Published in final edited form as:

Glia. 2008 September ; 56(12): 1339–1352. doi:10.1002/glia.20702.

PRODUCTION, CHARACTERIZATION AND EFFICIENT TRANSFECTION OF HIGHLY PURE OLIGODENDROCYTE PRECURSOR CULTURES FROM MOUSE EMBRYONIC NEURAL PROGENITORS

Carlos E. Pedraza, Raymond Monk, Junying Lei, Qi Hao, and Wendy B. Macklin

Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA

Abstract

Much current knowledge of oligodendrocyte biology, the myelin-forming cells in the central nervous system (CNS), comes from cell culture studies mainly from postnatal rat tissue but mouse cells have been much more difficult to produce in large quantities. We have developed a high yield protocol for production of oligodendrocyte precursor cells from mouse embryonic neural progenitors grown as neurospheres. Neurospheres can be maintained and expanded for long periods in culture in the presence of EGF. When floating neurospheres were plated on substrate-coated dishes in media supplemented with PDGF and bFGF, the spheres attached and generated migrating cells that were predominantly oligodendrocyte-lineage cells. Furthermore, cells in spheres could be shifted to the oligodendrocyte phenotype prior to plating on substrate, by incubation in suspension with PDGF/bFGF. Single cell suspensions plated after dissociation of either EGF-treated neurospheres or PDGF/bFGF-treated oligospheres had the bipolar, elongated morphology characteristic of oligodendrocyte precursor cells. mRNA and protein expression analysis of the cells generated by this method confirmed their oligodendrocyte lineage. Oligodendrocyte precursors generated by this method matured in response to ciliary neurotrophic factor treatment, producing cells with multiple processes and myelin-like membranes. The most important aspect of this protocol is the ability to generate very high numbers of relatively pure mouse oligodendrocyte progenitor cells, which can be easily transfected. These studies open up many kinds of investigations on transgenic and mutant mouse oligodendrocytes, thereby providing a valuable tool to study oligodendrocyte biology and development.

Keywords

Embryonic stem cells; neurospheres; mouse oligodendrocyte precursors; development; oligospheres

Introduction

During development, oligodendrocyte precursor cells arise in germinal zones, migrate and proliferate *in situ* to populate the brain and mature into myelinating oligodendrocytes, which is essential for normal nervous system development (Miller, 2002; Spassky, et al., 2001; de Castro and Bribian, 2005). In adults, oligodendrocyte precursors are present throughout the brain, they can migrate to damaged areas and to some extent remyelinate demyelinated lesions (Chang, et al., 2000, 2002). Given their importance, investigating oligodendrocyte regulation during development and in physiologic and pathologic conditions is crucial, but such investigations have been limited by the complexity of their study *in vivo* and therefore the need for efficient and reproducible methods for their analysis *in vitro* (Lopez-Cardoso, et al., 1889; Ness, et al., 2005).

To date, mixed glial cell cultures generated from neonatal tissue are among the best sources of oligodendrocyte precursor cells (McCarthy and deVellis, 1980; Armstrong, 1998; Yang, et al., 2005). Other approaches include obtaining cells by either immunopanning or the use of density gradients. However, these techniques use a high number of animals, longterm cell culturing and a large amount of panning antibody (Itoh, 2002; Bambakidis and Miller, 2004). Because of increased cell manipulation, these techniques are subject to significant variability and often the desired yield and purity of oligodendrocyte precursor cells is not high. Most importantly for current investigations, these techniques are particularly poor for generating large numbers of oligodendrocyte precursors from mouse, which seriously limits the types of experiments that can be done with the variety of mutant, transgenic and knockout mice currently being developed.

The *in vitro* production of neural and glial lineage cells from either embryonic stem cells or embryonic neural precursors has been described (reviewed in Blakemore and Franklin, 2000; Chandran and Compston, 2005). In such studies, the production of floating cell aggregates known as neurospheres (Nph) significantly increases cell yields. Such cultures were initially developed as aggregating cultures in which neural precursor development could be analyzed, studying cell/cell interactions and growth factor requirements (Matthieu et al., 1978, Monnet-Tschudi and Honegger, 1989). Neural precursors can be maintained long-term *in vitro* as floating Nphs. Nphs have been shown to proliferate efficiently in the presence of epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF), maintaining their multipotentiality (Monnet-Tschudi and Honegger, 1989; Akesson, et al., 2007; Dictus, et al., 2007). In general, after plating, Nphs produce predominantly astrocytes, along with some oligodendrocyte and neuron progenitors (reviewed in Bazan, et al., 2004; Campos, 2004).

In the current studies, we developed a high yield method of committing mouse EGF-Nphs to the oligodendrocyte precursor lineage. With this method we produced unlimited numbers of mouse oligodendrocyte precursors, which were a consistent, very pure cell population that proliferated as bipolar, elongated and highly migratory cells. These oligodendrocyte precursors responded to well known differentiation stimuli, showing morphological and gene expression changes characteristic of functional premyelinating oligodendrocytes. This

is the first report for such a large scale procedure for generating highly pure oligodendrocyte precursors from mouse.

Methods and Materials

Cell cultures

Neurospheres (Nphs) were prepared from embryos at 14.5 days of gestation obtained from timed pregnant females of either wild type mice or the *Plp*-EGFP transgenic mouse (Mallon, et al., 2002), as described elsewhere (Kornblum, et al., 2000; Zheng, et al., 2007). In brief, after separation of meninges and cerebellum, cerebrum tissue was mechanically triturated with a 1 ml Gilson pipette until total tissue disaggregation was achieved, filtered through a 70 μ m cell strainer (Fisher Scientific, Pittsburg, PA) and plated in 25 cm² plastic culture flasks (2 brains/flask). Nph proliferation media (NPM) was DMEM/F12, B27 neuronal supplement (Gibco, Baltimore, MD) and 10 ng/ml EGF (Sigma, St. Louis, MO). After 48–72 hr, floating Nphs were passaged at a 1:3 ratio in the same medium every 3–4 days.

To produce oligospheres (Olphs), passage 2–6 EGF-Nphs were mechanically dissociated into single cell suspensions and re-suspended in NPM, supplemented with 10 ng/ml platelet derived growth factor (PDGF, Peprotech, Rocky Hill, NJ) and 10 ng/ml bFGF (Peprotech), instead of EGF. After 72 hr, cell aggregates were passaged at a 1:2 ratio every 4–7 days, as with Nphs. Nphs and Olphs at passage 2–6 were used for subsequent experiments.

Oligodendrocyte precursors from either Nphs or Olphs were generated by either mechanical dissociation of spheres with a 1 ml pipette or by chemical disaggregation with the mouse NeuroCult dissociation kit (StemCell Technologies, Alberta, Canada). The single cell suspension was filtered through a 70 μ m cell strainer and plated on 6 or 24-well poly-D-Lysine (PDL)-coated plates at a cell density of 1.5×10^4 cells/cm². Cells were maintained in NPM supplemented with 10 ng/ml PDGF/bFGF. Bipolar cells were observed 24 hours after plating, which were highly proliferative when maintained in this medium.

Immunocytochemistry

Nphs, Olphs and oligodendrocyte precursors plated on PDL-coated dishes were initially fixed in 2 % paraformaldehyde (PFA) for 10 min at room temperature (RT) and then 4 % PFA for 15 min, washed three times with PBS and permeabilized with 0.1% Triton X100 for 10 min at RT. After blocking with 3% BSA in PBS for 60 min at RT, the spheres/cells were incubated with primary antibodies at the indicated concentrations overnight (ON) at 4°C. The cells were washed three times with cold PBS incubated with secondary antibodies for 45 min at RT, and washed again before being visualized on a Leica DMR (Wetzlar, Germany) or a Nikon Eclipse TE300 (Melville, NY) microscope. Primary antibodies used in this study included rabbit anti-NG2 proteoglycan (1:300, a kind gift from Dr. William Stallcup, Burnham Institute, San Diego, CA), mouse anti-glial acidic fibrillary protein (GFAP, 1:450, Sigma), rat anti-PLP/DM20 (1:50, clone AA3), mouse anti- β -III-tubulin (1:300, Chemicon, Temecula, CA), rabbit anti-PDGFR- α (1:300, Santa Cruz, CA), rat anti-PDGFR- α (1:300, Upstate, Lake Placid, NY). Anti-rat, anti-rabbit or anti-mouse AlexaFluor secondary antibodies (Gibco) were used at 1:700.

RNA extraction and real-time PCR (qRT-PCR)

Total RNA was isolated from spheres or single cell cultures using Tri-Zol reagent (Gibco). cDNA was generated from 1–3 µg total RNA by Retrotranscriptase II (Gibco) reaction and the selected transcripts were detected and amplified by qRT-PCR in a LightCycler machine (Roche, Indianapolis, IN) following the manufacturer's instructions. The specific primers used in this study to detect mRNA expression of indicated genes are listed in Table 1. The results are presented as specific mRNA expression relative to glyceraldehydes-3-phosphatase dehydrogenase (GAPDH), used as internal control. RT-PCR conditions were: first denaturation: 95 °C, 30 seconds, 1 cycle; annealing and amplification: 59°C, 5 seconds; 72°C, 20 seconds respectively, 40 cycles; melting curve: 65°C, 15 seconds, 1 cycle.

siRNA transfection of Nphs

Passage 2–6 EGF-Nphs were dissociated to single cell suspensions and transfected by electroporation with the Amaxa system according to the manufacturer's instructions for mouse neural precursors (Amaxa, Gaithersburg, MD). Cells (3×10^6) were transfected in each experiment with a transfection efficiency of 55–70% as assessed by GFP expression. To achieve effective inhibition of PDGFR α expression, a combination of three commercially available PDGFR α siRNAs (0.2 nm, based on PDGFR α NCBI GenBank accession number NM_011058) were obtained from Ambion (Austin, TX), whose sequences are based on their design algorithm. Sense 1: GGU ACA GCUUAUGGA UUA ATT; antisense 1: UUA AUC CAU AAG CUG UAC CTT; sense 2: GCG AGU UUA AUGUUU AUG CTT; antisense 2: GCA UAA ACA UUA AAC UCG CTG; sense 3: CCG AUU CCU UAG UCA UCG UTT; antisense 3: ACG AUG ACU AAG GAA UCG GTC. Control transfections were performed with an unrelated siRNA negative control (Ambion). Immediately after transfection, cells were plated in PDGF/bFGF to generate oligodendrocyte precursors.

Statistical analysis

For statistical analysis, paired Student *t*-test was conducted. The results are shown as mean $\pm$ SEM and mean $\pm$ SD as indicated.

Results

Differential impact of growth factors on cells generated from embryonic neural precursors

In order to generate oligodendrocyte lineage cells from Nphs, we analyzed the effect of different growth factors known to impact the development of oligodendrocyte precursor cells, separately or in combination. Thus, after mechanical dissociation of the EGF-Nphs, the EGF-containing culture medium was replaced with medium supplemented with either basic fibroblast growth factor (bFGF) or platelet derived growth factor (PDGF) or a combination of both, and the *de novo* formation of spheres was evaluated by phase contrast microscopy.

After 48 hours in media supplemented with bFGF, we observed cell aggregation similar to the EGF-Nphs, forming rounded, yellow spheres that proliferated rapidly and could be passaged and maintained for long periods (Figure 1C). PDGF alone on the other hand, was

unable to sustain either cell aggregation or survival. Only a few spheres survived after 48 h in media supplemented with this growth factor (Figure 1E). The addition of PDGF to the bFGF medium was able to induce *de novo* formation of spheres that were passaged several times and maintained *in vitro* for long periods (Fig 1G). Interestingly, after attachment of the PDGF/bFGF spheres to poly-D-lysine (PDL)-coated dishes, bipolar, elongated cells with an oligodendrocyte progenitor-like morphology were produced, which migrated from the spheres in greater numbers than those from bFGF-grown spheres (Figure 1H vs. 1D). Thus, the cells from bFGF-treated spheres showed some migratory behavior (Figure 1D), but this growth factor appeared insufficient for longterm survival of plated cells since a great number of dead, floating cells was observed (not shown). The PDGF-supplemented cells neither attached nor survived to any significant extent (Figure 1F). Since PDGF/bFGF-treated spheres had greater oligodendrocyte progenitor-like cells with increased migratory capabilities (see below), we called them oligospheres (Olph).

We took advantage of our *Plp*-EGFP transgenic mouse (Mallon, et al., 2002), which facilitated the identification of oligodendrocyte lineage cells generated by the neural progenitors. We found that a number of cells forming the primary EGF-Nphs as well as subsequent passaged EGF-Nphs showed green fluorescence indicative of *Plp* promoter activity (Figure 1I, J). Nevertheless, the *Plp* promoter activity was stronger in Olphs as early as 48 hours in PDGF/bFGF (not shown), and was greatly increased after 72 hr (Figure 1K, L). The few cells migrating from the EGF-Nphs and most of the cells migrating from the Olphs also showed *Plp*-EGFP expression (data not shown). These results suggest that the combination of PDGF/bFGF induced the commitment of the neural progenitors to the oligodendrocyte lineage, and that these oligodendrocyte lineage progenitors were highly motile.

Cells in oligospheres increased glial gene expression

We confirmed the glial and oligodendrocyte precursor commitment of cells in Olphs by analyzing mRNA expression patterns of specific astrocyte and oligodendrocyte lineage cell proteins, myelin proteins and transcription factors. Samples from Olphs in suspension were compared to EGF-Nphs by qRT-PCR. We first analyzed the expression of cell membrane receptors that might be implicated in the production of Olphs. As expected, we found a great increase in PDGFR α mRNA in Olphs (6 fold), while FGFR1 and 3 did not show significant variation as compared with EGF-Nph. Interestingly, FGFR2 expression appeared to be downregulated in Olphs, as was EGF receptor expression (EGFR, Figure 2A).

More importantly, expression of several cell-specific protein markers helped to identify the cell types in these samples. While the intermediate filament protein nestin, which is specific for undifferentiated progenitor cells (Jori, et al., 2003), showed little variation between Olphs and EGF-Nphs, the expression of the astroglial protein GFAP increased (3.5 fold). There was also a significant increase in expression in the mRNAs for the oligodendrocyte precursor cell marker NG2 proteoglycan (2.3 fold) and for the myelin protein PLP (2 fold). These increases were highly statistically significant.

The transcription factors described to actively regulate the generation, migration and maturation of oligodendrocyte precursors during development showed an interesting

expression pattern in Olphs. The increased expression of Olig1 (3-fold) and Olig2 (1.8-fold) along with Sox9 (3-fold) confirmed the oligodendrocyte lineage of cells in Olphs (Wu, et al., 2006; Jakovcevsky and Zecevic, 2005; Ligon, et al., 2006; Liu, et al., 2007; Stolt, et al., 2003). Additionally, the transcription factors of the homeobox family implicated in the regulation of differentiation of progenitors into oligodendrocyte precursors (Nkx2.1, Tekki-Kessaris, et al., 2001) and the maturation of oligodendrocyte precursors (Nkx2.2, Fancy, et al., 2004; Kitada and Rowitch., 2006) showed a significant increase (1.25- and 1.80-fold respectively; $p < 0.05$) (Figure 2C). Other transcription factors involved in oligodendrocyte production during development were also altered in expression (Corbin, et al., 2003; Wei, et al., 2005; Wang, et al., 2001; Liang, et al., 2005). The transcription factors GHS2, SP1, ID2 and ID4 showed significant increases in Olph (1.7-, $p < 0.01$; 1.5-, $p < 0.05$; 2.5-, $p < 0.01$; and 1.4-fold, $p < 0.05$, respectively), while EMX1 and SP3 expression had no statistically significant change relative to EGF-Nphs (Figure 2D). EMX1 has been shown to be expressed in oligodendrocyte progenitors arising in postnatal cortex (Kessaris et al., 2006). Presumably the progenitors derived in the current studies are not in the same pool as the EMX1-expressing cells that arise later in development. Interestingly, mRNA concentration for bone morphogenic protein 2 (BMP2), which drives cells to astrocyte differentiation at the expense of oligodendrocytes (Mabie, et al., 1999; Grinspan, et al., 2000; Gossrau, et al., 2007), did not change, although ID4 and particularly ID2 mRNAs were upregulated (1.4-, $p < 0.05$ and 2.6-fold, $p < 0.01$, respectively).

Oligodendrocyte precursor cells were highly migratory in spheres grown in PDGF/bFGF

In order to evaluate the glial lineage of the cells produced in EGF-Nphs and Olphs, we analyzed expression of PDGFR- α and GFAP as specific markers for early oligodendrocyte precursors and astrocytes respectively in plated spheres. After 48 hours of plating, EGF-Nphs plated in EGF produced for the most part non-migratory cells that accumulated around the sphere and a few migratory PDGFR- α positive cells (Figure 3A–C). It was noticeable that most of the cells within and immediately surrounding the sphere were GFAP-labeled (Figure 3B, C). On the other hand, the cells generated by Olphs actively migrated long distances from the sphere and, as judged by the generalized PDGFR- α staining, these cells were oligodendrocyte precursors (Figure 3G–I). The shift to highly migratory oligodendrocyte progenitor cells occurred quickly in the spheres. We evaluated the impact of treating EGF-Nphs with PDGF/bFGF immediately after plating on PDL-coated dishes. As shown in Figure 3(D–F), a large number of migratory cells were observed 48 hours after plating EGF-Nphs in PDGF/bFGF, which had oligodendrocyte precursor morphology and PDGFR- α positive staining (Figure 3D). We also detected a dense core of GFAP-positive cells indicating the conserved multipotentiality of these spheres regardless of the elevated number of oligodendrocyte precursors generated in response to PDGF/bFGF (Figure 3E, F). In all samples, the GFAP-positive cells remained in the dense core, while the oligodendrocyte precursors migrated away.

To characterize the migratory capabilities of cells originating from spheres, we analyzed the migration by time lapse for 25 hours. Migration from EGF-Nphs plated and maintained in EGF was not imaged, because migration was minimal, but cells from EGF-Nphs plated in PDGF/bFGF or cells from Olphs were highly migratory and were imaged. This allowed us

to observe behavioral differences between the cells produced under both conditions (Figure 4). EGF-Nph-derived cells, which were being shifted from EGF to PDGF/bFGF medium at plating, showed random motility, with cells migrating erratically back and forth around the sphere. After movement away from the sphere, we observed some cells undergoing cell division followed by continued unpredictably migration (Figure 4A, cell 2). On the other hand, the cells produced by Olphs, which had already been exposed for several days to PDGF/bFGF and already had increased expression of oligodendrocyte markers before plating (Figure 3), migrated in a slow, organized fashion heading directly out of the spheres in a radial movement (Figure 4B).

Dissociation of Oligospheres and EGF-Neurospheres into single cells cultured in PDGF/bFGF resulted in a highly pure oligodendrocyte precursor culture

In order to assess how efficient the conversion of multipotential progenitors to oligodendrocyte progenitors was, the relative number of oligodendrocyte progenitors in single cell preparations from dissociated Olphs or EGF-Nphs was quantified. Passage 2–6 Olphs or EGF-Nphs from our *Plp*-EGFP transgenic mouse were mechanically or chemically dissociated, filtered and plated in media supplemented with PDGF/bFGF. In dispersed cell suspensions from both types of spheres we observed attached cells of rounded to elongated morphology immediately after plating. Twenty four hours later, the cell population increased, forming a homogenous cell culture composed of bipolar, elongated cells that were already extending processes and showed *Plp*-EGFP expression (Figure 5A, B). The expression of PLP was further confirmed by immunocytochemistry with an antibody directed against PLP/DM20 showing colocalization with *Plp*-EGFP (Figure 5C, D). After 4 days, the cells approached confluence and a few cells showed multiprocess morphology typical of spontaneously maturing oligodendrocytes (Figure 5E, F).

Most cells derived from Olphs (Figure 5G, I) or EGF-Nphs plated in PDGF/bFGF (Figure 5H, J) were positive for PDGFR- α and NG2 (Figure 5G–J), confirming their oligodendrocyte lineage. We also detected some GFAP-positive cells, which appeared to be more abundant in EGF-Nph-derived oligodendrocyte precursor cultures (Figure 5H), relative to cells generated from Olphs (Figure 5G). By contrast, no cells expressing the neuronal markers β -III tubulin or phosphorylated neurofilament were detected in cultures derived from Olphs, although a few such cells were occasionally seen in cultures derived from EGF-Nph (data not shown). We quantified the purity of Olph- and EGF-Nph-derived oligodendrocyte progenitors, by counting NG2-positive cells as a percentage of the total number of cells. EGF-Nph-derived cultures contained 77.9 ± 11.2 % NG2+ cells, and this percentage increased to 96.8 ± 1.6 % in the oligodendrocyte progenitor cultures obtained after Olph dissociation. It should be noted that the current studies were done with Nphs obtained from E14.5 embryos. This was the optimal time point for highly pure oligodendrocyte production. For example, Nphs obtained from E15.5 embryos and treated in the same manner as these generated far more astrocytes (not shown). In general, while the purity of oligodendrocyte progenitor cells was higher in cells obtained from Olphs, the total number of cells tended to be fewer. This correlated with a slower sphere growth rate in Olph which required 4–7 days in culture before every passage while EGF-Nph needed to be subcultured every 3–4 days (data not shown). Thus, for high level production of

oligodendrocyte progenitor cells, plating of dissociated EGF-Nph cells in PDGF/bFGF was preferable where a small contamination of astrocytes was not a problem.

To assess gene expression in our single cell cultures, we performed qRT-PCR using specific primers for the cell membrane receptors PDGFR- α , EGFR and FGFR1, along with the transcription factors Olig1, Olig2, Sox9 and Nkx2.1. Total RNA was isolated from single cell preparations generated from EGF-Nphs plated in EGF, or EGF-Nphs and Olphs plated in PDGF/bFGF. We found that the cells obtained from EGF-Nph showed low, yet detectable mRNA expression levels of the genes studied, suggesting the presence of a small number of such cells in EGF-Nphs. Notably, Olph-derived oligodendrocyte precursors showed a remarkable increase in the expression of PDGFR- α and a clear increase in FGFR1 as compared to the cells from EGF-Nph plated in PDGF/bFGF. As expected, EGFR showed decreased expression as a consequence of the growth factor change from EGF to PDGF/FGF (Figure 6A).

The expression of the oligodendrocyte-specification transcription factor Sox9 was clearly upregulated in cells obtained from Olphs as well as the early oligodendrocyte precursor specific genes Olig1, Olig2 and Nkx2.1 (Figure 6B).

Effective RNA silencing of PDGFR α in oligodendrocyte precursors generated from neural progenitors

In order to assess the relative importance of the dramatic increase in PDGFR α after our cells were exposed to PDGF/bFGF, we studied the effect of siRNA-mediated knock-down of PDGFR α on the generation, proliferation and oligodendrocyte protein expression of oligodendrocyte precursors generated from Nphs.

Dissociated Nphs were transfected with a combination of three PDGFR α siRNAs, plated and grown for 7 days in PDGF/bFGF. PDGFR α knock-down was demonstrated by immunocytochemistry (Figure 7A, B). There was a decrease of 29.1 % in total cell numbers as compared to control siRNA transfected cells (Figure 7E). This resulted from reduced cell proliferation, since there was reduced BrdU incorporation in PDGFR α siRNA-transfected cells (61.8 ± 2.4 control siRNA-transfected versus 52.2 ± 3.2 PDGFR α siRNA-transfected BrdU⁺ cells as a percentage of total cells, a reduction of approximately 15% in proliferative rate, Figure 7F). The PDGFR α knock-down appeared to impact cell proliferation but not oligodendrocyte precursor specification. As observed in Figure 7F, the percentage of total cells that were NG2-positive and Olig2-positive cells was comparable for both control siRNA- and PDGFR α siRNA-transfected cells (Figure 7F). On the other hand, the morphology of the NG2-positive cells in PDGFR α siRNA-transfected cells appeared flattened, relative to control siRNA-transfected cells (Figure 7C, D arrows), suggesting that in addition to impacting oligodendrocyte progenitor cell proliferation, this growth factor receptor also impacted interaction of the cell with the substrate.

Oligodendrocyte progenitor cells obtained from embryonic Olphs and EGF-Nphs mature in response to ciliary neurotrophic factor

To further confirm the functionality of our highly pure mouse oligodendrocyte precursor cultures, we tested their ability to mature when exposed to ciliary neurotrophic factor

(CNTF), a well characterized inducer of oligodendrocyte maturation (Mayer et al, 1994; Stankoff et al., 2002). After 4 days in PDGF/bFGF, EGF-Nph- or Olph-derived oligodendrocyte precursors were treated with 10 ng/ml CNTF for 48 hours. In *Plp*-EGFP cells, clear morphological changes were induced by CNTF, which were characteristic of oligodendrocyte precursor maturation. These included the formation of thick, extended cell processes and the production of membranous structures characteristic of *in vitro* maturing oligodendrocytes. The morphological changes were accompanied by an increase in *Plp*-EGFP fluorescence, suggesting increased *Plp* gene expression induced by CNTF (Figure 8A, B). Immunocytochemistry after 7 days of CNTF treatments for the oligodendrocyte antigens O4 (Figure 8C, D) and myelin basic protein (MBP) (Figure 8E, F) further confirmed the maturation effect of CNTF on these oligodendrocyte progenitor cells.

Discussion

Understanding oligodendrocyte behavior in physiologic and pathologic conditions is fundamental to identifying new approaches to fight demyelinating diseases such as multiple sclerosis. In the current studies, we expanded on the extensive literature on *in vitro* production of neuronal and glial lineage cells from mouse embryonic neural progenitors and embryonic stem cells. These procedures involve production of Nphs prior to specification of cells into a defined lineage (reviewed in Blakemore and Franklin, 2000; Gottlieb, 2002). Several investigators have described the ability of Nphs to produce glial and neuronal lineage cells from Nphs in variable percentages depending on trophic stimuli (Murray et al., 2002; Bazan, et al., 2004; reviewed in Campos 2004; Chandran and Compston 2005). In the current work, we explored the impact of the known oligodendrocyte growth/survival factor, PDGF, on the fate of embryonic neural progenitors. PDGF has been used in combination with bFGF in oligodendrocyte progenitor cultures generated from rat mixed glia preparations to arrest the cells in the precursor stage and increase their proliferation (McKinnon, et al., 1990; Yim, et al., 2001; Baron, et al., 2000). Also, in studies on stem cell differentiation, PDGF has been shown to be necessary for the late specification of cultured embryonic stem cell lines to the oligodendrocyte lineage (Glasser, et al., 2004; Gibney and McDermott, 2007; Willerth et al., 2007). In contrast, recent reports describe a limited ability of PDGF to induce oligodendroglial specification of rat neural progenitors either alone or in combination with several other mitogenic factors (Gibney and McDermott, 2007; Chen, et al., 2007).

We found that PDGF itself was unable to sustain the formation of new spheres or the survival of dissociated cells from E14.5 mouse embryos. By contrast, bFGF alone allowed survival of cells in spheres but failed to induce the generation of oligodendrocyte progenitors in the efficient way observed for spheres grown in both bFGF and PDGF. The role of bFGF in early oligodendrocyte progenitor specification, migration and proliferation has been studied in a variety of *in vivo* and *in vitro* models (Fortin, et al., 2005; Bansal, et al., 2003a, b; Goddard, et al., 2001; Bansal and Pfeiffer, 1997). Additionally, bFGF is clearly of interest for its capacity to induce stem cell differentiation (reviewed in Dvorak, et al. 2006; Kashiwakura and Takahashi, 2005), in particular the specification of embryonic stem cells to neural cell types (reviewed in Fisher, 1997; Vaccarino, et al., 2001).

Despite low level expression, the FGF receptors expressed in these cells were essential for survival of the cells, since PDGF alone could not support sphere production. The expression of FGF receptors 1, 2 and 3, which was quite low in EGF-Nphs, did not increase in spheres converted to the oligodendrocyte lineage. An increase of FGFR1 mRNA expression was detected in single cells preparations plated in PDGF/bFGF (Figure 6), which is consistent with data demonstrating that FGF signaling is important to sustain the highly proliferative and migratory behavior of oligodendrocyte progenitors after neural precursor specification driven by PDGF/bFGF (McKinnon, et al., 1990; Yim, et al., 2001). On the other hand, FGFR2 mRNA concentration actually dropped as spheres shifted to the oligodendrocyte lineage in Olphs (Figure 2). This downregulation of FGFR2 is consistent with the studies by Bansal and coworkers (Bansal et al., 2003a, b; Fortin et al., 2005), who showed that the FGFR2 is expressed at low levels until oligodendrocytes mature. Thus, presumably shifting neural progenitors to the oligodendrocyte progenitor phenotype requires downregulation of FGFR2.

Exposure to PDGF/bFGF and conversion of cells to the oligodendrocyte lineage had a much more dramatic impact on PDGFR α gene expression in Olphs and single cells grown under the same conditions. PDGFR α mRNA was expressed at approximately the same low level as FGFR1 in Nphs, but it was significantly upregulated in Olphs, further confirming the relevance of signaling mediated by this receptor for the conversion of the neural progenitors to the oligodendrocyte lineage. The major role that PDGF plays in oligodendrocyte precursor survival and proliferation during the development has been extensively studied (Murtie et al., 2005; Paez et al 2006; reviewed in Dubois-Dalcq and Murray, 2000). In our system, the increased expression of PDGFR α in both Olphs and the single cell cultures suggests a major function for signaling mediated by this receptor. Our results from PDGFR α expression knock-down experiments confirm other studies (Bogler et al., 1990; McKinnon et al., 1993) indicating that indeed this receptor plays a major role in cell proliferation rather than in cell specification. The percentage of NG2 and Olig2-positive cells remains invariable in cells where the PDGFR α expression is abolished, while the number of proliferative cells is reduced significantly. Interestingly, the absence of this receptor appeared to induce morphological changes in oligodendrocyte progenitors. This would be consistent with the concept that the PDGFR α interacts with integrins (Baron et al., 2002, 2003), and that the interaction changes the affinity for PDGF needed by the cells.

Most interestingly, the response of cells in EGF-Nphs to switch to the oligodendrocyte lineage was very rapid, occurring as early as a few hours after switching to medium containing PDGF/bFGF. Motility of cells changed rapidly and it was initially quite random in direction reflecting the remarkable change of cell function induced by the new stimuli. Similarly, when single cell suspensions were generated from EGF-Nphs and plated in PDGF/bFGF supplemented medium, the appearance of fusiform, bipolar oligodendrocyte precursor cells was observed only a few hours after plating. In contrast to the long-term protocols required to induce specific cell specification in several stem cell populations, the quick response to a defined oligodendrocyte precursor differentiating stimuli we observed in these neural progenitors, positions these stem cells as a prime source for the study of oligodendrocyte biology and cell-based therapies.

Most mRNAs changes were consistent with the conversion of these cells to the oligodendrocyte lineage. It was of some interest that ID2 and ID4 mRNAs also increased in these cells. These mRNAs have been shown to be regulators of oligodendrocyte differentiation. In excess, they induce astrocyte differentiation, but their absence induces premature oligodendrocyte differentiation (Kondo and Raff; 2000; Wang et al., 2001). Thus, their increased expression in cells in Olphs is indicative of cells being maintained as progenitor cells in the spheres. By contrast, BMP2 did not increase in these cells. Thus, astrocyte differentiation was not being increased at the expense of oligodendrocyte production (Adachi, et al., 2005; Samanta and Kessler, 2004).

A number of studies have used B104 conditioned media to enhance commitment to the oligodendrocyte lineage. In early reports, Avellana-Adalid et al. (1996) coined the term oligospheres for aggregates of postnatal rat brain cells grown in B104 neuroblastoma conditioned media and low serum, which generated large amounts of oligodendrocyte progenitors. Zhang and co-workers, using a similar approach, described the commitment of rat neuronal precursors to the oligodendrocyte lineage in Nph cultures maintained in B104 neuroblastoma conditioned media (Zhang, et al., 1998a, 1998b). The cells generated from those Nphs were mostly oligodendrocyte progenitors after plating in the absence of fetal bovine serum and they became type 2 astrocytes in its presence. The first report of oligospheres from mouse also used B104-conditioned media to commit early postnatal EGF-Nphs to the oligodendrocyte lineage, as determined by mRNA expression of PDGFR α and FGFR (Vitry et al., 1999). Additionally, Lu and coworkers describe a neurosphere method to generate rodent oligodendrocyte precursors, in which the use of B104-conditioned media is recommended over a combination of growth factors (Cheng et al., 2007). On the other hand, B104-conditioned media was shown to induce oligodendrocyte precursors only very poorly from E-18-derived rat Nphs (Gibney and McDermott, 2007). Unfortunately, the specific factors present in B104-conditioned media that induce neural precursor commitment to the oligodendrocyte lineage have not been identified. The relative purity of our single cells derived from EGF-Nphs or Olphs in media supplemented with PDGF/bFGF shows that this growth factor combination is sufficient to induce the commitment of most multipotent cells in mouse E14.5 Nphs to the oligodendrocyte lineage.

This is an extremely efficient and reproducible method to produce mouse oligodendrocyte precursor cultures with high purity and extended proliferation as bipolar, immature oligodendrocyte progenitors, although as noted above, the age at which the embryonic cells are obtained impacts the purity of the cultures. The oligodendrocyte progenitors produced by this method respond appropriately to oligodendrocyte maturation/differentiation stimuli, acquiring the multi-process morphology of pre-myelinating oligodendrocytes and forming significant membranous structures. These oligodendrocyte progenitors are suitable for studies requiring elevated number of cells such as high through-put screenings and transplantation studies. The degree of oligodendrocyte purity assured by this method may be valuable for survival, migration, proliferation and remyelination after cell transplantation in experimental models of multiple sclerosis.

Acknowledgments

This study was supported by NS25304, BRTT/WCI State of Ohio, Collaborative MS Research Center of Excellence and National Multiple Sclerosis Society (NMSS) grants to W. Macklin. C. Pedraza is the recipient of a NMSS postdoctoral fellowship.

References

- Adachi T, Takanaga H, Kunimoto M, Asou H. Influence of LIF and BMP-2 on differentiation and development of glial cells in primary cultures of embryonic rat cerebral hemisphere. *J Neurosci Res.* 2005; 79:608–615. [PubMed: 15678513]
- Akesson E, Piao JH, Samuelsson EB, Holmberg L, Kjaeldgaard A, Falci S, Sundstrom E, Seiger A. Long-term culture and neuronal survival after intraspinal transplantation of human spinal cord-derived neurospheres. *Physiol Behav.* 2007; 92:60–66. [PubMed: 17610915]
- Armstrong RC. Isolation and characterization of immature oligodendrocyte lineage cells. *Methods.* 1998; 16:282–292. [PubMed: 10071067]
- Avellana-Adalid V, Nait-Oumesmar B, Lachapelle F, Baron-Van Evercooren A. Expansion of rat oligodendrocyte progenitors into proliferative “oligospheres” that retain differentiation potential. *J Neurosci Res.* 1996; 45:558–570. [PubMed: 8875321]
- Bambakidis NC, Miller RH. Transplantation of oligodendrocyte precursors and sonic hedgehog results in improved function and white matter sparing in the spinal cords of adult rats after contusion. *Spine J.* 2004; 4:16–26. [PubMed: 14749190]
- Bansal R, Pfeiffer SE. FGF-2 converts mature oligodendrocytes to a novel phenotype. *J Neurosci Res.* 1997; 50:215–228. [PubMed: 9373031]
- Bansal R, Magge S, Winkler S. Specific inhibitor of FGF receptor signaling: FGF-2-mediated effects on proliferation, differentiation, and MAPK activation are inhibited by PD173074 in oligodendrocyte-lineage cells. *J Neurosci Res.* 2003; 74:486–493. [PubMed: 14598292]
- Bansal R, Lakhina V, Remedios R, Tole S. Expression of FGF receptors 1, 2, 3 in the embryonic and postnatal mouse brain compared with Pdgfralpha, Olig2 and Plp/dm20: implications for oligodendrocyte development. *Dev Neurosci.* 2003; 25:83–95. [PubMed: 12966207]
- Baron W, Metz B, Bansal R, Hoekstra D, de Vries H. PDGF and FGF-2 signaling in oligodendrocyte progenitor cells: regulation of proliferation and differentiation by multiple intracellular signaling pathways. *Mol Cell Neurosci.* 2000; 15:314–329. [PubMed: 10736207]
- Baron W, Shattil SJ, French-Constant C. The oligodendrocyte precursor mitogen PDGF stimulates proliferation by activation of alpha(v)beta3 integrins. *EMBO J.* 2002; 21:1957–1966. [PubMed: 11953315]
- Baron W, Decker L, Colognato H, French-Constant C. Regulation of integrin growth factor interactions in oligodendrocytes by lipid raft microdomains. *Curr Biol.* 2003; 13:151–155. [PubMed: 12546790]
- Bazan E, Alonso FJ, Redondo C, Lopez-Toledano MA, Alfaro JM, Reimers D, Herranz AS, Paino CL, Serrano AB, Cobacho N, Caso E, Lobo MV. In vitro and in vivo characterization of neural stem cells. *Histol Histopathol.* 2004; 19:1261–1275. [PubMed: 15375770]
- Blakemore WF, Franklin RJ. Transplantation options for therapeutic central nervous system remyelination. *Cell Transplant.* 2000; 9:289–294. [PubMed: 10811401]
- Bogler O, Wren D, Barnett SC, Land H, Noble M. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. *Proc Natl Acad Sci U S A.* 1990; 87:6368–6372. [PubMed: 2201028]
- Campos LS. Neurospheres: insights into neural stem cell biology. *J Neurosci Res.* 2004; 78:761–769. [PubMed: 15505793]
- Chandran S, Compston A. Neural stem cells as a potential source of oligodendrocytes for myelin repair. *J Neurol Sci.* 2005; 233:179–181. [PubMed: 15907942]
- Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J Neurosci.* 2000; 20:6404–6412. [PubMed: 10964946]

- Chang A, Tourtellotte WW, Rudick R, Trapp BD. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *N Engl J Med*. 2002; 346:165–173. [PubMed: 11796850]
- Chen Y, Balasubramanian V, Peng J, Hurlock EC, Tallquist M, Li J, Lu QR. Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc*. 2007; 2:1044–1051. [PubMed: 17546009]
- Corbin JG, Rutlin M, Gaiano N, Fishell G. Combinatorial function of the homeodomain proteins Nkx2.1 and Gsh2 in ventral telencephalic patterning. *Development*. 2003; 130:4895–4906. [PubMed: 12930780]
- de Castro F, Bribian A. The molecular orchestra of the migration of oligodendrocyte precursors during development. *Brain Res Brain Res Rev*. 2005; 49:227–241. [PubMed: 16111552]
- Dictus C, Tronnier V, Unterberg A, Herold-Mende C. Comparative analysis of in vitro conditions for rat adult neural progenitor cells. *J Neurosci Methods*. 2007; 161:250–258. [PubMed: 17207861]
- Dubois-Dalcq M, Murray K. Why are growth factors important in oligodendrocyte physiology? *Pathol Biol (Paris)*. 2000; 48:80–86. [PubMed: 10729915]
- Dvorak P, Dvorakova D, Hampl A. Fibroblast growth factor signaling in embryonic and cancer stem cells. *FEBS Lett*. 2006; 580:2869–2874. [PubMed: 16516203]
- Fancy SP, Zhao C, Franklin RJ. Increased expression of Nkx2.2 and Olig2 identifies reactive oligodendrocyte progenitor cells responding to demyelination in the adult CNS. *Mol Cell Neurosci*. 2004; 27:247–254. [PubMed: 15519240]
- Fisher LJ. Neural precursor cells: applications for the study and repair of the central nervous system. *Neurobiol Dis*. 1997; 4:1–22. [PubMed: 9258907]
- Fortin D, Rom E, Sun H, Yayon A, Bansal R. Distinct fibroblast growth factor (FGF)/FGF receptor signaling pairs initiate diverse cellular responses in the oligodendrocyte lineage. *J Neurosci*. 2005; 25:7470–7479. [PubMed: 16093398]
- Gibney SM, McDermott KW. Differentiation of oligodendrocytes in neurospheres derived from embryonic rat brain using growth and differentiation factors. *J Neurosci Res*. 2007; 85:1912–1920. [PubMed: 17526011]
- Glaser T, Perez-Bouza A, Klein K, Brustle O. Generation of purified oligodendrocyte progenitors from embryonic stem cells. *FASEB J*. 2005; 19:112–114. [PubMed: 15486057]
- Goddard DR, Berry M, Kirvell SL, Butt AM. Fibroblast growth factor-2 inhibits myelin production by oligodendrocytes in vivo. *Mol Cell Neurosci*. 2001; 18:557–569. [PubMed: 11922145]
- Gossrau G, Thiele J, Konang R, Schmandt T, Brustle O. Bone morphogenetic protein-mediated modulation of lineage diversification during neural differentiation of embryonic stem cells. *Stem Cells*. 2007; 25:939–949. [PubMed: 17218404]
- Gottlieb DI. Large-scale sources of neural stem cells. *Annu Rev Neurosci*. 2002; 25:381–407. [PubMed: 12052914]
- Grinspan JB, Edell E, Carpio DF, Beesley JS, Lavy L, Pleasure D, Golden JA. Stage-specific effects of bone morphogenetic proteins on the oligodendrocyte lineage. *J Neurobiol*. 2000; 43:1–17. [PubMed: 10756062]
- Itoh K. Culture of oligodendrocyte precursor cells (NG2(+)/O1(-)) and oligodendrocytes (NG2(-)/O1(+)) from embryonic rat cerebrum. *Brain Res Brain Res Protoc*. 2002; 10:23–30. [PubMed: 12379434]
- Jori FP, Galderisi U, Piegari E, Cipollaro M, Cascino A, Peluso G, Cotrufo R, Giordano A, Melone MA. EGF-responsive rat neural stem cells: molecular follow-up of neuron and astrocyte differentiation in vitro. *J Cell Physiol*. 2003; 195:220–233. [PubMed: 12652649]
- Kashiwakura I, Takahashi TA. Fibroblast growth factor and ex vivo expansion of hematopoietic progenitor cells. *Leuk Lymphoma*. 2005; 46:329–333. [PubMed: 15621822]
- Kessaris N, Fogarty M, Iannarelli P, Grist M, Wegner M, Richardson WD. Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat Neurosci*. 2006; 9:173–179. [PubMed: 16388308]
- Kitada M, Rowitch DH. Transcription factor co-expression patterns indicate heterogeneity of oligodendroglial subpopulations in adult spinal cord. *Glia*. 2006; 54:35–46. [PubMed: 16673374]
- Kondo T, Raff M. Basic helix-loop-helix proteins and the timing of oligodendrocyte differentiation. *Development*. 2000; 127:2989–2998. [PubMed: 10862737]

- Kornblum HI, Yanni DS, Easterday MC, Seroogy KB. Expression of the EGF receptor family members ErbB2, ErbB3, and ErbB4 in germinal zones of the developing brain and in neurosphere cultures containing CNS stem cells. *Dev Neurosci.* 2000; 22:16–24. [PubMed: 10657694]
- Liang Y, Bollen AW, Nicholas MK, Gupta N. Id4 and FABP7 are preferentially expressed in cells with astrocytic features in oligodendrogliomas and oligoastrocytomas. *BMC Clin Pathol.* 2005; 5:6. [PubMed: 16018821]
- Ligon KL, Fancy SP, Franklin RJ, Rowitch DH. Olig gene function in CNS development and disease. *Glia.* 2006; 54:1–10. [PubMed: 16652341]
- Liu Z, Hu X, Cai J, Liu B, Peng X, Wegner M, Qiu M. Induction of oligodendrocyte differentiation by Olig2 and Sox10: evidence for reciprocal interactions and dosage-dependent mechanisms. *Dev Biol.* 2007; 302:683–693. [PubMed: 17098222]
- Lopes-Cardozo M, Sykes JE, Van der Pal RH, van Golde LM. Development of oligodendrocytes. Studies of rat glial cells cultured in chemically-defined medium. *J Dev Physiol.* 1989; 12:117–127. [PubMed: 2696749]
- Mabie PC, Mehler MF, Kessler JA. Multiple roles of bone morphogenetic protein signaling in the regulation of cortical cell number and phenotype. *J Neurosci.* 1999; 19:7077–7088. [PubMed: 10436062]
- Mallon BS, Shick HE, Kidd GJ, Macklin WB. Proteolipid promoter activity distinguishes two populations of NG2-positive cells throughout neonatal cortical development. *J Neurosci.* 2002; 22:876–885. [PubMed: 11826117]
- Matthieu JM, Honegger P, Trapp BD, Cohen SR, Webster HF. Myelination in rat brain aggregating cell cultures. *Neuroscience.* 1978; 3:565–572. [PubMed: 211463]
- Mayer M, Bhakoo K, Noble M. Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. *Development.* 1994; 120:143–153. [PubMed: 8119123]
- McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol.* 1980; 85:890–902. [PubMed: 6248568]
- McKinnon RD, Matsui T, Dubois-Dalcq M, Aaronson SA. FGF modulates the PDGF-driven pathway of oligodendrocyte development. *Neuron.* 1990; 5:603–614. [PubMed: 2171589]
- McKinnon RD, Smith C, Behar T, Smith T, Dubois-Dalcq M. Distinct effects of bFGF and PDGF on oligodendrocyte progenitor cells. *Glia.* 1993; 7:245–254. [PubMed: 8454310]
- Miller RH. Regulation of oligodendrocyte development in the vertebrate CNS. *Prog Neurobiol.* 2002; 67:451–467. [PubMed: 12385864]
- Monnet-Tschudi F, Honegger P. Influence of epidermal growth factor on the maturation of fetal rat brain cells in aggregate culture. An immunocytochemical study. *Dev Neurosci.* 1989; 11:30–40. [PubMed: 2653794]
- Murray K, Calaora V, Rottkamp C, Guicherit O, Dubois-Dalcq M. Sonic hedgehog is a potent inducer of rat oligodendrocyte development from cortical precursors in vitro. *Mol Cell Neurosci.* 2002; 19:320–332. [PubMed: 11906206]
- Murtie JC, Zhou YX, Le TQ, Vana AC, Armstrong RC. PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination. *Neurobiol Dis.* 2005; 19:171–182. [PubMed: 15837572]
- Ness JK, Valentino M, McIver SR, Goldberg MP. Identification of oligodendrocytes in experimental disease models. *Glia.* 2005; 50:321–328. [PubMed: 15846801]
- Paez PM, Garcia CI, Soto EF, Pasquini JM. Apotransferrin decreases the response of oligodendrocyte progenitors to PDGF and inhibits the progression of the cell cycle. *Neurochem Int.* 2006; 49:359–371. [PubMed: 16621163]
- Samanta J, Kessler JA. Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. *Development.* 2004; 131:4131–4142. [PubMed: 15280210]
- Spassky N, Olivier C, Cobos I, LeBras B, Goujet-Zalc C, Martinez S, Zalc B, Thomas JL. The early steps of oligodendrogenesis: insights from the study of the plp lineage in the brain of chicks and rodents. *Dev Neurosci.* 2001; 23:318–326. [PubMed: 11756747]

- Stankoff B, Aigrot MS, Noel F, Wattilliaux A, Zalc B, Lubetzki C. Ciliary neurotrophic factor (CNTF) enhances myelin formation: a novel role for CNTF and CNTF-related molecules. *J Neurosci*. 2002; 22:9221–9227. [PubMed: 12417647]
- Stolt CC, Lommes P, Sock E, Chaboissier MC, Schedl A, Wegner M. The Sox9 transcription factor determines glial fate choice in the developing spinal cord. *Genes Dev*. 2003; 17:1677–1689. [PubMed: 12842915]
- Tekki-Kessaris N, Woodruff R, Hall AC, Gaffield W, Kimura S, Stiles CD, Rowitch DH, Richardson WD. Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon. *Development*. 2001; 128:2545–2554. [PubMed: 11493571]
- Vaccarino FM, Ganat Y, Zhang Y, Zheng W. Stem cells in neurodevelopment and plasticity. *Neuropsychopharmacology*. 2001; 25:805–815. [PubMed: 11750175]
- Vitry S, Avellana-Adalid V, Hardy R, Lachapelle F, Baron-Van Evercooren A. Mouse oligospheres: from pre-progenitors to functional oligodendrocytes. *J Neurosci Res*. 1999; 58:735–751. [PubMed: 10583906]
- Wang S, Sdrulla A, Johnson JE, Yokota Y, Barres BA. A role for the helix-loop-helix protein Id2 in the control of oligodendrocyte development. *Neuron*. 2001; 29:603–614. [PubMed: 11301021]
- Wei Q, Miskimins WK, Miskimins R. Stage-specific expression of myelin basic protein in oligodendrocytes involves Nkx2.2-mediated repression that is relieved by the Sp1 transcription factor. *J Biol Chem*. 2005; 280:16284–16294. [PubMed: 15695521]
- Willerth SM, Fixel TE, Gottlieb DI, Sakiyama-Elbert SE. The effects of soluble growth factors on embryonic stem cell differentiation inside of fibrin scaffolds. *Stem Cells*. 2007; 25:2235–2244. [PubMed: 17585170]
- Wu S, Wu Y, Capecchi MR. Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. *Development*. 2006; 133:581–590. [PubMed: 16407399]
- Yang Z, Watanabe M, Nishiyama A. Optimization of oligodendrocyte progenitor cell culture method for enhanced survival. *J Neurosci Methods*. 2005; 149:50–56. [PubMed: 15975663]
- Yim SH, Hammer JA, Quarles RH. Differences in signal transduction pathways by which platelet-derived and fibroblast growth factors activate extracellular signal-regulated kinase in differentiating oligodendrocytes. *J Neurochem*. 2001; 76:1925–1934. [PubMed: 11259511]
- Zhang SC, Lundberg C, Lipsitz D, O'Connor LT, Duncan ID. Generation of oligodendroglial progenitors from neural stem cells. *J Neurocytol*. 1998; 27:475–489. [PubMed: 11246488]
- Zhang SC, Lipsitz D, Duncan ID. Self-renewing canine oligodendroglial progenitor expanded as oligospheres. *J Neurosci Res*. 1998; 54:181–190. [PubMed: 9788277]
- Zheng XS, Yang XF, Liu WG, Shen G, Pan DS, Luo M. A novel method for culturing neural stem cells. *In Vitro Cell Dev Biol Anim*. 2007; 43:155–158. [PubMed: 17619224]

Figure 1. The combination of PDGF and bFGF sustains the production and viability of oligospheres

Embryonic cells were incubated in NPM for several passages and then dissociated to single cells to generate new spheres either in EGF (A, B, I, J), bFGF (C, D), PDGF (E, F), PDGF/bFGF (G, H, K, L). Spheres exposed to EGF (A), bFGF (C), PDGF (E) or PDGF/bFGF (G) for 48 hr, were then plated on PDL-coated plates and incubated for additional 48 hr with NPM containing EGF (B), bFGF (D), PDGF (F) or PDGF/bFGF (H), and cell migration from spheres was analyzed. Spheres were prepared from *Plp*-EGFP mice (I, J, K, L) in order to assess the expression of the *Plp* promoter in these cells. PDGF alone was not sufficient for Nph survival (E, F) but in combination with bFGF produced cell aggregation and *de novo* sphere formation (G). Generation of migratory oligodendrocyte precursor-like cells was observed from plated spheres in medium supplemented with bFGF (D) and more abundantly in PDGF/bFGF (H). Plated EGF-Nphs produced limited numbers of migratory cells (B) and PDGF alone was incapable of maintaining cell viability (F). While some cells in EGF-Nphs obtained from the *Plp*-EGFP transgenic mouse showed green fluorescence indicative of *Plp* promoter activity (I, J), *Plp*-EGFP expression was increased in cells in the spheres grown in PDGF/bFGF (Oligospheres, Olph, K, L). Bar; 50 μ m

Figure 2. Gene expression profiles of cells in oligospheres

Total RNA obtained from EGF-Nph or Olph samples was analyzed by real time PCR for cell-specific marker expression. Data are presented for expression of membrane receptors (A), myelin and glial proteins (B) and transcription factors involved in the developmental regulation of oligodendrocyte progenitor generation, migration and maturation (C, D). The results are presented as mean $\pm$ SEM quantifying samples from 3–5 independent sphere cultures. Each comparison uses specific mRNA expression levels in Olphs, quantified initially relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression, and then presented relative to expression in EGF-Nph as 100%. (** $P < 0.01$; * $P < 0.05$).

Figure 3. Migratory cells in spheres are oligodendrocyte precursors

In order to compare cell populations in Nphs and Olphs, Nphs were plated in EGF (A–C) or PDGF/bFGF (D–F) and Olphs were plated in PDGF/bFGF (G–I). Samples were fixed and immunostained 48 hr later. Cells were analyzed for expression of the specific oligodendrocyte precursor marker, PDGFR- α (A, C, D, F, G, I) and the astrocyte marker, GFAP (B, C, E, F, H, I). The generation of migratory cells positive for PDGFR- α was remarkably elevated in either Nphs or Olphs plated in PDGF/bFGF, as compared to Nphs plated in EGF (A–C). GFAP-positive cells were also detected in all the three conditions, but there was a clear decrease in the number of these cells in plated Olphs (B, E, H). Bar: 50 μ m

Figure 4. Migratory cells generated by plated EGF-Nph and Olph present differential motility behavior

Spheres were plated in PDGF/bFGF and imaged for 25 hr. Images taken at 5 hr intervals are presented. Analyzed cells are in yellow. The movement of each cell during the past 5 hr is in black and the movement of each cell during the next 5 hr is in red with an arrow. Plated EGF-Nph in media supplemented with PDGF/bFGF migrated erratically around the plate (A) and often moved back to their origin (A, 10 hr or 15 hr). Note cell 2 which divides between 20 and 25 hours. Cells from Olphs, on the other hand, showed a radial, oriented migration that appeared organized in a unidirectional manner (B).

Figure 5. Single cells obtained from dissociated EGF-Nph and Olph plated in PDGF/bFGF generate a highly pure oligodendrocyte precursor culture

Olphs and EGF-Nphs were mechanically dissociated into single cell suspensions and plated on PDL-coated dishes in medium supplemented with 10 ng/ml PDGF/bFGF. Bipolar cells expressing *Plp*-EGFP were detected after 24 hours (A, B). Cultures of bipolar cells were essentially confluent after 4 days although they contained some spontaneously maturing cells (E, F). Immunocytochemistry was performed to identify PLP/DM20 expressing cells (C) which co-localized with *Plp*-EGFP (D). This cell population was relatively homogeneous for expression of PDGFR α (G, H) and NG2 (I, J) in both growth conditions, although a few GFAP- positive cells were more easily found in EGF-Nph-derived oligodendrocyte progenitor cell cultures. Total cell number and NG2-positive cell number were determined for Olph and EGF-Nph-derived cultures to assess oligodendrocyte progenitor purity. At least 500 cells per field were counted in 5–8 fields and the results are shown as mean $\pm$ SD of a representative experiment (K). Bars: 50 μ m

Figure 6. Increased expression of specific oligodendrocyte precursor mRNAs in single cell preparations from spheres confirms their oligodendrocyte lineage
 Real time PCR with specific primers for the indicated cell membrane receptors (A) and specific oligodendrocyte lineage transcription factors (B) was performed. Single cells preparations were generated from EGF-Nphs dissociated and plated either in EGF or in PDGF/bFGF or from Olphs dissociated and plated in PDGF/bFGF for 4 days. Results are presented as mean $\pm$ SD of two separate experiments. Expression levels of the indicated genes are relative to GAPDH.

Figure 7. siRNA-mediated knock-down of PDGFR α expression in Nph-derived cells affects oligodendrocyte precursor cell proliferation

Passage 2–6 Nphs were dissociated to single cell suspensions, transfected with PDGFR α siRNA (B, D) or control siRNA (A, C) and plated. After 7 days in culture, cells were stained for PDGFR α (A, B), NG2 (C, D) or Olig2 (not shown). PDGFR α knock-down was confirmed by immunostaining with two different antibodies against the receptor (see methods). A and B show PDGFR α staining with a rabbit polyclonal anti-PDGFR α . Total cell numbers were quantified by DAPI stain in 3 independent experiments (E, average $\pm$ SEM). BrdU incorporation in NG2-, Olig2- and PDGFR α -positive cells was quantified and the number of BrdU-positive cells in control siRNA-transfected cells (white bars) or PDGFR α siRNA-transfected cells (gray bars) is expressed as a percentage of total cells from 2 independent experiments (F). * $p < 0.05$; ** $p < 0.01$ according to Student's t test. Bar: 20 μ m

Figure 8. Oligodendrocyte precursor cells generated from Olphs and EGF-Nph can differentiate into oligodendrocytes

Four day oligodendrocyte progenitor cultures obtained from either Olphs (A, B) or EGF-Nphs (C–F) were grown for 48 hr in PDGF/bFGF and then for another 48 hr in the absence (A, C, E) or presence of 10 ng/ml of ciliary neurotrophic factor (CNTF; B, D, F). *Plp*-EGFP expression is shown in A and B. Cells were stained for O4 (C, D) or MBP (E, F). Bar: 20 μ m.

Table 1

Oligonucleotides used for qRT-PCR

Protein	Accession No.	Primer Forward 3'-5'	Primer Reverse 3'-5'
GAPDH	M32599	TGAAGGTCGGTGTGAACGGATTGGC	CATGTAGGCCATGAGGTCCACCAC
PDGFR-a	M84607	ACCTTGACAATAACGGGAG	GAAGCCTTTCTCGTGGACAG
EGFR	AF275367	GGCTGCAGCATCCTCTGGTT	CGGATTACCGCTCCTGATAGG
FGFR1	BC033447	CAACAAGACAGTGGCCTGGG	CCGTGCAATAGATAATGATC
FGFR2	BC091652	AATGCCTCCACGGTGGTC	CTCCAGTAATCTGGGGAAG
FGFR3	AF277717	GGAGTTCCACTGCAAGG	CCCCGTAGCTGAGGACGCC
Nestin	BC062893	AGTCAGAGCAAGTGAATGG	AGAAACAAGATCTCAGCAGG
GFAP	BC103571	CAAGAACGAGTCCCTAGAGC	CCTTCTGACACGGATTGGT
NG2	AF352400	AAGAGGAGGAAGAGGGAAGC	CTCAGAGTCATCGCGTGGT
PLP/DM20	NM011123	GTATAGGCAGTCTCTGCGCTGT	AAGTGGCAGCAATCATGAAGG
OLIG1	BC046316	GACCTCAGCCAATCTTCC	TAACACCCTTGATGTTGTACC
OLIG2	BC051967	TCAGAGCACAGGAGCAAGC	AACGACACAGAAAGAAAACAGC
SOX9	Z18958	TGGCAGACCAGTACCCGCATCT	TCTTCTTGCTGACCGCGC
NKX2.1	NM009385	GCCCTACAGTTTCAGTCCAG	ACTGGGACTGGGGTTCTTTT
NKX2.2	U31566	CATTCGCTACAAGATGAAACG	AGAGGCAAAGAAGCAAAGC
NKX6.1	NM144955	GCGCTCCTTGCCTGTA	TCCCATCTTTGTCAAACAA
EMX1	X68881	CCGAGACGCAGGTGAAGGTG	TCCGGCCCTTCCTCTTCCAG
GSH2	S79041	CACTCAGTTACGGATTGGTTTTT	ATCAACCCTGGTTTTTCCTG
SP1	X60136	AAGGAGGCCAGGTGTAG	GGAAAGACGGGCAATAAC
SP3	AF062567	TACTCGCCTCTGGAACACCT	GCAGACCAAGAGGCACATTAG
ID2	BC053699	AAAACAGCCTGTCGGACCAC	CTGGGCACCAGTTCCTTGAG
ID4	AF077859	CAGTGCGATATGAACGACTGC	GACTTTCCTGTTGGGCGGGAT
BMP2	AF041421	GGACTGCGTCTCCTAAA	GCTCAAACCTCAACTCCGAC