AN IMPROVED METHOD FOR GENERATING OLIGODENDROCYTE PROGENITOR CELLS FROM MURINE INDUCED PLURIPOTENT STEM CELLS

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Table of Contents

List of Tables

List of Figures

INTRODUCTION

The oligodendrocyte is a crucial structural and functional element of the central nervous system (CNS). A variety of disorders affect these cells, which may lead to their loss, resulting in neurological deficits, suffering, and death. Clinical therapies are lacking in virtually all of these diseases. Stem cell technologies and cell transplantation offer a potential avenue of treatment for the broad spectrum of oligodendrocyte pathologies.

Oligodendrocytes & Oligodendrocyte Pathology

The major role of oligodendrocytes is the production of lipid rich myelin sheaths that segmentally envelop axons, serving as insulation, and organizing distribution of voltage gated channels. They so enable saltatory conduction of signals throughout the neuraxis [1]. In addition, the oligodendrocyte provides protection and trophic factors of vital importance to the survival of axons, such as proteolipid protein (PLP) and sirtuin 2 [2-5]. Without these provisions, the CNS is not able to operate properly.

Dysmyelination results in loss of organization of voltage-dependent channels on the axolemma [4, 5]. Initially, this causes conduction block, but subsequently, by a compensatory mechanism, voltage gated sodium channels NaV 1.2, are inserted into the previously internodal axonal membrane [1]. These

channels, which are ordinarily expressed on neurons only prior to myelination, enable propagation of action potentials due to their small sodium conductance [6, 7]. However, NaV 1.6 channels, normally expressed only at the nodes of Ranvier following myelination, also become widely distributed across the axolemma [8]. Their high sodium conductance leads to a pathological increase in the cytoplasmic concentration of sodium, causing protease activation and axonal degeneration.

Myelin can also play a protective role as it shields the axon from inflammatory mediators. During an inflammatory process, such mediators are released into the axonal environment, where in absence of myelin they deplete ATP and disturb mitochondrial function, resulting in malfunction of oxidative metabolism within the axon itself. The Na^{+}-K^{+} ATPase that is required for extruding sodium from the axon fails, leading to high sodium concentrations [9]. This in turn increases the activity of the Na^{+}-Ca^{2+} exchange channel, which requires influx of more calcium ions, to counter the efflux of sodium [10]. High intra-axonal calcium concentrations in turn activate axonal proteases, which lead to fragmentation of neurofilament, thus disintegrating axonal structure and disturbing axonal transport [11].

Several oligodendrocyte factors have been identified that appear to have a primary role of maintaining axonal stability, rather than being responsible for myelin formation. Transgenic mice lacking proteolipid protein (Plp) for example, an integral myelin sheath protein, show long term axonal degeneration in spite of

normal myelin formation [3]. Similar conclusions can be made from observations in human demyelinating conditions, such as multiple sclerosis (MS), where remyelination prevents axonal degeneration [12], or Pelizaeus-Merzbacher disease where lack of myelin leads to axonal loss [2].

Disorders affecting oligodendrocytes are numerous and invariably result in profound morbidity, many ultimately leading to death. They can be divided into two main categories: primary and secondary – based on the relationship between oligodendrocytes and the initial locus of pathology [13]. Primary demyelination results either from inborn errors of metabolism within, or direct inflammatory damage to oligodendrocytes. Genetic disorders affecting myelination can be grouped into those affecting lysosomal function (metachromatic leukodystrophy and Krabbe's disease), those impairing peroxisomal function, such as adrenoleukodystrophy, and those resulting in deficient or abnormal myelin proteins (Pelizaeus-Merzbacher disease) [14]. Although relatively rare, with onset in early infancy, these disorders are a cause of great suffering and early death.

MS is the prototypical inflammatory disorder leading to loss of myelin, and the most common inflammatory disorder of the CNS. With a lifetime risk of 1 in 400 and onset in young adulthood, this disabling disease represents a significant burden on the population of the northern latitudes [15]. The cost of the disease is estimated to be $ 47,215 per patient per year in the United States [16], and in the European Union alone, the yearly cost of this disease is estimated to total 9

billion euros [17]. While our understanding of the underlying pathophysiology is very limited, it ultimately results in loss of oligodendrocytes and subsequent degeneration of axons, the cause of permanent neurological sequelae [13].

Secondary demyelination occurs after non-selective insults to all elements of the CNS, where oligodendrocytes are bystanders in the way of generalized pathology – such as ischemia, infection, or trauma.

A significant subset of patients with cerebral palsy includes those with periventricular leukomalacia. This disorder is characterized by loss of oligodendroglia and/or their precursors in the later stages of fetal development, due to a variety of possible insults in utero [13]. Absence of myelinated white matter is the direct cause of debilitating features of this disorder.

Progressive multifocal leukoencephalopathy (PML) is an infectious disorder, related to immune suppression and is seen almost exclusively in patients with HIV/AIDS. It is related to reactivation of the polyomavirus JC which induces a lytic infection of oligodendrocytes. The disease is fatal in most cases, but survivors are left with permanent neurological deficits due to the subcortical demyelinating process [18]. Another etiology of myelin loss is acute demyelinating encephalomyelitis. This rare and poorly understood process involves an immune attack directed against oligodendrocytes, in the wake of a generalized viral or bacterial infection. It has been associated with measles, varicella, infectious mononucleosis, and non-specific febrile illness. Although most patients recover completely, some are left with permanent disability due to

loss of myelin [19].

Stroke, one of the most common causes of neurological disability, is caused by non-discriminatory loss of CNS tissue due to ischemia. Among all cell types that make up the CNS are oligodendrocytes and their precursors, which are lost after their nutrient supply is cut off. Specifically, stroke can occur in areas of white matter, thus leading to demyelinating lesions.

Traumatic injury to the CNS, especially of spinal cord, leads to loss of tissue due to direct mechanical disruption. The initial area of injury however is broadened, regardless of location in the CNS axis, by secondary injury mechanisms. Disruption of vasculature and the blood-brain-barrier leads to infiltration of elements – molecules and cells of the immune system – that are harmful to the neighboring non-injured tissue. Extravasation of blood into the nervous tissue triggers activation of the clotting cascade needed for coagulation. Subsequent activation of complement and release of cytokines by a variety of the components of injured tissue cause an influx of leukocytes and macrophages, further augmenting the inflammatory reaction. All these processes lead to generation of free radicals and reactive oxygen species that cause more damage in the previously intact region bordering the epicenter of injury. Furthermore, prohibitive to regeneration are myelin debris itself and formation of an astrocytic scar [20, 21]. Excitotoxicity and direct cellular damage lead to apoptosis of both neurons and oligodendrocytes. Loss of oligodendrocytes and myelin around intact axons exposes them to injury, dysfunction and disintegration, as described

above. Indeed, it is this loss of myelin that contributes to deterioration of neurological function following initial spinal cord injury [22].

Evidence is starting to emerge implicating oligodendrocyte pathology in certain neurodegenerative and psychiatric disorders, such as ALS [23], schizophrenia and bipolar disorder [24, 25]. The relationship of the diseased oligodendrocytes in those conditions however is not yet known.

Endogenous Remyelination - The Oligodendrocyte Progenitor Cell

The adult CNS retains the ability to regenerate oligodendrocytes that are lost due to a variety of pathological mechanisms. Such regenerated oligodendrocytes are able to functionally remyelinate the denuded axons left in the wake of the insult. The process of remyelination occurs in a variety of experimental settings in adult animals, such as the cuprizone induced demyelination model [26], after direct delivery of lysolecithin or ethidium bromide [27], and in experimental autoimmune encephalomyelitis [13]. Evidence for remyelination exists also in cases of traumatic injury, specifically after spinal cord injury [28], where myelin can arise from Schwann cells of peripheral nerve and central progenitor cell origin [29]. In the adult human, remyelination is commonly seen in cases of MS [30].

The degree to which surviving mature oligodendrocytes can contribute to remyelination is not completely resolved, but existing evidence indicates that this

is little or none. Instead, oligodendrocyte proliferation and remyelination are linked to the adult oligodendrocyte progenitor cell (OPC), a pluripotent glial precursor found in the CNS [13]. OPCs are found in both white and gray matter, representing approximately 5-8% of the cell population (similar to the density of microglia) [31]. They are multi-processed cells, characterized by the expression of markers such as the proteoglycan NG2, the platelet-derived growth factor receptor-α (PDGFR-α), the bHLH transcription factors Olig1 and Olig2, and O4 (13, 31 – 33). The evidence suggesting OPCs as the primary mediators of remyelination is indirect, but multifaceted, and based mainly on rodent studies [13, 32]. Indirect retroviral and autoradiographic tracing has demonstrated that cells dividing within the white matter can give rise to remyelinating oligodendrocytes [33, 34]. Focal areas of demyelination tend to harbor OPCs before the appearance of oligodendrocytes, with a spatial and temporal pattern supporting the idea that OPCs repopulate damaged white matter and lead to remyelination [31, 35]. Finally, OPCs that are transplanted into demyelinated areas restore the lost myelin with great efficiency [13].

In response to injury, astrocytes and microglia secrete a variety of signals likely to activate the endogenous OPC population [36-38]. OPCs undergo a transition from a quiescent state to proliferation, changing morphology [31] and upregulating expression of genes that are associated with their development. In fact, many parallels can be drawn between the normal developmental process of myelination and remyelination following oligodendrocyte pathology. The guiding

cellular mechanisms in both instances involve the same molecular players and are summarized in Figure 1 [36].

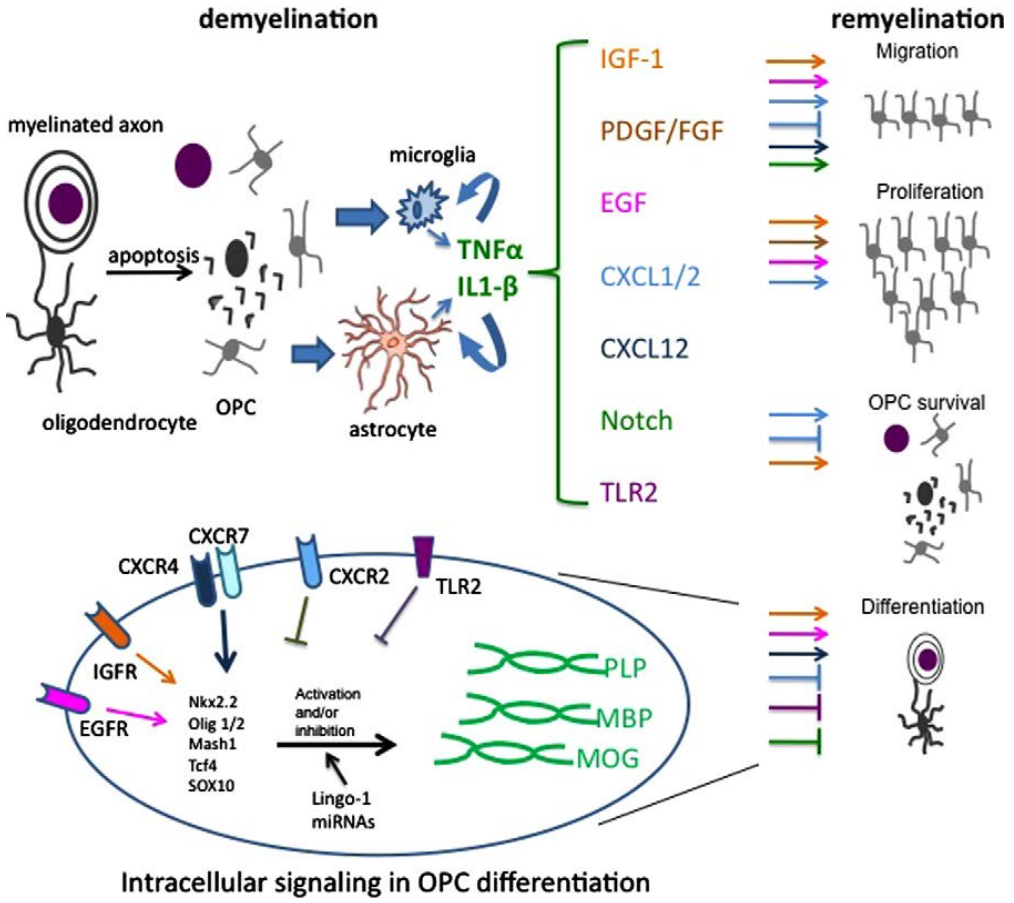


Figure 1. Putative remyelination mechanisms, adapted from Patel and Klein ,2011 [36]. Depicted are roles for cytokines, chemokines, growth factors, transcription factors and other signaling proteins in remyelination of demyelinated lesions in MS. During demyelination, oligodendrocytes undergo apopotosis, leaving debris, which may contribute to the activation of microglia and astrocytes, which then express cytokines (TNF-a, IL-1b). Cytokines alter expression of chemokines (CXCL1, CXCL2, CXCL12) and growth factors (IGF-1, PDGF, FGF,EGF), whose activation or inhibition of various aspects of remyelination are depicted via color-coding of arrows and signs of blockade, respectively, for each biologic process (migration, proliferation, OPC survival and differentiation). For certain molecules, such as CXCL1/2, opposing effects on OPCs have been observed, depending on the animal model used. Growth factors differentially impact on cell survival and proliferation while chemokines contribute to migration and differentiation, depending on OPC position.TLR2 activation inhibits OPC differentiation. Notch-1 also blocks OPC differentiation to promote migration. Molecules that regulate OPC maturation are depicted in the context of intracellular signaling during differentiation. Included are chemokine (CXCR4/7, CXCR2) and growth factor (IGF-1R, EGFR) receptors, miRNAs, Lingo-1 and several transcription factors (Nkx2.2, Olig1/2, Mash1, Tcf4, SOX10) which may negatively or positively impact on the expression of myelin proteins including proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG).

Development of the Oligodendrocyte Progenitor Cell

OPCs arise in the ventral aspect of the neural tube, during the middle period of neurogenesis. Sonic hedgehog (SHH) signaling is of crucial importance for the induction of this progenitor population. From their ventral origin they migrate dorsally, causing a ventral to dorsal myelination gradient. This temporal and spatial relationship of the initial OPC wave is seen throughout the entire neuraxis, in humans as well in rodents. In the telencephalon, the initial OPC spread originates from the medial and lateral ganglionic eminences, emerging in the rostral migratory pathway. Later on however, OPCs are generated in the dorsal cortical subventricular zone (SVZ). There is further evidence suggesting multiple origins of OPCs throughout the developing CNS, especially during human fetal development [32, 39].

OPCs from the SVZ are characterized by their expression of glial fibrillary acidic protein (GFAP), and give rise to both oligodendrocytes and astrocytes, and are capable of self-renewal. OPCs residing in the hippocampus however, are also able to generate neurons [13]. The different origins of OPCs may suggest redundancy, or that they differ in other aspects as well, such as the type of axon or pathway they go on to myelinate. The common thread however is their widespread migration and differentiation into myelinating oligodendrocytes. Furthermore, if one of the OPC origins is destroyed, the others are capable of taking over, ultimately resulting in intact myelination of the entire CNS [40].

Failure of Endogenous Remyelination and Rationale for Exogenous Therapy

In spite of ample evidence that remyelination is possible after a variety of insults to the white matter, it is insufficient in most instances, thus failing to rescue the affected organism from neurological deficit. MS and traumatic injury are prime examples of this scenario, among others mentioned above. This fact lays down the foundation for a rationale for exogenous oligodendrocyte replacement and remyelination therapies.

Endogenous OPCs fail to remyelinate for a number of reasons. Age has been shown to correlate inversely with the efficiency of remyelination, as it decreases the efficiency of OPC recruitment and their ability to differentiate [41-43]. Histone deacetylases, which play an important role in OPC differentiation, are impaired in older animals [44, 45]. A disparity has also been observed between the different sexes, with decline in remyelination capability occurring faster in male than female rats [46]. The aging of the host environment could also be responsible for the observed trend. Macrophages are slower to react as they age, and thus the inflammatory response to the pathological insult is delayed, resulting in delayed clearance of debris that inhibits remyelination [47].

Combined with these generic factors, each disease affecting oligodendrocytes may exert specific influence interfering with remyelination. In MS for example, an abnormality in expression of semaphorins appears to inhibit

proper migration of OPCs to the sites where they are needed [48]. Moreover, the disease process can affect OPCs directly, as specific antibodies directed against the OPC proteoglycan NG2 raise the possibility of an immune attack on this cell type [49].

In the light of inadequate endogenous remyelination, two avenues of treatment are possible. One option is to address the failure of, and enhance the intrinsic mechanism. Proponents of this method argue that since complete remyelination is possible, there ought to be a way to identify its shortcomings when it fails, and address those with non-invasive methods. No meaningful progress has however been made in this area to date [13].

On the other hand, transplantation methods aimed at establishing remyelination have been studied extensively over the past three decades [50-52]. Transplantation of oligodendrocytes themselves did not yield any success likely due to the inability of such mature cells to myelinate [53]. A variety of other cell types, including OPCs [54-56], have all been able to restore myelin after transplantation into different models of demyelination. Such cells include Schwann cells, for which a clinical trial addressing spinal cord injury is under way [50, 57, 58], and olfactory ensheathing cells [59-61]. Neural stem cell lines [62], and embryonic stem cell derived glial precursors [63] have also shown new myelin formation, probably due to their differentiation into OPCs and eventually oligodendrocytes. These studies serve as proof of principle that the cell therapy approach may be feasible.

OPC transplantation and iPS cell derived OPCs

Significant leaps have been made in the field of cell based therapy aimed at remyelination. Embryonic stem cell derived glial precursors have been applied in phase I clinical trials for Pelizaeus-Merzbacher disease, and in spinal cord injury. Although the latter study was abandoned due to financial reasons, the former demonstrated a safe profile, at least during the first year following transplantation [64, 65].

Despite such progress, many questions remain relating to both cell type and optimizing their delivery. Cell numbers are limited, as is their expansion potential, thus necessitating repeated harvesting of tissues that are very difficult to come by. Utilizing human embryonic stem cells to culture glial progenitor cells, and OPCs in particular, is an attractive method, however requires immunosuppression of the host, due to allogenicity [66].

The advent of induced pluripotent stem cell technology is promising in several regards. iPS cells are generated by forced expression of several transcription factors which lead to reprogramming of the cell's genome activity and revert it to a state of pluripotency. Such reprogrammed cells are defined by their ability to form all three germ layers, teratomas, infinite division under defined culture conditions, and expression of markers of pluripotency [67]. This approach enables us to generate pluripotent cells from adult somatic sources, thus eliminating any concern for limited supply. Also, it is possible to generate stem

cells on an individual basis, circumventing the issue of immune rejection, should they be applied in transplantation therapies.

OPCs have already been derived from mouse iPS cells but the published protocols have very low yields [68, 69]. Although OPCs can be generated from human iPS cells [70], the mouse cell remains a valuable model in which to further the understanding of mechanisms of their differentiation. The generation of iPS cells currently requires genome manipulation. The risk of insertional mutagenesis that is inherent to retroviral approaches currently prevents these cells from being eligible for clinical trials. As better methods are rapidly developed, such as non-integrating viral vectors, and non-viral methods of genome editing, the iPS cell could reach clinical potential. Further human trials may be underway sooner than anticipated, but the ability to analyze tissue post-transplant remains a great advantage of animal studies. Such studies may enable us to gain further insights into remyelination, and possibly open doors to further improvement and enhancement of the process. Published protocols designed to direct differentiation of mouse derived stem cells into OPCs share several aspects, but diverge mostly in timing, as outlined in Table 1. A discrepancy in yields is observed between protocols designed for mouse iPS cells and those for embryonic stem cells. With that in mind, we sought to improve upon the existing protocols attempting to differentiate OPCs from iPS cells.

Study:	**Tokumoto et al.**	**Czepiel et al.**	**Jiang et al.**	**Najm et al.**
Stem Cell	*Mouse iPS*	*Mouse iPS*	*Mouse ES*	*Mouse EpiSC*
Day 1	EB form, ES medium	EBs form, ES medium	EBs form, KSR medium	Cells, KSR + NOGG + DORS
Day 2	ES	ES	KSR	N2 + NOGG + DORS
Day 3	ES	ES	KSR	N2 + NOGG
Day 4	ES	ES	KSR + RA	N2 + NOGG + RA + SHH
Day 5	ITS/fibronectin medium	ES	KSR + RA + PUR	Dissoc. PolyO + Laminin N2+ FGF2 + PDGF-AA + SHH
Day 6	ITS/fibronectin	ES	N2 + RA + PUR	N2+ FGF2 + PDGF-AA + SHH
Day 7	ITS/fibronectin	ES	N2 + RA + PUR	N2+ FGF2 + PDGF-AA + SHH
Day 8	ITS/fibronectin	Dissociation, N2 + FGF2 + EGF	Dissociation, N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 9	ITS/fibronectin	N2 + FGF2 + EGF	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 10	ITS/fibronectin	N2 + FGF2 + EGF	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 11	Dissociation, N2 + FGF2 PolyO + Fibronectin	N2 + FGF2 + EGF	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 12	N2 + FGF2	N2 + FGF2 + EGF	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 13	N2 + FGF2	N2 + FGF2 + EGF	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 14	N2 + FGF2 + EGF	N2 + FGF2 + EGF	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 15	N2 + FGF2 + EGF	N2 + FGF2 + EGF	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 16	N2 + FGF2 + EGF	Laminin coat, N2 + PDGF-AA	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 17	N2 + FGF2 + EGF	N2 + PDGF-AA	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day 18	N2 + FGF2 + PDGF-AA	N2 + PDGF-AA	N2 + FGF2	N2+ FGF2 + PDGF-AA + SHH
Day ...	...	...	...	...
Yield:	12.6%	18%	96.4%	90%

Table 1. Summary of published protocols for directed differentiation of stem cells into OPCs and oligodendrocytes: Tokumoto et al. [69], Czepiel et al. [68], Jiang et al. [72], Najm et al. [73]. ES: embryonic stem cell; EpiSC: Epiblast stem cell; EB: embryoid body; ES medium: embryonic stem cell medium with serum; KSR: Knockout serum replacement medium; N2: N2 supplemented neurobasal medium; PolyO: poly-L-ornithine coating; RA: Retinoic acid; PUR: purmorphamine; NOGG: noggin; DORS: dorsomorphin; SHH: sonic hedgehog.

MATERIALS AND METHODS

General Experiment Outline

Due to the high yields achieved with mouse embryonic stem cells, and the characteristics they share with iPS cells, we initially set out to apply the protocol devised for ES cell differentiation into OPCs [72] on mouse iPS cells. After failing to achieve comparable results, the latter, OPC expansion part of the protocol devised by Nejm et al. [73] was added, leading to much improved results as described below.

iPS Cell Reprogramming and culture

Mouse iPS cell lines were generated and characterized as described previously[71]. Three cell lines expressing green fluorescent protein were subjected to the main experiments: 3F10, JG2, and JBl6. The 3F10 line harbors tamoxifen-inducible Cre recombinase MerCreMer, under the control of the endogenous Oct4 locus, as well as the double fluorescent mT/mG Cre reporter. This construct allows us to replace expression of tdTomato with enhanced green fluorescence protein (eGFP) via exposure to tamoxifen [71]. iPS cells were cultured on irradiated embryonic mouse fibroblasts in mES media (Knockout-

DMEM (Gibco) containing: 20% fetal bovine serum (HyClone), 1X NEAA, 1X L-Glutamine, 100µM 2-mercaptoethanol (Sigma) supplemented with LIF (1000u/ml Millipore) at 37^{o}C in 5% CO_2.

OPC differentiation protocol

Day Zero: The iPS cells and feeder cells were disaggregated from plates using TrypLE Express (Gibco). They were subsequently transferred into the KnockOut Serum Embryoid Body media: MEM/EBSS (HyClone), supplemented with 20% KnockOut SR (Gibco), 1mM sodium pyruvate (Sigma), 1 % MEM non- essential amino acids (Gibco), 0.1 mM 2-Mercaptoethanol (Sigma), and 1 % Anti-Anti (Gibco).

The cells were left in suspension in Ultra-Low Attachment Surface 6 well plates (Costar), at a density of 500,000 cells per well, where they were left to aggregate into embryoid bodies. They were kept in the KnockOut Serum Embryoid Body media for 3 days, with daily change of media starting on day two.

On day 4, the media was supplemented with 0.2 micromolar Retinoic Acid (Sigma), and on day five, Retinoic acid and 1 micromolar Purmorphamine (Cayman Chemical).

On day six, the embryoid bodies were switched to the N-2 supplemented media: MEM/EBSS (HyClone), supplemented with N-2 Supplement (Gibco), 1mM sodium pyruvate (Sigma), 1 % MEM non- essential amino acids (Gibco), 0.1 mM

2-Mercaptoethanol (Sigma), and 1 % Anti-Anti (Gibco). That media was changed daily until day 8.

On day 8, the embryoid bodies were treated with TrypLE Express (Gibco), and transferred into the OPC selecting media: DMEM / F12 (Gibco), supplemented with 1x B-27 Supplement (Gibco), 1x N-2 MAX Media Supplement (R&D Systems), 20 ng/mL of FGF-2, 20 ng/mL of rhPDGF-AA, 200 ng/mL of rhShh (R&D Systems), and 1 % Anti-Anti (Gibco).

The embryoid bodies were plated onto tissue culture plates coated with Poly-L-ornithine (Sigma), and the media was changed every other day. They were left to grow to confluence, and passaged in a 1:2 ratio.

Immunocytochemical Characterization

Cultured cells were fixed using 10% formalin for 10 minutes and then washed three times with PBS. Where necessary, cells were permeabilized using 1% Tween in PBS for 5 minutes. The cells were incubated with blocking buffer (1% bovine serum albumin (BSA) in PBS with 0.1%Tween 20) for 30 minutes. Cells were incubated overnight at 4°C with primary antibodies diluted in blocking buffer. After washing three times in PBS-T the cells were incubated with secondary antibodies diluted in blocking buffer for 2 hours at room temperature before final washing. For the primary antibody directed against NG2, the permeabilization step was substituted by antigen retrieval (5 minutes in L.A.B.

Solution, 24310, Polysciences Inc.), and all detergent was omitted from the blocking and washing solutions.

Primary antibodies: glial fibrillary acidic protein (MAB360, 1/400, Millipore), β-tubulin III (T8660, 1/400, Sigma-Aldrich), MBP (AB7349, Abcam), A2B5 (MAB312, 1/250, Millipore), Olig2 (AB9610, 1/250, Millipore), PDGFRα (sc-338, 1/400, Santa Cruz Biotechnology), Olig 1 (MAB5540, 1/100, Millipore), Nestin (NES, Aves Labs), and NG2 (AB5320, 1/100, Millipore).
Secondary antibodies: Alexa Fluor 555 F(ab')2 fragment of goat anti-mouse IgG (H+L) (Invitrogen A21425), Alexa Fluor 555 donkey anti-rabbit IgG (H+L) (Invitrogen A31572), Alexa Fluor 555 goat anti-mouse IgM (µ chain) (Invitrogen A21426), and Alexa Fluor 633 goat anti-mouse IgM (µ chain).
Nuclear contrast was performed with Hoechst nuclear stain (Thermo Scientific).

Fluorescence Microscopy

Direct fluorescence imaging of live cells, fixed cells and fixed and sectioned tissues was performed using DMI 6000B inverted microscope (Leica) equipped with an automated stage and cell culture chamber. Images were captured using a Retiga 2000R camera (QImaging) using IP Lab software (BD Biosciences). EGFP and dTomato expression was detected directly in live and fixed cells.

Total RNA extraction and analysis

Total RNA was prepared from EBs and cells using the RNeasy MiniKit (Qiagen). Genomic DNA was removed from RNA by DNase (Promega) treatment for 1h at 37C. cDNA was generated by reverse transcription from 2 μg total RNA using SuperScript III Reverse Transcriptase and oligo(dT)20 primers (Invitrogen). Quantitative gene expression was analyzed in an Eppendorf lightcycler using predesigned primers (IDT) for the following genes: APC, CNP, GAPDH, GFAP, MBP, Nanog, Nestin, Oct4, Pax 6, Olig1, Olig2, PDGFRa, Nkx 2.2, Syn1, Tuj 1. Samples from three biological replicates were analyzed and results are indicated as means in bar graphs on a logarithmic scale, with standard error bars.

Cell Transplantation

Shiverer mice were obtained from the Jackson Laboratories. Mice were anesthetized with ketamine-xylazine and a burr hole was used as the access for stereotactic injection of cells. Injection coordinates were measured from the bregma: 0.4 mm anterior, 2.2 mm lateral, and at depths of 2.5 and 3.0 mm. Cells were injected at 25,000 per microliter, with a total of 10 microliters. The animals were maintained until 4 and 6 weeks post injection, at which point they were sacrificed, and their brains processed for immunohistochemical analysis and electron microscopy.

Immunohistochemical analysis of transplanted cells

After sacrifice, mice were perfused transcardially with 4% formaldehyde. The brains were removed and stored in 30% sucrose at 4°C until they were embedded in O.C.T. (Tissue-Tek), and frozen. 7-9 □M sections were Leica CM 3050S cryostat. Transplanted cells were identified by eGFP expression under fluorescence microscopy. Primary antibodies: glial fibrillary acidic protein (MAB360, 1/400, Millipore), β-tubulin III (T8660, 1/400, Sigma-Aldrich), and MBP.

RESULTS

Generation of OPCs

The initial attempt of applying the protocol devised by Jiang et al. [72] did not yield successful results. The embryoid bodies failed to attach following the initial eight days of the protocol, and ultimately cells failed to expand. Only after all the factors inducing OPC proliferation were combined [73], did we see survival and expansion of the OPC population beyond eight days.

Our protocol for generating OPCs from mouse iPS cells is outlined in Figure 2. Mouse iPS cells are initially left in KSR media to aggregate into embryoid bodies in ultra-low attachments plates. Subsequently, the media is changed daily, with addition of retinoic acid on the fourth, and purmorphamine on the fifth day. On

the sixth day, KSR media is substituted by N2 supplemented media, retaining retinoic acid and purmorphamine. On day 8, the embryoid bodies are treated with trypleE, and plated on poly-L-ornithine coated tissue culture plates, where they expand in OPC media containing PDGF-AA, sonic hedgehog, and FGF.

Three cell lines were subjected to the protocol: 3F10, JBl6, and JG2. 3F10 and JBl6 were from transgenic animals expressing eGFP.

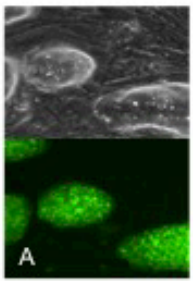
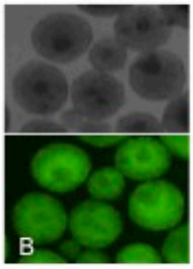
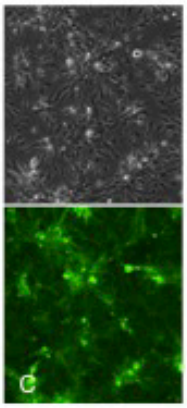
Stage 1 iPS cells		DAY 0 Undifferentiated mouse iPS cells, with transmembranous GFP iPS cell media with LIF
Stage 2 Neuroglial Patterning		Embryoid body formation DAY 1 – 3 KSR media DAY 4 KSR media + RA DAY 5 KSR media + RA + Pur DAY 6 – 8 N2 media + RA + Pur
Stage 3 OPC Expansion		DAY 9 – 25 OPC expansion OPC media with PDGF-AA, SHH, FGF

Figure 2. Modified differentiation protocol for generating OPCs from pluripotent mouse iPS cells. The protocol is divided into three stages: iPS cell maintenance, neuroglial patterning, and OPC expansion. The conditions in each stage are summarized in the right column of the table. Representative micrographs are shown, in phase contrast and green fluorescence imaging, in the middle column.

Characterization of OPCs

Quantitative RT PCR was used to assess expression of key marker genes at various stages of the OPC differentiation protocol (Figure 3). Gene expression was compared to controls of wild type animals at embryonic day 14, lacking mature oligodendrocytes, and those old three weeks, when myelination is actively happening. A continuous decrease was seen in the expression of Oct 4 and Nanog, genes of pluripotency, while genes associated with neuroglial precursors are gradually upregulated (Figure 3A). Upregulation of genes associated with the OPC identity, such as Olig 2, Olig1, and PDGFRα, is noted starting on day 8 (Figure 3B). Genes ordinarily expressed in mature oligodendrocytes, are found at minimal levels throughout the protocol, as are those characteristic of neurons (Figure 3C).

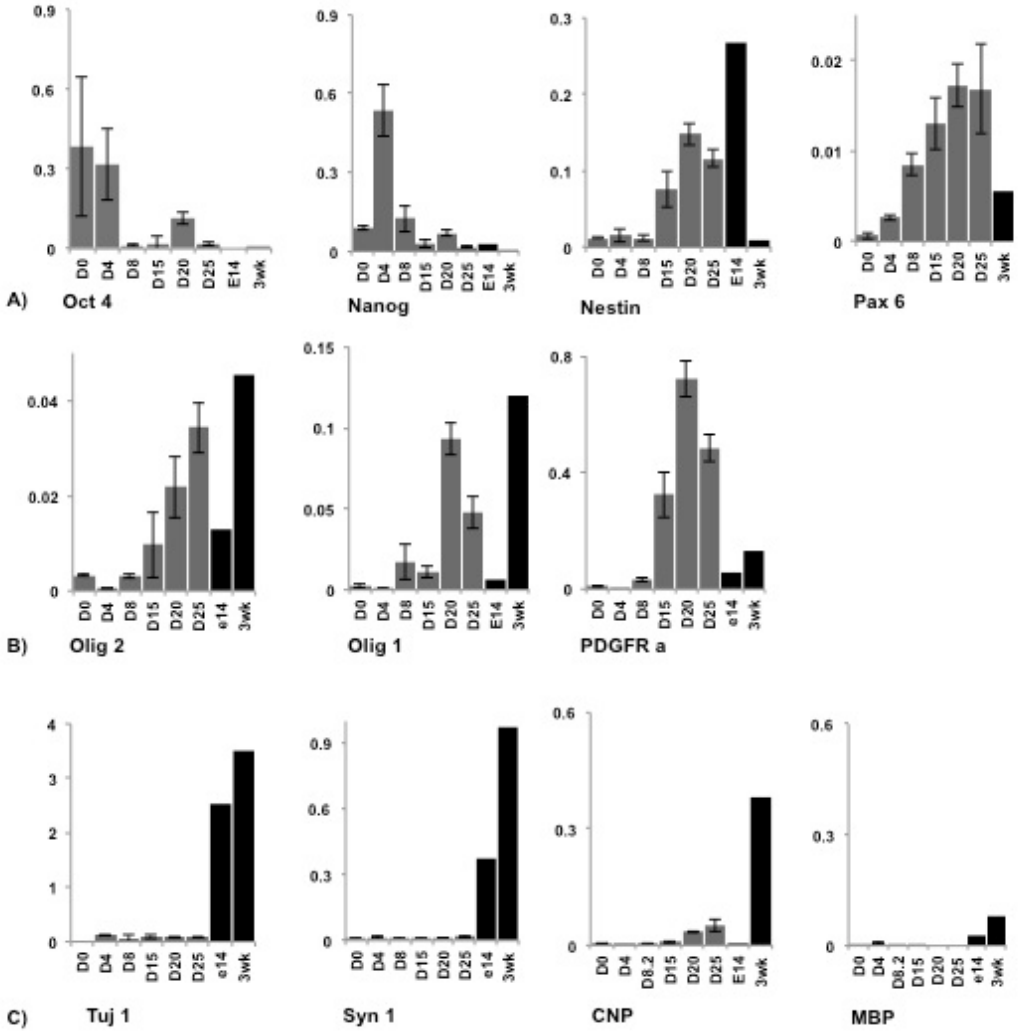


Figure 3. Quantitative analysis of gene expression during differentiation of mouse iPS cells into OPCs. Gene expression on specific days of the differentiation protocol is shown for specific genes. Gene expression is normalized to GAPDH and expressed relative to GAPDH expression. E14 and 3wk represent gene expression in brain tissue from embryonic day 14 and 21 days post natal mice.

Immunocytochemistry confirmed the presence of a variety of OPC markers including Olig 2, Olig 1, PDGFRα, NG 2, A2B5, and Nestin. Markers of the neuronal and astrocytic lineages were also seen, albeit with very low frequency (Figure 4). Markers characteristic of mature oligodendrocytes were not observed, along with markers of pluripotency. We used immunocytochemistry to quantify

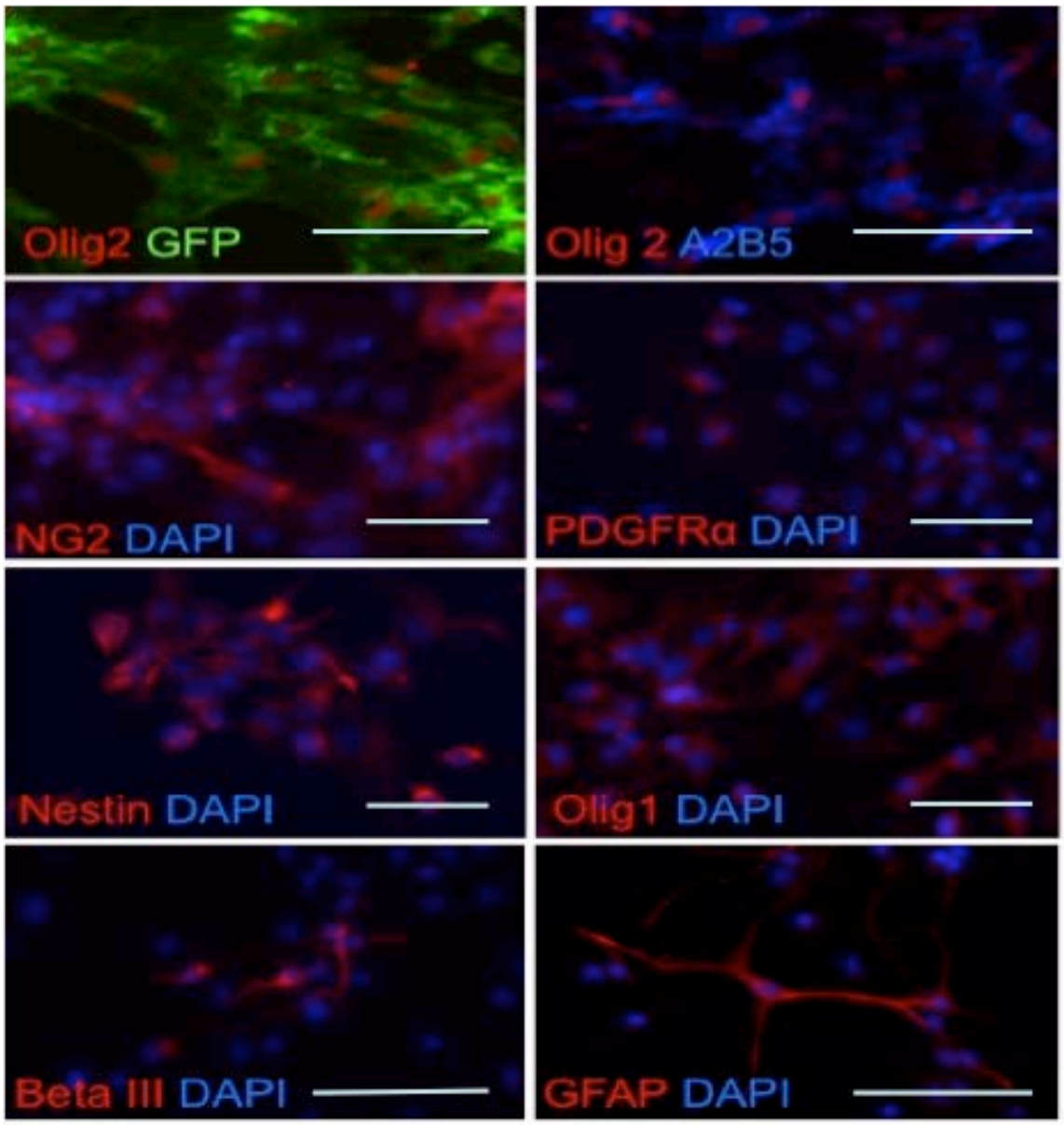


Figure 4. Immunocytochemical analysis of d25 OPCs. Immunocytochemistry demonstrating presence and co-labeling of OPC markers Olig 2, A2B5, as well as Olig 1, NG 2, PDGFRα, and nestin. Occasional presence of neuronal (Beta III), and astrocytic (GFAP) markers was observed. All scale bars represent 50 μm.

OPCs and other cell types in culture following 25 days of the protocol (Figure 5). Two of the three cell lines that were subjected to the protocol yielded 88 and 82 % of Olig 2 positive cells, and both had greater than 70 % double positivity for Olig 2 and A2B5. The third cell line, JG2, demonstrated a more modest yield of 47 % Olig 2 expressing cells (Table 2).

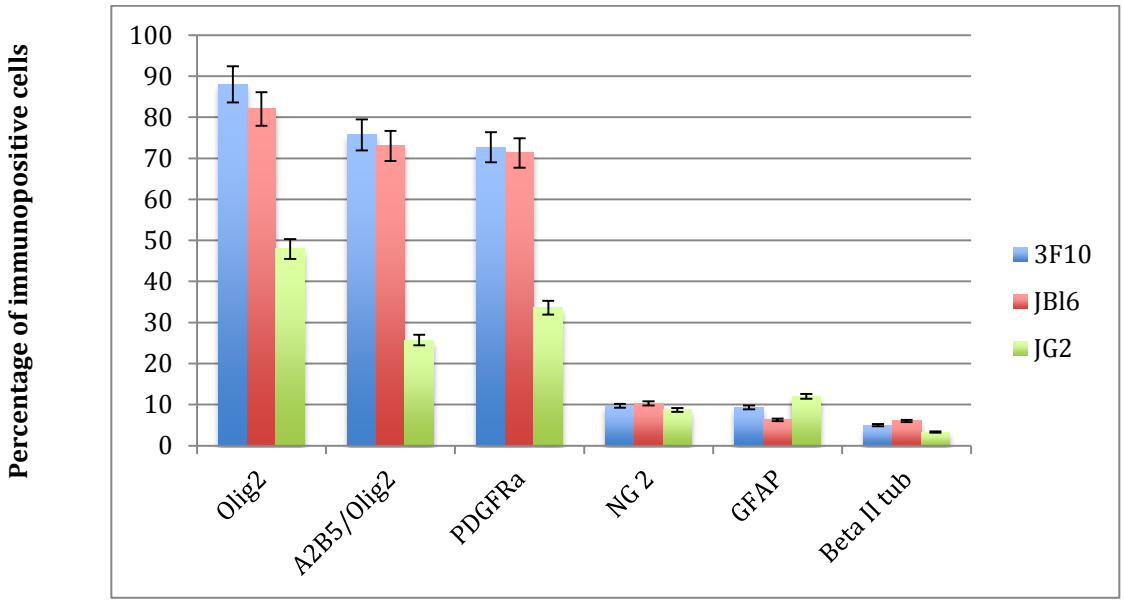


Figure 5. Expression of OPC, neural, and glial markers following 25 days of OPC differentiation protocol. Cultures were immunostained for Olig 2, A2B5, PDGFRα, NG 2, GFAP, and beta III tubulin. The proportion of immunopositive cells was scored for each marker and each cell line: 3F10, JBl6, and JG2. At least three repeats were performed in each group. Data are provided as means ± SEM.

	3F10 Day 25
Olig 2⁺	88 ± 1.3
A2B5⁺ Olig 2⁺	75.7 ± 3.8
PDGFR α⁺	72.7 ± 3.7
NG 2⁺	9.7 ± 0.8
GFAP⁺	9.3 ± 3.1
Beta III tub⁺	5.0 ± 2.5

	JBl6 Day 25
Olig 2⁺	82 ± 6.1
A2B5⁺ Olig 2⁺	73 ± 2.0
PDGFR α⁺	71.3 ± 1.9
NG 2⁺	10.3 ± 2.6
GFAP⁺	6.3 ± 2.4
Beta III tub⁺	6.0 ± 1.5

	JG2 Day 25
Olig 2⁺	47.9 ± 2.1
A2B5⁺ Olig 2⁺	25.7 ± 2.0
PDGFR α⁺	33.6 ± 1.8
NG 2⁺	8.7 ± 1.2
GFAP⁺	12.0 ± 1.5
Beta III tub⁺	3.3 ± 0.7

Table 2. Expression of OPC, neural, and glial markers following 25 days of OPC differentiation protocol. Percentage of cells scoring positive for detection of respective antigen by immunocytochemistry. Percentages illustrated in Figure 5.

In vivo myelination

In order to assess functionality of OPCs generated via our protocol *in vivo*, they were transplanted into brains of *shiverer* mice – a model of congenital dysmyelination, lacking mature oligodendrocytes and myelin basic protein. The mice were maintained for four weeks following transplantation and their brains were subsequently obtained for immunohistochemical analysis. The transplanted OPCs demonstrate ability to migrate, as eGFP positive cells appear in a circumferential distribution surrounding the initial site of transplantation (Figure 6. A, D). Furthermore, myelin basic protein is observed, indicating presence of mature oligodendrocytes (Figure 6. B, C).

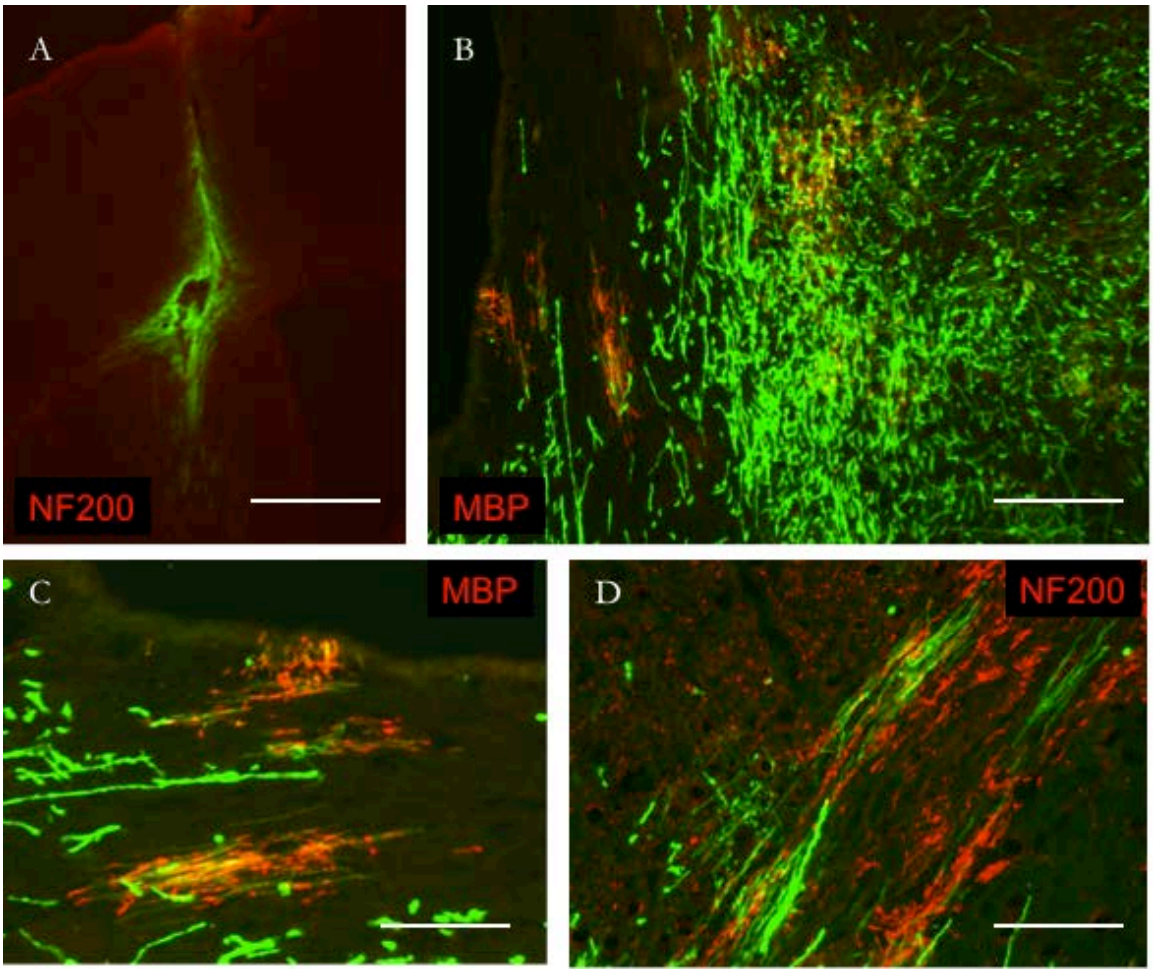


Figure 6. Functional analysis of OPCs following transplantation into *Shiverer* mouse brains. A, B. Differentiating OPCs 28 days following transplantation, eGFP and Neurofilament (red). C, D. Myelin expression by OPCs 28 days after transplantation, eGFP and MBP (red). Scale bars (A) 500 μm, 200 μm (B), 50 μm (C), 100 μm (D).

Discussion

In the present study, we demonstrate a novel protocol for derivation of OPCs from mouse iPS cells. Although two methods have been previously described, they report rather low efficiencies, with OPC and oligodendrocyte yields ranging from 12 to 18%[68, 69]. In contrast, our yield exceeds 80%. Our protocol was devised through optimization and combination of elements from existing protocols intended for OPC derivation from mouse embryonic and epiblast stem cells [72, 73].

We initially set out to replicate the protocol devised by Jiang et al. with the hypothesis that similarities between embryonic stem cells would lead to parallel results. Following the initial eight days however, the cells failed to attach in culture and proliferate. After the addition of OPC media, as outlined by Najm et al., containing Shh, PDGF-AA, and FGF, cell survival, attachment, and proliferation improved significantly.

The protocol consists of several transitions through defined media, aimed to replicate the developmental environment favoring differentiation of OPCs. Stage two of the protocol was adopted from Deng et al. [72], guiding the initial cohort of iPS cells to a neuroglial fate over a period of 8 days. Retinoic acid, added on day 4, supports neural specification whilst inhibiting mesodermal differentiation via blockade of the Nodal signaling pathway[74]. Subsequently, the

SHH pathway is activated by addition of purmorphamine (reference), and directs neural progenitors toward the OPC fate by upregulating genes essential for OPC specification, such as Sox 10, Olig 2, and Nkx 2.2 [75-77].

The third stage utilizes PDGF-AA, a factor known to promote proliferation and differentiation of pre-OPC progenitors into OPCs. FGF is a potent mitogen that aids expansion of OPCs, along with the support of SHH which potentiates the drive toward the OPC fate. Thus, during this stage, the cell population is enriched with OPCs through targeted proliferation of this cell type [78].

Characterization of OPCs was accomplished utilizing a panel of established OPC markers via immunocytochemistry, as well as RT-PCR. Immunostaining revealed presence of all the OPC markers in comparable amounts, with the exception of NG 2. Lower detection of this surface proteoglycan molecule may be explained by technical difficulty of its preservation through the fixation and staining process, as has been described previously in literature[39]. It certainly proved to be a challenging task to optimize the immunostaining protocol for this particular marker.

On the other hand, the sequence and timing of expression and co-expression of OPC markers still remains to be defined. The differentiation pathway of an OPC toward a mature oligodendrocyte, although a continuous spectrum, may harbor a variety of pre-OPC subtypes [78]. Thus, it is possible that NG 2 may not be highly expressed during all stages of the OPC lifetime.

RT-PCR revealed an increase in expression of genes characteristic for OPCs, such as Olig 1 and 2, Nkx 2.2, and PDGFRα. This not only corroborates our immunocytochemical findings, but also reveals a temporal profile of gene activity during the various stages of the protocol. While there is a gradual increase in expression of OPC defining genes, there is a decline in expression of genes related to pluripotency, such as Oct 4 and Nanog. An expected increase in expression of Nestin and Pax 6, characteristic of neuroepithelial precursors, is seen as well. Markers of mature oligodendrocytes, MBP, APC, and CNP, do not demonstrate a significant expression. Markers of neurons and astrocytes however are expressed in trace amounts, as observed by the presence of those cell types in culture.

In contrast to the previously reported studies, we used three different iPS cell lines to demonstrate efficiency and robustness of our protocol. While comparable results were achieved with two of the cell lines, 3F10 and JBl6, the yield from JG2 was a modest 47%, as judged by Olig 2 expression. Nevertheless, the number is significantly greater in comparison to previous reports in literature. This discrepancy however may be due to several reasons, the major concern of which would be underlying difference between iPS cell lines that lies beyond the reach of our standard characterization methods. Further research into the nature of iPS cells and epigenetic phenomena of cell reprogramming may shed more light on this subject.

The ultimate confirmation of OPC functionality comes from *in vivo* transplantation studies. Of the two previously published protocols for differentiation of mouse iPS cells to OPCs, only one demonstrated such evidence[68]. The *shiverer* mouse carries an autosomal recessive mutation (*shi*) that results in lack of myelin and MBP expression. As such, it is an excellent model of congenital dysmyelination. By transplanting OPCs into brains of *shiverer* mice it is possible to assess the capability of OPCs to differentiate into mature oligodendrocytes. Such oligodendrocytes will give rise to MBP that is undetectable in control animals. OPCs generated with our protocol were indeed able to give rise to MBP, and thus we can infer mature oligodendrocytes. The fact that the transplanted cells express eGFP facilitated their observation under fluorescence microscopy. Furthermore, co-localization of MBP and eGFP signals provides evidence that the formed myelin stems from the transplanted cell population.

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