNA sequencing Core).

2.7 Embryonic Stem cell transfection

Mouse ES cell were maintained on the gamma irradiated feeder layer in ES medium plus LIF. When they reached confluency, cells were trypsinized, counted, and then 1×10^6 cells per 35 mm plate were plated for transfection the same day. The cells were transfected with plasmids using LipofectAMINE Reagent (Gibco/BRL) including transferrin (Sigma, 4mg/ml in DMEM) plus 0.1-5 ug of selected DNA. For 35 mm dishes, a mix of 6.25 ul of lipofectamine (2 mg/ml) plus 2 ul transferrin (8 ug) and DMEM were diluted to a final volume of 63 ul then incubated at room temperature for 45

min. The mouse ES cells were washed with 1X PBS and, at the end of the incubation period, the transfection mix was added to the plates and incubated at 37° C, 5% CO₂ for up to 4 hours. For tetracycline inducible expression vectors, we used the Clontech pTRE-2 vector with our cDNA inserts plus the rtTA (Tet-ON) trans-activator plasmid. These DNAs were co-transfected at a 1:10 ratio of regulatory to response plasmid, which was previously shown to be optimal for these transfections. For antibiotic selection, complete media containing 400 ug/mL G418 (Gibco) was added 24 hrs post transfection and the media was replaced twice weekly until resistant colonies appeared, generally after 12 days of selection. For maintenance, the cells were cultured in the same media containing 200ug/mL G418.

2.8 Total RNA extraction

Total RNA from 7AC5 cells was isolated using Trizol Reagent (Invitrogen), following the manufacture's protocols. Briefly, 1 ml Trizol was added per 3.5 cm plate and allowed to sit for 5 min at room temperature to allow complete cell dissociation. The homogenized sample was then transferred to a sterile tube and chloroform was added (0.2 ml/ 1ml Trizol). The sample tubes were securely capped and vigorously shaken by hand for 15 sec, then allowed to sit for 2-3 min at room temperature, then centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and mixed with 0.5 ml isopropanol, allowed to sit for 10 min then centrifuged at 12,000 x g for 10 min at 4° C. Supernatant was removed and the RNA pellet was washed once with 1 ml 75% ethanol by vortexing then centrifuged at 7,500 x g for 5 min at 4° C. The RNA pellet was

then dissolved in 50 μ l of double distilled water. RNA yields were determined by measuring the optical density (A_{260}).

2.9 RT-PCR (Reverse Transcription- Polymerase Chain Reaction)

RNA was reversed transcribed into first strand cDNA using M-MLV Reverse Transcriptase. A 20 μ l reaction volume consisting of 1 μ l oligo (dT)₁₂₋₁₈ (500 μ g/ml, Invitrogen), 1 μ g RNA of interest, and sterile H₂O was added to a PCR microcentrifuge tube and heated at 65°C for 5 min in a water bath, then chilled at 4° for 10 min. Then, 4 μ l of 5X first strand buffer, 2 μ l of 0.1M DTT (Invitrogen, Carsband CA), 1 μ l of 10 mM 2'-deoxynucleoside 5'-triphosphate mix (dNTP; Invitrogen), and 1 μ l (200 units) of M-MLV RT were added and incubated at 37° C for 60 min. The yields of cDNA were generally equal to the amount of input RNA.

For PCR reactions, 2 μ l of complementary DNA (cDNA) were amplified in a reaction mixture containing 5 μ l of 10x PCR buffer (200 mM Tris-HCL pH 8.4, 500 mM KCl), 1.5 μ l of 50 mM MgCl, 1 μ l of 10 mM dNTP mix, 0.5 μ l (2.5units/ μ l) of Taq DNA polymerase, 2 μ l of forward and reverse amplification primers (0.4 μ M each) specific to the gene of interest, and 38 μ l H₂O in a final volume of 50 μ l. The reaction mix was amplified using a Stratagene Mastercycler gradient thermocycler set for the following parameters: 94°C, 3 min then (94° 1 min, 55° 1 min, 72° 2 min) for 30 cycles, followed by an extension reaction at 37° C for 7 min. A portion of the PCR reaction (15 μ l) was analyzed by electrophoresis on a 1.5% agarose gel in Tris-Acetate-EDTA buffer (0.04M Tris-Acetate, 2 mM EDTA), containing 0.5 μ g/ml of ethidium bromide to visualize the DNA products under UV illumination. As an internal control, PCR amplification of the

housekeeping gene GAPDH was used. GAPDH products were used to normalize the input cDNA levels in order to estimate the relative mRNA levels for other genes of interest in each sample.

Table 2. PCR Primers for RT-PCR

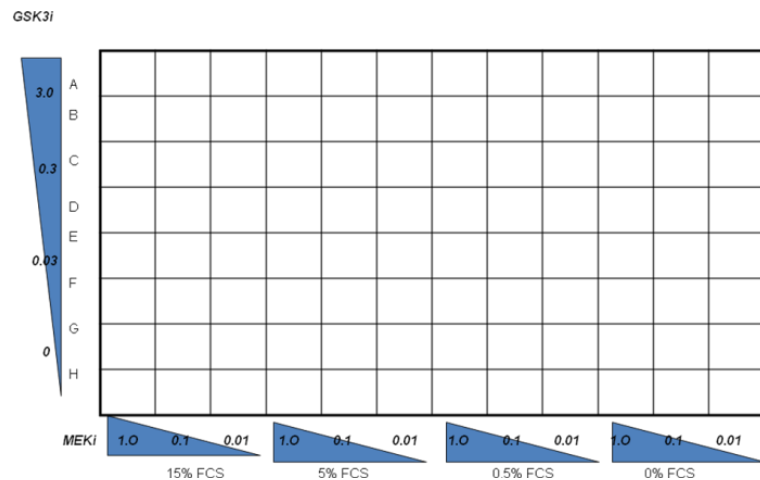
| GENE | Primer Name | List of Primers for RT-PCR | GenBank |
|-----------------|------------------------|-------------------------------------|-------------|
| Nanog | Nanog.581f | 5'-TCCAGCAGATGCAAGAACTCTC-3' | |
| | Nanog.719r | 5'-TTCTGCGTCACACCATTGCT-3' | |
| OCT4/3 | mOct3.423f | 5'-GAGGAGTCCCAGGACATGAA-3' | BC068268 |
| | mOct3.576r | 5'-AGATGGTGGTCTGGCTGAA-3' | |
| SOX2 | hSOX2.910f | 5'- GCACATGAACGGCTGGAGCAACG-3' | NM_003106 |
| | hSOX2.1116r | 5'- TGCTGCGAGTAGGACATG CTGTAGG-3' | |
| hPDGFR α | hPDGFR α .212 | 5'-CTTTCATTACCCTCTATCCTTCC-3' | NM_006206.3 |
| | hPDGFR α .474r | 5'-TAAATGTGCCTGCCTTC-3' | |
| hPDGFR α | alphaR3181f- | 5'-GACAGTGGCTACATCATTCCTC-3' | |
| | hPDGFR α .3292r | 5'-AAGGTGGAAGTCTGGAACCCGT-3' | |
| Vimentin | h&mVim.1001F | 5'-TCC AAG TTT GCT GAC CTC TCT G-3' | NP_035831 |
| | h&mVim.1216R | 5'-GCG GCC AAT AGT GTC TTG GT-3' | |
| hPDGFR α | hPDGFR α .3121f | 5'-AACGAGGAAGACAAGCTGAAG-3' | |
| | hPDGFR α .3292r | 5'-AAGGTGGAAGTCTGGAACCCGT-3' | |
| hPDGFR α | alphaR3181f- | 5'-GACAGTGGCTACATCATTCCTC-3' | |
| | CMRr | 5'-CMR ? | |
| GAPDH | hGAPDH 447f | 5'AGCCAAAAGGGTCATCATCTC-3' | NM_002046 |
| | GAPDH643r | 5'-CTGTGGTCATGAGTCCTTCCA-3' | |
| Tet-ON (rtTA) | rtTA.845f | 5'-GCCTGACGACAAGGAAACYCG-3' | |
| | rtTA.1044r | 5'-AGAGCACAGCGGAATGACTTG-3' | |
| Tet-ON (rtTA) | CMV571f | 5'-CACCAAAATCAACGGGACTT-3' | |
| | rtTA.1044 | 5'-AGAGCACAGCGGAATGACTTG-3' | |
| mOlig2 | h.mOlig2.427f | 5'-CACCAAGAAGGACAAGAAGCA -3' | AB028697 |
| | h.mOlig2.689r | 5'-GTAGATCTCGCTCACCAGTCG -3' | |

3. RESULTS

3.1 Optimizing mouse ES cell culture conditions

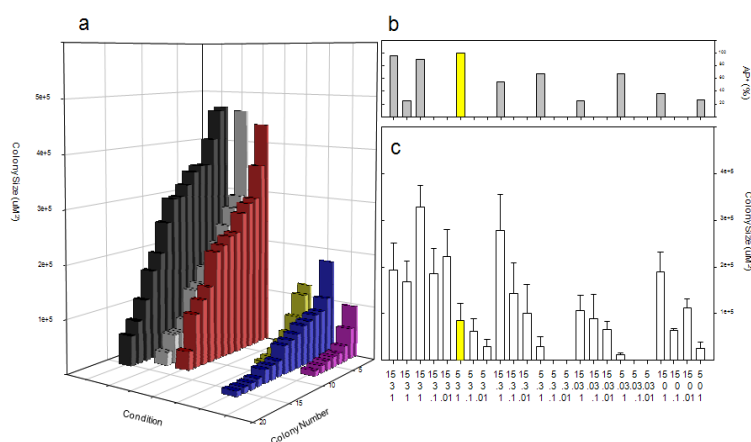
In order to maintain undifferentiated mouse ES cell lines in vitro, it is important to provide the ES cells with an active LIF molecule. This requires daily refeeding, which is expensive, time consuming and cultures are more prone to contamination. Exciting new results suggest that we can improve mES cell cultures conditions, which would make ES engineering easier. Based on recent studies (Buehr et al., 2008; Li et al., 2008) we examined the effects of inhibitors of the Wnt effector GSK3 (CHR9402) and the MAP kinase effector MEK (PD325901) on maintaining the undifferentiated state of mouse ES cells in the absence of LIF. For convenience, these inhibitors will be termed GSKi and MEKi. We plated the mouse ES cell line 7ac5 (Hadjantonakis et al., 1998) on irradiated MEFs in a 96 well plate. The 7ac5 cells have a yellow fluorescent protein (YFP) transgene, which provides a convenient marker for identifying ES cells in these mixed cultures. The 7ac5 cells were plated at clonal density (20 cell per well) and we monitored ES colony growth over 9 days (Figs. 5, 6). The culture conditions for this study are shown in Fig. 5.

Figure 5. Template for mES cell culture conditions. The format for addition of GSKi, MEKi and FBS to mouse ES (7ac5) cells growing in a 96-well BD/Falcon culture dish. See text for details.



We divided the plate into four groups of cells that were exposed to 0, 0.5, 5 or 15% FBS. These groups were further subdivided into distinct combinations of MEKi and GSK3i. The cells were fed three times a week. At 1 day post plating the 7ac5 cells had attached and we observed small colonies with the characteristic undifferentiated morphology of ES colonies (not shown). On day 3 the first colony count was performed under phase and fluorescence to look for the expression of YFP, and this was repeated on day 6 to look for the survival of these colonies. On day 9 the cells were fixed in 4% PF and stained for the ES specific marker alkaline phosphatase (AP), and the total number of positively stained colonies were counted (Fig. 6a, b). We also measured the total size (microns²) and compared to the total number of colonies.

Figure 6. 2i5s media for ES cell self-renewal. ESCs cultured for 9 days in the indicated conditions of 5-15% fetal bovine sera, 0-3 uM GSK inhibitor, and .01-1 uM MEK inhibitor. Cells were fixed and processed for AP activity then counted for (a) AP⁺ colony number and individual colony size (uM²), (b) percent AP⁺ per total colonies, and (c) mean colony size. Optimal conditions for maintaining ESC self renewal were 3 uM CHIR99021, 1 uM PD0325901 and 5% FBS (yellow bars).



colonies that remained ES-like (AP^+ , undifferentiated) decreased (Fig. 6b). In other words, there was more differentiation, suggesting that GSK3i is a potent inhibitor of ES differentiation in these culture conditions (Fig. 6a-6c). For cells growing in 5% FBS, with high concentration of GSKi plus decreasing concentration of MEKi, the colony size decreased with lower concentrations of MEKi (Fig. 6c). In 5% FBS average colony size was 1/3 that of colonies growing in 15% FBS. While the cell growth decreased in cultures with 5% FBS, the colonies that did grow were all AP^+ (Fig. 6c). Thus FBS provides a very significant cue for ES cell growth, and ES cells require the presence of two inhibitors to stay undifferentiated in cultures lacking LIF. Based on these findings we chose 5% FBS, 3.0 μ M of GSK3i, plus 0.1 μ M of MEKi (now named 2i5S) as our optimal media for mouse ES self renewal. This provides sufficient growth of undifferentiated cells, eliminates daily refeed and there is no need to provide LIF. Thus 2i5S reduces the costs of maintaining mouse ES cell in culture.

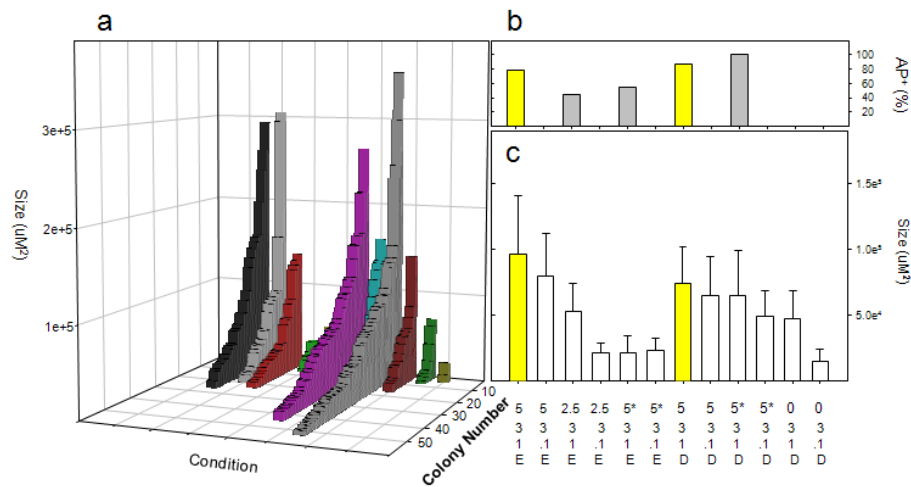


Figure 7. ES cell self-renewal in minimal sera conditions. ESCs cultured for 9 days in the indicated conditions of either ES media (E) or D/N media (D) with 3 μ M GSK inhibitor, .1-1 μ M MEK inhibitor, and the indicated concentrations of fetal bovine sera (s) or sera replacement (s*). Cells were fixed and processed for AP activity then counted for (a) AP^+ colony number and individual colony size (μ M²), (b) percent AP^+ per total colonies, and (c) mean colony size. Optimal conditions for ESC self renewal in 2i (3 μ M CHIR99021, 1 μ M PD0325901) were 5% sera or sera replacement (yellow bars).

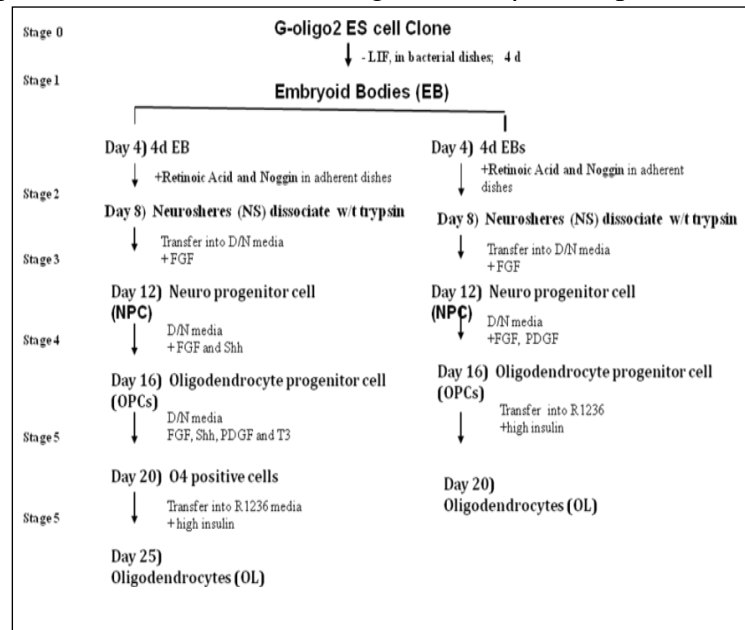
Having established the efficient concentration of both inhibitors, we wanted to test if we could replace FBS with Serum Replacement (SR) in our cultures. This would ultimately be the best culture condition for growing human ES cells to be used clinically. We set up another 96 well plate with 7ac5 cells (20 cells/ well) and divided the plate into 6 groups. Each group contained a set concentration of inhibitors plus different concentration of either FBS or SR. Cells were cultured in either ES base media as in Figure 6 or in D/N media (Fig. 7). Again, the cells were cultured for 9 days and data was collected as described for Figure 6. In these low Sera/SR conditions, ES cells grew better in D/N media (colony size and colony number), with better growth in 5% FBS than in 5% SR. In ES media conditions, 5% FBS was necessary to maintain AP⁺ ES colonies. In D/N media conditions, both 5% FBS or 5% SR were sufficient to maintain AP⁺ ES colonies (Fig. 7b). We concluded that for ES self renewal we need either 5% FBS or a media base D/N that contains a rich source of supplements of (B27 and N2). For our studies we have used the ES media base with either 2i5S (with media replacement every 3 days), or ES media base with LIF (with daily media replacement).

3.2 Optimizing mouse ES to OL differentiation

After completing the experiments to generate optimal ES cell self-renewal conditions, we next focused on manipulating culture conditions for neuronal differentiations. Murine ES cells can be differentiated into oligodendrocytes (OL) in vitro in the presence of a combination of specific signal molecules. We used several distinct ES lines including 7ac5 (Hadjantonakis et al., 1998) and G-olig2 (Xian et al., 2003; Xian et al., 2005) and Ainv15 cell lines (Kyba et al., 2002). The ES cells were grown in 0.1% gelatin-coated dishes on a monolayer of neomycin (G418) resistant MEF feeder cells. For our initial

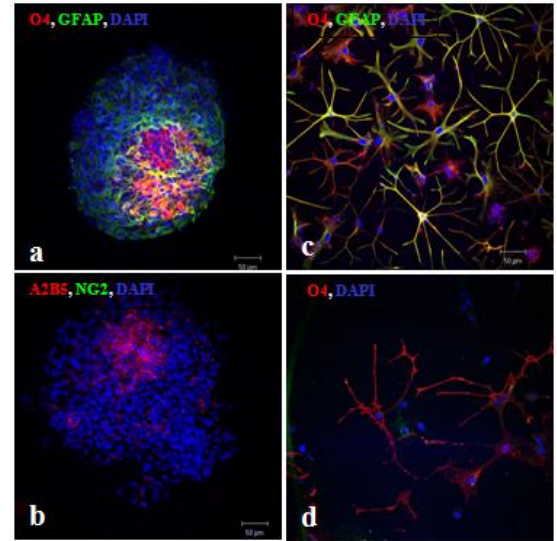
studies, these ES lines were amplified in ES media containing DMEM, β -mercapto-ethanol, non-essential amino acids, 15% FCS plus LIF. ES cells were then differentiated into OPCs by sequential induction process (Fig 8).

Figure 8. ES to OL differentiation scheme. Schematic of the culture conditions used to generate OLs from ES cells in vitro. See text for details.



First, to induce formation of embryoid bodies (EBs), ES cells (5×10^6 cells) were plated on non-adherent bacterial dishes without LIF. These EBs were positive for multiple markers including GFAP (Fig. 9a). On day 4, Retinoic Acid plus Noggin was added and cultures were maintained for 4 days to promote neural development. Using immunohistochemistry analysis at this point the ES progeny expressed Nestin and GFAP antigenic markers. On day 8, the ES cell media was replaced with D/N media (50/50 mixture of DMEM-F12 plus N2 supplement, and Neurobasal medium plus B27 supplement) plus FGF2 for 4 days, to promote neuroepithelial cell formation. Immunohistochemistry of these cultures revealed A2B5 (Fig. 9b) and NG2 positive cells, indicating the cultures underwent neural development. The cells continued to be selected in FGF plus Sonic Hedgehog (Shh); after 4 days the first PDGFR α positive OPCs were detected. These cells were also stained for the OPC marker O4 (Fig. 9d). After 12 days the cells were kept in previous media plus PDGF and T3 to promote differentiation of OPC into OLs (Fig. 3, 9d).

Figure 9. Histochemical analysis of mES-derived cells. Representative confocal images of ES cells growing in cultures under conditions that promote formation of EB, NS, OPC and OL cultures, as described in the text. a-d shows staining of O4, A2B5, GFAP, NG2 neural markers with a Dapi.

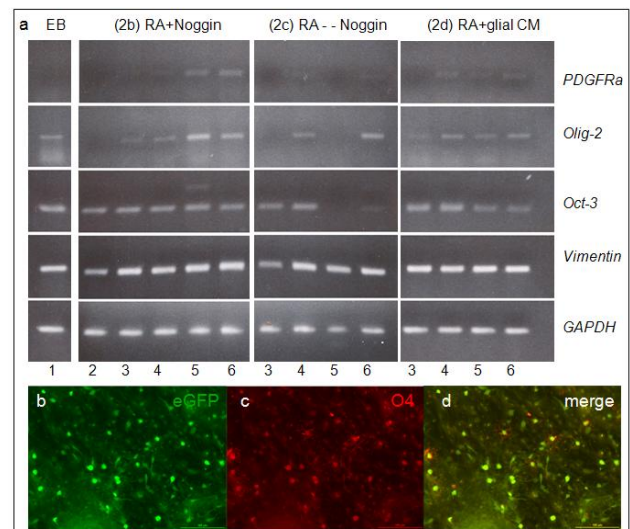


Our studies to date indicate that ES cells can be programmed into the OL lineage in vitro. Since this programming is not as

efficient as seen with myeloid or adipocyte differentiation, we set up another set of experiments using the G-Olig2 cell line to test combination of different ligands at various time points to establish a more efficient differentiation protocol. We first determined the effects of the signaling molecule Noggin on ES differentiation by changing the culture condition at stage 2. The cells were either cultured in the presence of RA with Noggin, RA then Noggin or RA plus glial cell conditioned media. The culture conditions after stage 2 were kept the same as previously described. RNA was collected at each stage and we examined the expression of lineage-specific markers in these cultures. There was a significant increase in the expression of OPC markers after RA plus Noggin in (Fig. 10 condition 2b).

Figure 10. mouse ES cells-to-OPC/OL differentiation in vitro

(a) RT-PCR analysis of lineage marker expression. Lane numbers correspond to culture stage in Figure 3, and the culture conditions at step 2 are detailed above each group in panel a; glial CM, conditioned media from primary rat brain glial cultures. (b-d) GFP expression from the olig2 locus, co-localized in O4⁺ cells at stage 6 of ES-to-OL differentiation.



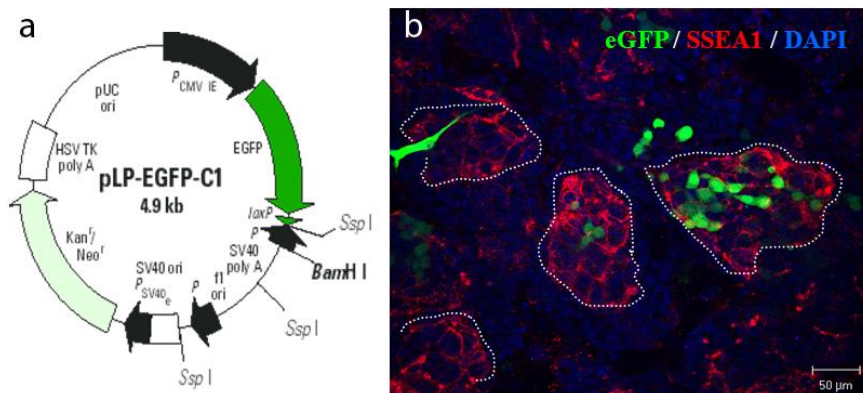
The expression levels of Olig2 can be seen at stage 3 and PDGFR α is first seen at stage 5. We also detected a very strong level of the GFP transgene in these cultures (Fig. 10b). In contrast, Oct-3 expression levels stayed constant throughout all the 6 stages of the differentiation. In cultures with sequential RA (2days) then Noggin (2days), (condition 2c), there was only weak expression of PDGFR α present and Olig-2 is only clearly expressed at the last stage of the differentiation. In cultures with RA plus glial cell condition media (Fig. 10, condition 2c) the gene expression was very similar to condition with RA then Noggin. These results indicate that Noggin caused a subsequent increase in neural differentiation and the number of expanding Olig2 cells, compared to the parallel cultures. This suggests an increase in the percentage of Olig2 positive cells in the culture population (Fig. 10). Together our results have defined an efficient and rapid protocol for ES-to-OPCs differentiation in vitro.

Noggin turned out to be an important signaling cue for obtaining large numbers of differentiated oligodendrocytes from mouse ES cells. We then focused on the following stages of the protocol and tested if we could reduce the incubation time of any of these molecules within our cultures described above. More specifically, we tested if we could eliminate any of the signaling cues from our protocol and still obtain the same end result. After removing Shh from our cultures (data not shown) the number of OPCs did not differ when compared to the control condition (Fig. 8). In addition, there was no significant change in number of differentiated OLs in the cultures omitting T3 plus Shh when compared to the control conditions. Altogether this data supports the new improved differentiation protocol that eliminated one stage and reduced in time of incubation to generate a significant number of differentiated OLs in our cultures.

3.3 DNA transfection of mES cells in vitro

Having optimized culture conditions for ES-to-OL differentiation, we next focused on engineering ES cells to generate OPCs with enhanced PDGFR α signalling migration. Our studies focused on PDGF and its trans-membrane receptor PDGFR α , which directs OPC migration during brain development. Previous studies suggest that PDGF directs migration by a rheostat that activates P13K and PLC γ pathways at different ligand levels. This model suggests that if we could administer PDGF into CNS lesions, and if we could generate OPCs that express the PDGFR α that is uncoupled from PLC γ , these OPCs would migrate into those lesions and promote myelin repair. To test this we attempted to engineer 7ac5 ES cells to express wild-type and signaling mutant versions of PDGFR α transgenes (PI3K-defective and PLC γ defective).

Figure 11. Green Fluorescent protein eGFP: transient transfection. (a) Clontech eGFP-N1 expression vector. **(b)** G0lig2 mES cells (GFP⁻, G418^r) transiently expressing eGFP (green) and stained with the ES marker SSEA1. The GFP expression is from the introduced transgenes.



To test the efficiency of transfection, we used pCMV-eGFP.N1 (Fig. 11a) and G0lig-2 mouse ES cells, using transferrin-enhanced Lipofectamine (McKinnon et al., 2005). ES cells were maintained in the ES media 15% FCS plus LIF, and we monitored transgene expression by epifluorescence. GFP⁺ cells were visible within ES colonies as soon as 24hr after transfection (Fig. 11b). At 48hrs post transfection, the eGFP⁺ cells and the total number of cells was counted and recorded. The efficiency was approximately

60% as determined by transient eGFP expression (Fig. 11). This data was then used in manipulating and improving transfection experiments using PDGFR α expression vectors.

Next we attempted to isolate clonal derived 7ac5 cells, stably expressing the pMo- α R constructs. Our transfection vector pMo.PDGFR α -ires-Neo (Osterhout et al., 1997) is bicistronic, encoding PDGFR α and a selectable (G418^r) marker. We have a series of these vectors encoding wild-type PDGFR α or mutated versions of PDGFR α , each with a single mutation at a specific tyrosine residue in the signaling domain (Y-to-F substitutions) that prevent PDGF-activation of one signaling pathway. We focused on mutations that uncouple PDGFR α from PI3-kinase (Y731F Y742F) and from PLC γ (Y1018F). Stable transformants were selected by expanding ES colonies on MEF feeder layers in ES media plus LIF with 400ug/mL G418. After 12 days including daily refeeding with fresh media, colonies were visible. When they reached a large size, these colonies were isolated and replated on fresh feeder layer coated dishes for expansion as clones.

Table 3. ES (7ac5) stable transformants (G418^r)

| Plasmid | Day 10 ¹ | Day 15 ² | Day 25 |
|--|---------------------|---------------------|--------|
| 1. eGFPN1 | 201(1.00) | 42(1.00) | Ø n.d. |
| 2. pMo α R ^{wt+} | 113(.56) | 18(.43) | 64 |
| 3. pMo α R ^(731/742) | 89(.44) | 29(.69) | 105 |
| 4. pMo α R ⁽¹⁰¹⁸⁾ | 87(.43) | 27(.64) | 73 |

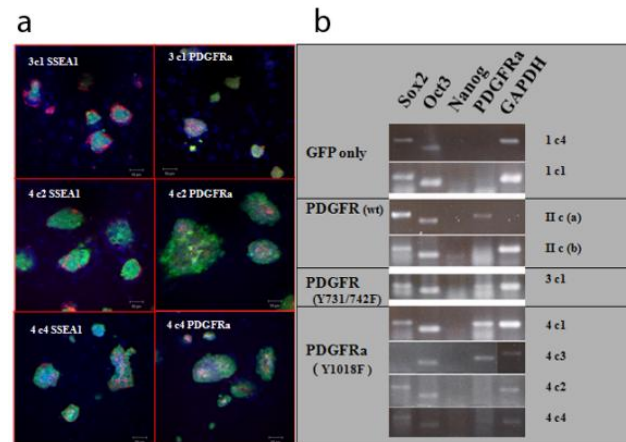
1. 466 fields (YFP⁺) (10x objective) for each condition.
2. Phase contrast count of entire dish for each condition
3. YFP⁺ cells, entire dish.

These studies amplified the G418 sensitive ES cell lines 7ac5. The frequency of G418^r stable transformants is shown in Table 3. After 10 days, there were approximately one half as many colonies in each of the pMo α R wild type dishes. After 15-25 days, the

number of pMoαR wild type was significantly less than pMoαR^{mutant}. Relative to the eGFP-Neo controls, pMoαR w⁺ transformants had 60% fewer colonies, while PI3K and PLCγ mutant receptors had 40% fewer colonies. These results suggest that pMoαR wild type over expression somehow effects the growth of 7ac5 cells.

Figure 12. Stable transgene expression in 7ac5 ES cells.

(a) 7ac5 ES cells clonally isolated (G418^r) and stained with the ES marker SSEA1. (b) RT-PCR analysis of ES subclones for expression of ES markers (Sox2, Oct3, Nanog), transgenic PDGF receptors, and the house keeping control GAPDH. PDGFRα PCR primers specifically recognize the human receptor transgene and not the murine cDNA. Both Nanog and PDGFRα showed variable expression in the different subclones.

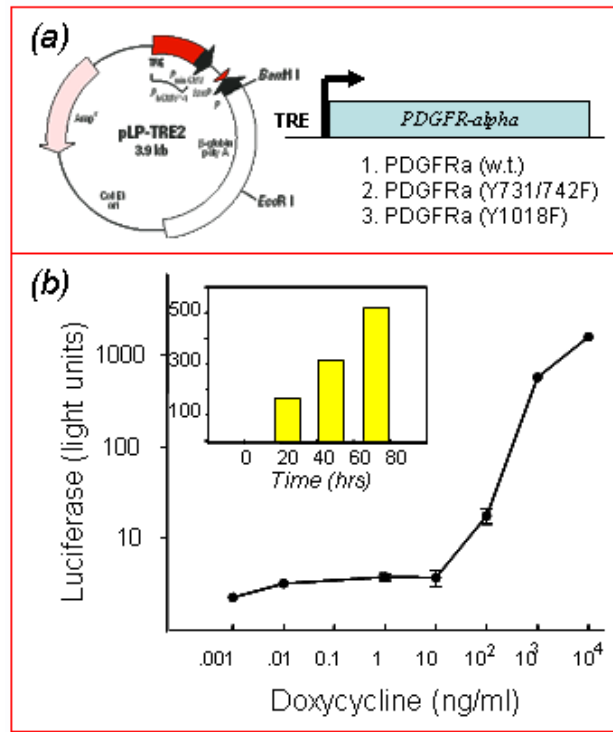


We next examined individual subclones for the expression of our PDGFRα transgenes as well as ES markers. ES lines were expanded, then cells were harvested for histochemistry (Fig. 12a) and RT-PCR analysis (Fig. 12b). We also froze down an aliquot of each line for future studies. Molecular characterization of ES derivatives and eGFP controls revealed expression of ES markers (Sox2, Oct3/4, Nanog), and most importantly, expression of the PDGFRα transgene. The eGFP controls showed high frequency of rapidly expanding ES subclones, and confocal images revealed SSEA1-positive ES colonies. In contrast, all three PDGFRα transgenes gave reduced numbers of ES colonies that were distinct from 7ac5 in morphology and growth rates. Wild-type PDGFRα gave the lower frequency of SSEA-1 and PDGFRα positive colonies relative to signal transduction mutant PDGFRα transgenes. Also, the Y731/732F (PI3K activation defective) and the Y1018F (PLCγ activation defect) versions of PDGFRα expanded more rapidly than wild-type transgenes, again suggesting that premature expression of

PDGFR α transgenes somehow altered the ES colonies. Furthermore, all PDGFR α expressing ES subclones were distinct from 7ac5 in morphology and growth rates, indicating that transgene expression alters the ES cells.

Figure 13. Luciferase (cre): tetracycline-inducible (Tet-ON) expression

(a) Schematic representation of the tetracycline-responsive element (TRE) plasmid vector pLP-TRE2 and recombinant versions containing PDGFR α wild type (w.t.) and tyrosine-substitution mutants Y742F and Y1018F. (b) TRE-dependent expression of the reporter construct firefly luciferase in transiently transfected U2-OS-Luc cells which express constitutive rtTA, the tetracycline-sensitive transactivator protein. After transfection with TRE-Luc, the cells were exposed to the indicated levels of the rtTA inducer doxycycline and 20-70 hrs later the cell extracts were assayed for Luciferase enzymatic activity. Luc activity was dependent on Dox levels (>10 ng/mL) and time post transfection.



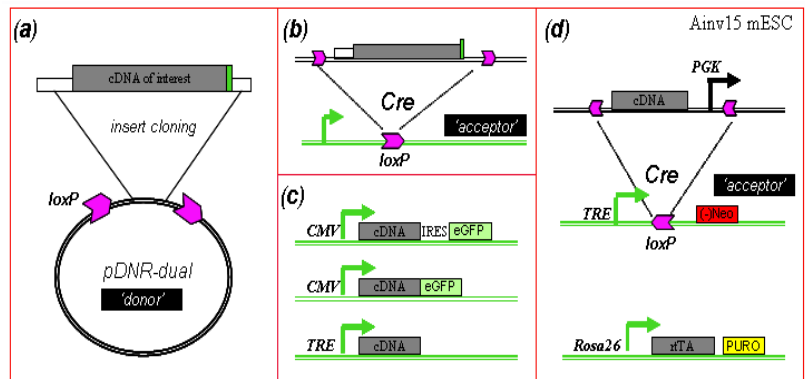
3.4 Inducible (Tet-ON) gene expression in mouse ES cells in vitro

Given the possibility that constitutive PDGFR α expression is toxic to ES cells, we decided to switch to a tetracycline inducible vector to control transgene expression. We employed a second set of vectors (TRE.PDGFR α) with a tetracycline inducible promoter (Fig 13a). Preliminary studies with doxycycline-induced expression of a reporter (luciferase) showed strict regulation (Fig. 13b). In cells expressing TRE-luciferase and its Tc-responsive transactivator (rtTA), Luc expression was undetectable in absence of induction and 1500-fold higher levels after 48 hrs in the presence of 1mM Doxycycline (Dox). Thus the inducible (Tet-ON) promoter will allow controlled expression of the PDGFR α transgene only in the presence of Dox. To mimic the endogenous expression pattern of OPCs to OL differentiation, we transfected plasmids of PDGFR α under the

control of TRE-inducible promoter plus pTet-ON (rtTA) advanced plasmid expressing trans-activator protein into 7ac5 cell line. Pilot studies determined the 1:10 ratio of Tet-ON and TRE-PDGFR α plasmids respectively to be the optimal ratio for the transfection experiments (data now shown). At 24 hrs post transfection we started the selection by incubation with 400ug/mL G418. Colonies were initially present, but we failed to isolate colonies as it appeared all the 7ac5 cells were resistant to G418. We do not know why our ES line was no longer drug sensitive. After multiple attempts, we switched to another ES cell line called Ainv15 (Fig. 14d). One of the benefits of this cell line is that it contains the reverse tetracycline trans-activator (rtTA) within the constitutively active ROSA26 locus. The ROSA26 rtTA transgene allows the Tet-activation protein to be expressed ubiquitously at moderate levels, and thus ensures a stable level of Tet-ON (rtTA) gene expression. Ongoing studies are in progress, using Ainv15 cell line to isolate clones with an inducible promoter to express four versions of PDGFR α for future in vivo

experiments.

Figure 14. TRE-PDGFR α : Cre-mediated recombination. Schematic representation of (a) pDNR-dual vector, (b) loxP acceptor vectors, and (c) Cre recombinase site in Ainv15 cells. See text for details.

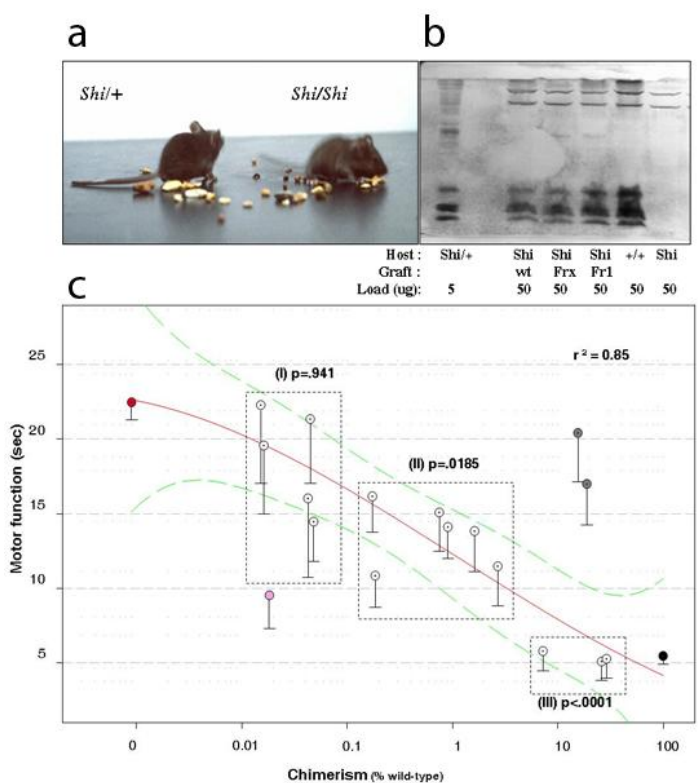


3.5 ES cell therapy: rescue of Shiverer mice with mES cells in vivo

Finally, we also initiated pilot studies to test whether our ES cell lines have therapeutic effects in vivo. To address whether stem cells can rescue CNS myelin degeneration, we used the dysmyelinated Shiverer (Shi) mouse (Fig 15a), which has a genomic deletion that spans the gene encoding Myelin Basic Protein (MBP). These mice

have uncompacted CNS myelin which results in tremors, motor impairment and shortened life span. We wanted to determine whether ES cells could rescue Shi, and how many ES cells are required to rescue the Shi phenotype. ES cells were injected into day e3.5 Shi blastocysts then chimeric pups were raised through adulthood (Kiel et al., 2008). We used genomic DNA (tail biopsy) to determine the number of shiverer and wild type cells in each pup (chimerism). When they turned two weeks old, we examined their motor function.

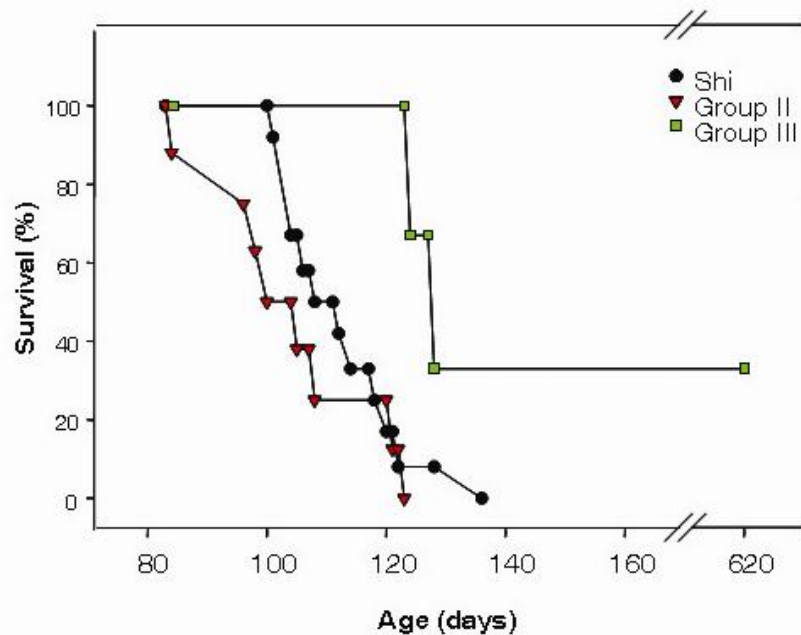
Figure 15. ES cell rescue of Shiverer mutant mice. (a) Adult Shi/+ heterozygous and Shi/Shi homozygous mice. Mutant animals have a pronounced tremor, as shown by the blurred tail in the image. (b) Western blot analysis of MBP isoforms in whole brain extracts from wild-type (+/+), Shi/+, and Shi/Shi hosts grafted at day 2 with either wild-type OPCs or OPCs expressing versions of FGF receptor transgenes. (c) Relationship between chimerism and motor function. Chimerism was determined by cycle threshold (C_T) using Q-PCR analysis of genomic DNA, with *mbp* levels normalized to *gapdh* then compared to normalized *mbp* in wild-type animals. Non-grafted controls include *mbp*-null Shiverer (red) and wild-type mice (black symbol). Motor function was determined by obtaining the time for exit from a shallow tray of water in the home cage. Motor function was scored in double-blind format with genotype analysis decoded post hoc. Red circle, shi/shi (n=12); black circle, wild-type (n=6); open circles, individual chimeras; dashed lines indicate 95% confidence interval, and shaded circles represent outliers. Shi mutants lack balance and coordination, while Shi-chimeras with >7% wild-type ES chimerism showed significant improved locomotor function.



In the motor function test, we placed individual animals into a small tray containing water and then recorded the time for their complete exit from the tray (Fig. 16b). Shi mutants performed poorly with an exit time of 25 sec, while wild type mice had no

problem with an exit time of 5 sec. In contrast, chimeric mice showed a range of mobility and their performance correlated with chimerism. Shi mutants performed poorly while chimeric mice were more mobile and exit time correlated with chimerism. Animals with 0.1-5% wild type cells had 33% faster exit speed, and chimeras with greater than 7% wild-type cells had 75% faster exit speeds from the water tray. Chimeric animals also had an extended life span relative to Shi mutants (Fig. 16). Therefore, ES cells have great potential for myelin cell therapy.

Figure 16. Survival of MBP^{shi}:Olig2^{GFP} chimeras. Age of natural death for MBP^{shi} (circles) and for MBP^{shi}:Olig2^{GFP} chimeras from group II (triangles) and III (square symbols). Group III chimeras with >20% extended life span relative to MBP^{shi} controls retained a mild tremor.



4. DISCUSSION

Collectively, the data have demonstrated four important findings for the genetic engineering of ES cells for brain therapy. First, we describe an improved and effective medium named “2i5S media” that can support the long-term expansion of mouse ES cell lines. We were able to generate a cocktail of supplements to maintain the expansion of undifferentiated mouse ES cells in the absence of LIF. Second, we describe an efficient program for the generation of oligodendrocytes from mES cells *in vitro*. Prior work suggested this could take as long as 3-4 weeks in culture, and our studies now show that this differentiation scheme *in vitro* can be as efficient and on the same time frame (14 days) as *in vivo* differentiation. Third, we initiated studies aimed at directing the migration of ES-derived cells after transplant into the brain.

Our initial studies indicated that such ES cell genetic engineering must employ transgene vectors with controlled (inducible) gene expression, and we identified an effective Tet-ON system that allows for such controlled studies. Finally, we have initiated studies designed to test the utility of our genetically engineered ES cells in myelin mutant animals *in vivo*. Pilot studies with wild-type ES cells transplanted into myelin mutant shiverer mouse blastocysts demonstrate the formation of graft-derived myelin, and chimeric mice with greater than 7% wild-type (ES-derived) oligodendrocytes had improved motor function and longevity. In total our studies form the basis for future studies which will examine transplants of ES cells expressing forms of growth factor receptors which are designed to enhance the therapeutic benefits of these cells. The

current work thus represents a small step forward towards stem cell regenerative therapeutics for myelin diseases such as multiple sclerosis.

ES cell self renewal : 2i5s

We reexamined ES cell media supplements based on published data of several groups who have derived ES cells from species other than mice (Buehr et al., 2008). To date it has not been possible to generate rat ES lines using culture conditions established for mouse ES cell lines. It has been thought that differences in early embryos, and the differentiation potential of non mouse species, underlie prior unsuccessful experimental results (Buehr et al., 2003). Recently two groups were able to efficiently generate rat ES cell lines (Buehr et al., 2008; Li et al., 2008; Ying et al., 2008). In their previous studies Smith's group discovered that to maintain mouse ES pluripotency and self renewal it is necessary to repress differentiation signals. Some of the commitment cues include fibroblast growth factor 4 (FGF4), glycogen synthase kinase 3 (GSK3) and the mitogen-activated protein kinase (MEK) pathway. Culturing the rat ES cell lines with specific inhibitors of these signaling molecules, they were able to efficiently derive and propagate long-term self-renewing, pluripotent rat ES lines. These ES cells were able to generate teratomas, and most importantly they have the capacity to colonize all three germ layers and produce a high rate of chimerism (Buehr et al., 2008; Li et al., 2008; Ying et al., 2008).

The discovery of the role these inhibitors play in ES cell self-renewal suggested that we could still optimize mouse ES culture conditions in order to efficiently perform ES

gene manipulation experiments *in vitro*. It would be ideal to generate cocktail combination where we could completely eliminate FBS from our cultures, as this would ultimately be the best culture condition for growing human ES cells to be used clinically. We investigated whether D/N media with N2 and B27 supplements was able to replace serum altogether, and combining it with inhibitors would be capable of sustaining self-renewal and preserve ground state pluripotency of ES in culture. Our studies have indeed established an ES culture media, termed '2i5s' which fulfills these criteria. We now routinely maintain mouse ES cell lines in this media formulation, and ongoing studies are exploring optimal conditions for the growth of human ES lines, including the NIH-approved WA09 (H9) cells. The ability to grow mES cells in 2i5s media without daily refeeding has resulted in a very substantial reduction in the cost, and daily work effort, of maintaining these cells during DNA transfection studies.

ES – to – OL differentiation in vitro

The second focus of our studies was to generate a more efficient programming sequence from mouse ES cells into OPCs and OLs in cultures. Various studies have shown that there is a very dynamic combination of transcription factors expressed during oligodendrocyte development. These TFs all have an importance in OL specification and maturation, and serve as downstream targets or effectors of extracellular ligands and intracellular signalling pathways (Collarini et al., 1992; Lu et al., 2002). We found that addition of the signaling molecule Noggin at a specific step of the mouse ES cell cultures process increased the number of mouse ES cells to progress towards OPCs. Previous work shows that the pathway of oligodendrocyte programming appears to be regulated by

Bone Morphogenetic Protein (BMP) and Noggin at multiple steps. More specifically, BMP was found not only to inhibit OLs development in vitro by shifting them to mature into astrocytes (Mabie et al., 1999; Mehler et al., 2000), but it also inhibited the expression of multiple myelin protein expressed by immature OLs. In other words, it seems as though BMP affects cell lineage decisions at earlier stages and later down the pathway it inhibits cell specialization (See et al., 2004). Therefore, when cultures were incubated in the presence of Noggin it resulted in a 1.7 fold increase in generation of oligodendrocytes, suggesting that Noggin acts as an antagonist for BMP (Mehler et al., 2000; See et al., 2004). Addition of Noggin to the cultures at step 2 showed an increase in generating OPCs to progress towards mature OLs. Analyzing isolated RNA constructs from these cultures showed an up-regulation in the expression levels of Olig-2 as well as PDGFR α .

OPC Migration – the PDGFR Rheostat

A third focus of our studies was OPC migration. A number of experiments support the idea that migration of OPCs is modulated by other secreted factors rather than PDGF and its receptor. In the spinal cord, it is proposed that OPCs migration is influenced by a ventrally produced gradient of sonic hedgehog (Shh) and a dorsally derived gradient of bone BMP. Several very useful and well studied models for OL development are the vertebrate optic nerve (ON) and the developing spinal cord. In the spinal cord, Miller's group observed that the first OPCs appear at the ventral midline. In the ON, OPC migration is dependent on retinal axon projections into the brain. Interestingly, retinal axons induce the OPCs developments in vitro and vivo, but only in the presence of Sonic

Hedgehog (Shh) and neuregulin. This data suggests that optic nerve OPCs development is dependent on cues generated from axons, but it also shows that Shh is required for the specification of these cells (Gao and Miller, 2006). Using the same system model, additional research results clearly illustrated the importance of the Shh in optic nerve oligodendrocyte precursor specification. In fact, the molecular events which guide the migration of optic nerve OPCs from preoptic area to colonize the entire nerve is induced by the Shh. In vitro experiments using optic nerve explants and over interference with Shh, strongly suggests that Shh acts as a chemoattractant and mitogen for migration of ON OPCs, which is independent from netrin-1 and FGF-2 (Merchan et al., 2007).

Myelin therapy – rescue of shiverer mice

Finally, a fourth area of investigation was to determine the myelin-forming ability of ES-derived OLs. Pilot experiments in vivo were performed to evaluate the amount of cell transplants that are required for therapeutic tissue repair purposes. Our main focus was to test if stem cells can rescue CNS myelin degeneration, therefore we used dysmyelinating shiverer mice as our model. The shiverer animal model has a very strong phenotype, with almost no variations between individual shi/shi animals. Most importantly the pathology of Shi is distributed throughout the CNS. After generating chimeric mice, we were able to determine the quantity of shi/wild type cells present in each pup, by using Q-PCR to identify wild and mutant type MBP intron 1. The results showed that there is a strong relationship between levels of ES^{wt} derived cells present in each pup and motor function rescue. The data showed that with as little as 1% of ES^{wt} derived cells present in

a pup we saw a small improvement in their motor function and anything above 7% seemed to rescue the phenotype of the chimeric animals.

Myelin therapy – human pluripotent stem cells for brain repair

The fact that pluripotent ES cells are derived from blastocysts creates a wide range of ethical controversies, as embryos are destroyed in generating these stem cells. These issues have hindered the study of human ES cells. To eliminate this problem, recent studies have identified four specific transcription factors (Sox2, Oct3/4, Klf4 and c-Myc) which can reprogram a mouse or human fibroblast into induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). These factors when introduced into fibroblasts by retrovirus-mediated transfection give rise to iPS cells which are remarkably similar to ES cells in morphology, proliferation, gene expression and their potential to differentiate into all germ layers (Okita et al., 2007; Wernig et al., 2007) and are able to generate chimeric mice (Wernig et al., 2007). The generation of iPS cells from human fibroblasts has also very recently been reported (Takahashi et al., 2007). These recent discoveries have attracted much attention, due to their potential to advance human stem cell research while no longer invoking ethical issues. Furthermore, iPS cell have the potential to generate patient-specific ES cells for therapy. This will improve the quality of therapeutic approaches of cell transplantation by eliminating one of the biggest concerns, which is immune rejection.

5. REFERENCES

- Armstrong, R., Friedrich, V.L., Jr., Holmes, K.V., and Dubois-Dalcq, M. (1990). In vitro analysis of the oligodendrocyte lineage in mice during demyelination and remyelination. *J Cell Biol* 111, 1183-1195.
- Battiste, J., Helms, A.W., Kim, E.J., Savage, T.K., Lagace, D.C., Mandyam, C.D., Eisch, A.J., Miyoshi, G., and Johnson, J.E. (2007). Ascl1 defines sequentially generated lineage-restricted neuronal and oligodendrocyte precursor cells in the spinal cord. *Development* 134, 285-293.
- Bauman, T.M., and Kasper, L.H. (2004). Novel approaches and cutting edge immunotherapies in multiple sclerosis. *Front Biosci* 9, 2302-2322.
- Billon, N., Jolicoeur, C., Ying, Q.L., Smith, A., and Raff, M. (2002). Normal timing of oligodendrocyte development from genetically engineered, lineage-selectable mouse ES cells. *J Cell Sci* 115, 3657-3665.
- Blight, A.R. (2002). Miracles and molecules--progress in spinal cord repair. *Nat Neurosci* 5 Suppl, 1051-1054.
- Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 309, 255-256.
- Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., and McKay, R.D. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285, 754-756.
- Buehr, M., Meek, S., Blair, K., Yang, J., Ure, J., Silva, J., McLay, R., Hall, J., Ying, Q.L., and Smith, A. (2008). Capture of authentic embryonic stem cells from rat blastocysts. *Cell* 135, 1287-1298.
- Buehr, M., Nichols, J., Stenhouse, F., Mountford, P., Greenhalgh, C.J., Kantachuvesiri, S., Brooker, G., Mullins, J., and Smith, A.G. (2003). Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. *Biol Reprod* 68, 222-229.
- Cai, J., Qi, Y., Hu, X., Tan, M., Liu, Z., Zhang, J., Li, Q., Sander, M., and Qiu, M. (2005). Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. *Neuron* 45, 41-53.
- Chen, C.P., Kiel, M.E., Sadowski, D., and McKinnon, R.D. (2007). From stem cells to oligodendrocytes: prospects for brain therapy. *Stem Cell Rev* 3, 280-288.

- Collarini, E.J., Kuhn, R., Marshall, C.J., Monuki, E.S., Lemke, G., and Richardson, W.D. (1992). Down-regulation of the POU transcription factor SCIP is an early event in oligodendrocyte differentiation in vitro. *Development* 116, 193-200.
- Connor, J.R., and Menzies, S.L. (1996). Relationship of iron to oligodendrocytes and myelination. *Glia* 17, 83-93.
- Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154-156.
- Filbin, T.T. (2003). Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nature Reviews Neurosci.* 4, 703-713.
- Franklin, R.J., and Blakemore, W.F. (1997). To what extent is oligodendrocyte progenitor migration a limiting factor in the remyelination of multiple sclerosis lesions? *Mult Scler* 3, 84-87.
- Frohman, E.M., Racke, M.K., and Raine, C.S. (2006). Multiple sclerosis--the plaque and its pathogenesis. *N Engl J Med* 354, 942-955.
- Fruttiger, M., Karlsson, L., Hall, A.C., Abramsson, A., Calver, A.R., Bostrom, H., Willetts, K., Bertold, C.H., Heath, J.K., Betsholtz, C., *et al.* (1999). Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development* 126, 457-467.
- Gao, L., and Miller, R.H. (2006). Specification of optic nerve oligodendrocyte precursors by retinal ganglion cell axons. *J Neurosci* 26, 7619-7628.
- Glaser, T., Perez-Bouza, A., Klein, K., and Brustle, O. (2005). Generation of purified oligodendrocyte progenitors from embryonic stem cells. *FASEB J* 19, 112-114.
- Hadjantonakis, A.K., Gertsenstein, M., Ikawa, M., Okabe, M., and Nagy, A. (1998). Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* 76, 79-90.
- Kiel, M.E., Chen, C.P., Sadowski, D., and McKinnon, R.D. (2008). Stem cell-derived therapeutic myelin repair requires 7% cell replacement. *Stem Cells* 26, 2229-2236.
- Kyba, M., Perlingeiro, R.C., and Daley, G.Q. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 109, 29-37.
- Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R.E., Schulze, E.N., Song, H., Hsieh, C.L., *et al.* (2008). Germline competent embryonic stem cells derived from rat blastocysts. *Cell* 135, 1299-1310.

- Lowell, S., Benchoua, A., Heavey, B., and Smith, A.G. (2006). Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol* 4, e121.
- Lu, Q.R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, C.D., and Rowitch, D.H. (2002). Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* 109, 75-86.
- Mabie, P.C., Mehler, M.F., and Kessler, J.A. (1999). Multiple roles of bone morphogenetic protein signaling in the regulation of cortical cell number and phenotype. *J Neurosci* 19, 7077-7088.
- Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78, 7634-7638.
- McKinnon, R.D., Waldron, S., and Kiel, M.E. (2005). PDGF alpha-receptor signal strength controls an RTK rheostat that integrates phosphoinositol 3'-kinase and phospholipase Cgamma pathways during oligodendrocyte maturation. *J Neurosci* 25, 3499-3508.
- Mehler, M.F., Mabie, P.C., Zhu, G., Gokhan, S., and Kessler, J.A. (2000). Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. *Dev Neurosci* 22, 74-85.
- Merchan, P., Bribian, A., Sanchez-Camacho, C., Lezameta, M., Bovolenta, P., and de Castro, F. (2007). Sonic hedgehog promotes the migration and proliferation of optic nerve oligodendrocyte precursors. *Mol Cell Neurosci* 36, 355-368.
- Miyamoto, Y., Yamauchi, J., and Tanoue, A. (2008). Cdk5 phosphorylation of WAVE2 regulates oligodendrocyte precursor cell migration through nonreceptor tyrosine kinase Fyn. *J Neurosci* 28, 8326-8337.
- Noble, M., Murray, K., Stroobant, P., Waterfield, M.D., and Riddle, P. (1988). Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. *Nature* 333, 560-562.
- Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* 448, 313-317.
- Osterhout, D.J., Ebner, S., Xu, J., Ornitz, D.M., Zazanis, G.A., and McKinnon, R.D. (1997). Transplanted oligodendrocyte progenitor cells expressing a dominant-negative FGF receptor transgene fail to migrate in vivo. *J Neurosci* 17, 9122-9132.

- See, J., Zhang, X., Eraydin, N., Mun, S.B., Mamontov, P., Golden, J.A., and Grinspan, J.B. (2004). Oligodendrocyte maturation is inhibited by bone morphogenetic protein. *Mol Cell Neurosci* 26, 481-492.
- Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336, 688-690.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872.
- Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663-676.
- Vallstedt, A., Klos, J.M., and Ericson, J. (2005). Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. *Neuron* 45, 55-67.
- Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448, 318-324.
- Xian, H.Q., McNichols, E., St Clair, A., and Gottlieb, D.I. (2003). A subset of ES-cell-derived neural cells marked by gene targeting. *Stem Cells* 21, 41-49.
- Xian, H.Q., Werth, K., and Gottlieb, D.I. (2005). Promoter analysis in ES cell-derived neural cells. *Biochem Biophys Res Commun* 327, 155-162.
- Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* 21, 183-186.
- Ying, Q.L., Wray, J., Nichols, J., Battle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453, 519-523.