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**Platelet-Derived Growth Factor Enables Direct Derivation of
Oligodendrocyte Progenitors from CNS Stem Cells**

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Medicine

by
Rajesh Chalamalasetty Rao

2007

PLATELET-DERIVED GROWTH FACTOR ENABLES DIRECT DERIVATION OF OLIGODENDROCYTE PROGENITORS FROM CNS STEM CELLS

Rajesh C. Rao and Ronald D. McKay. Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD. (Sponsored by Diane S. Krause, Department of Laboratory Medicine, Yale University School of Medicine).

Oligodendrocytes derived in the laboratory from stem cells have been proposed as a treatment for acute and chronic injury to the central nervous system (CNS). Platelet-derived growth factor-receptor alpha (PDGFR α) signaling is known to play an important role for regulation of oligodendrocyte progenitor cell numbers both during development and adulthood. Here, we analyze the effect of PDGFR α signaling on CNS stem cells derived from embryonic day 13.5 murine cortex and cultured in monolayer. Fetal and adult CNS stem cells express PDGFR α , and PDGF-AA treatment increases viability and proliferation of these cells. In the absence of insulin, this effect of PDGF-AA is very clear. Consistent with this result, PDGF-AA strongly stimulates glycolytic rate. PDGF-AA treatment rapidly induces morphological changes in the cells although the cells maintain expression of a wide range of precursor markers. We show that a brief exposure to PDGF-AA rapidly and efficiently induces oligodendrocytes from CNS stem cells. Our data suggest that phosphoinositide kinase-3 (PI3K)/Akt, mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal-related kinase (MEK/Erk), mammalian target of rapamycin (mTOR) regulate survival, proliferation, glycolytic rate, and oligodendroglialogenesis induced by PDGF-AA. By treating with PDGF-AA, progenitor cells directly from embryonic cortex can be expanded and differentiated into oligodendrocytes with high efficiency. Our results show that PDGF-AA promotes oligodendrocyte progenitor generation from CNS stem cells and supports their survival and proliferation. The derivation of oligodendrocytes demonstrated here may support the safe and effective use of stem cells in the development of new therapies targeting this cell type.

Acknowledgements

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Introduction

Oligodendrocyte progenitors are dividing, lineage-restricted precursors that exist in both the developing and adult central nervous system (CNS) and differentiate into oligodendrocytes, the cell type responsible for myelination of CNS axons (1). *In vivo*, oligodendrocyte progenitors arise in distinct temporal waves starting from embryonic day 12.5 (E12.5) in the ventral forebrain, which then migrate laterally and dorsally, arriving in the cortex by E16 (2). Historically, the isolation of oligodendrocyte progenitors has involved extensive, multistep purification procedures from postnatal tissues of the forebrain and optic nerve (3, 4). The transplantation of this cell population has been proposed as a therapeutic approach to treat demyelinating diseases such as multiple sclerosis, and spinal cord injury (5-7).

Multipotent stem cells that are the precursors to oligodendroglial, astroglial, and neuronal progenitors can be readily derived from the fetal and adult brain of many mammals and expanded to large numbers (8, 9). Neural stem cells can also be efficiently generated from embryonic stem (ES) cells (10, 11). Stem cells have been proposed as a source of oligodendrocytes to treat demyelinating disease and transplantation of human ES cell derived oligodendrocyte progenitors in spinal cord injury is imminent (12-14). Cytokine signaling regulates the production of differentiated cell types from stem cells *in vitro* (15). Transplantation shows that the *in vitro* system generates functional oligodendrocytes but there is no study that explicitly defines the transition from stem cell to oligodendrocyte progenitor.

Platelet-derived growth factor (PDGF) was initially identified because of its role in the proliferation of glial cells and its oncogenic function in sarcoma virus (16-18). The subsequent identification of the PDGF receptor has had an important role in defining membrane events that trigger proliferation (19-21). PDGF and its receptors are expressed in several different cell types in the developing and adult CNS (22). PDGF ligands A, B, C, and D occur as homodimers and as a heterodimer (AB) and bind two tyrosine kinase cell surface receptors, PDGFR α and PDGFR β . Most of the ligands (AA, AB, BB, CC) bind PDGFR α while a subset (BB, DD) bind PDGFR β . Ligand binding to PDGF receptor induces receptor dimerization, followed by activation of tyrosine kinase domains, and autophosphorylation of tyrosine residues on the cytoplasmic domain of the receptor (22). Docking proteins and signal adaptors interact with the phosphorylated tyrosine residues to initiate downstream signaling cascades important for cell metabolism, growth and survival, including the phosphoinositide kinase-3/Akt/ mammalian target of rapamycin (PI3K/Akt/mTOR) and mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-related kinase (MEK/Erk) pathways (23-25).

In the CNS, PDGFR α is expressed by neuroepithelial cells as early as E8.5, retinal astrocytes, oligodendrocyte progenitors, developing and adult neurons, and adult neural stem cells in the subventricular zone (SVZ) (26-30). PDGFR α -null mice are not viable; lethality is seen between embryonic day 9.5 and 16 (E9.5-16). These embryos show multiple CNS defects, including failure of neural tube closure and neural crest abnormalities (31, 32). Several defects in oligodendrocyte development related to

impaired PDGFR α signaling are observed. PDGFR α haploinsufficient animals, which survive long enough for oligodendroglialogenesis to occur, display precocious differentiation of oligodendrocytes progenitors *in vivo* and *in vitro* (32). The PI3K/Akt pathway is a particularly important downstream effector of this receptor as knockin mice harboring mutations in PDGFR α that selectively eliminate its capacity to activate PI3K die perinatally and display several defects, including impaired migration of oligodendrocytes progenitors and generalized hypomyelination (32, 33). Conditional inactivation of PDGFR α in adult SVZ neural stem cells results in a severe reduction in stem cell-derived oligodendrocytes (29).

PDGF-A plays a specific role in the developing CNS. PDGF-A knockout mice, when compared to wild-type or PDGF-B null mice, have reduced numbers of oligodendrocytes, display severe hypomyelination and a dysmyelinating phenotype (tremor), which argues for an essential role of the ligand in oligodendrocyte development (34). *In vitro*, PDGF-AA is generated by astrocytes and is a more potent mitogen for oligodendrocyte progenitors than are other isoforms (35-38). In the adult brain, infusion of exogenous PDGF-AA shifts cell production of SVZ neural stem cells from neurogenesis to oligodendroglialogenesis *in vivo* (29). In the mouse embryo, PDGF-A and PDGFR α are expressed in adjacent layers; PDGF-A is the predominant isoform expressed in the developing CNS (38, 39).

Apart from these developmental roles, PDGF signaling has been implicated in CNS tumorigenesis. Autocrine PDGF signaling—most commonly overexpression of the ligand

and/or receptor—is found in essentially 100% of human gliomas (40). Indeed, gene transfer of PDGF-AA, has been shown to promote tumor-like formation *in vivo* (41, 42). Continuous, *in vivo* ventricular infusion of PDGF-AA into the adult brain induces the formation of large, SVZ stem cell-derived hyperplasias with some features of glioma (29).

In this manuscript, we report the effects of PDGF-AA on neural stem cells derived from the fetal cerebral cortex. Continuous treatment of CNS stem cells with PDGF-AA maintains expression of neural precursor markers and increases viability, proliferation, nestin⁺ cell number and glycolytic rate. The increased cell number and glycolytic rate observed with PDGF-AA treatment is regulated by PI3K/Akt, MEK/Erk, and mTOR pathways. Brief exposure to PDGF-AA rapidly induces oligodendroglialogenesis from CNS stem cells through MEK/Erk and PI3K/Akt pathways. Using PDGF-AA, we describe a one-step method to derive large numbers oligodendrocyte progenitors directly from embryonic cortex and CNS stem cells. These findings demonstrate that PDGF-AA regulates CNS stem and progenitor cell number, metabolism, and fate through particular intracellular signaling pathways. The efficacy and safety of the oligodendrocyte progenitor populations derived here can now be simply assessed.

Statement of Purpose, Specific Hypothesis, and Specific Aims

Despite the imminent use of stem cell-derived oligodendrocytes in clinical therapy of spinal cord injury, there is no study that explicitly defines the transition from stem cell to oligodendrocyte progenitor (43, 44). In this study, our purpose was to define this transition by using multipotent neural stem cells derived from the fetal cortex, which clonally generate oligodendrocytes, neurons, and astrocytes. Given that platelet derived growth factor-AA (PDGF-AA) plays an essential role in oligodendrocyte development, we hypothesized that the cytokine would induce oligodendroglialogenesis from CNS stem cells (45). Our specific aims were to generate a rapid, serum-free method to generate oligodendrocytes from CNS stem cells and to determine which cell signaling pathways regulate the specification of the oligodendroglial fate.

Materials and Methods

Cell culture. Fetal cortices were dissected free of meninges from E13.5 CD-1 mouse embryos (Charles River, Wilmington, MA) in Hank's buffered saline solution (HBSS; Mediatech, Herndon, VA). Cortices were dissociated by brief mechanical trituration and resuspended in serum free medium containing Dulbecco's modified Eagle medium (DMEM/F12, Mediatech) with N2 supplement (expansion medium) and fibroblast growth factor-2 (FGF2, 20 ng/ml, added daily, R&D Systems, Minneapolis, MN). N2 supplement contains 25 µg/ml insulin, 100 µg/ml human apotransferrin, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite (all from Sigma-Aldrich, St. Louis, MO) (46). CNS stem cells from passage 1 were passaged at ~60% confluence by briefly incubating in HBSS and gently scraped with a cell scraper. CNS stem cells from passage 1 were plated at 5,000-25,000 cells/cm². FGF2 was included throughout our experiments unless otherwise stated. To derive, propagate and expand oligodendrocyte progenitors from E13.5 cortical cells or CNS stem cells, cells were cultured in PDGF-AA (30 ng/ml, added daily) without FGF2 (20 ng/ml, R&D Systems) in expansion media. To rapidly induce oligodendroglialogenesis from CNS stem cells, passage 1 stem cells were re-plated at 25,000 cells/cm² in PDGF-AA containing medium for 12 hours, and cultured in serum-free differentiation media consisting of Neurobasal media with B-27 supplement (Invitrogen, Carlsbad, CA) for a differentiation period of 4-5 days. To induce differentiation of oligodendrocyte progenitors derived from CNS stem cells or primary E13.5 embryonic cortical cells, PDGF-AA was withdrawn and expansion media was replaced with differentiation media.

Immunocytochemistry. Cultured CNS cells were fixed with 4% paraformaldehyde and processed as described (47, 48). Cells were stained with primary antibodies against the following proteins: A2B5 (mouse monoclonal, 1:500, Chemicon, Temecula, CA), NG2 (rabbit polyclonal, 1:100, Chemicon), O4 (mouse monoclonal, 1:50, Chemicon), BrdU (rat monoclonal, 1:400, Accurate, Westbury, NY), cleaved caspase-3 (rabbit polyclonal, 1:200, Cell Signaling, Danvers, MA), nestin (rabbit serum 130, 1:100, McKay Lab), Sox2 (goat polyclonal, 1:50, R&D Systems), PDGFR α (rabbit polyclonal, 1:500, Santa Cruz Biotech, Santa Cruz, CA), Olig2 (rabbit polyclonal, 1:5000, kind gift of H. Takebayashi). Appropriate secondary antibodies (Alexa Fluor 488 and 568, 1:200, Molecular Probes, Invitrogen) were applied and incubated for 1 hour at room temperature. Nuclei were stained with DAPI (0.25 μ g/ml, Sigma, St. Louis, MO). Images were captured, using fluorescent filters, with a Zeiss Axioplan microscope and Zeiss Axiocam HR camera (Carl Zeiss Inc, Thornwood, NY).

Measurement of glycolytic rate. CNS stem cells were plated at 6.25×10^4 cells/cm² and cultured for 24 or 48 hours, and treated as indicated in the Figure legends. Cells were then incubated in expansion media supplemented with 10 mM glucose and 20 μ Ci/ml [5-³H]glucose (Perkin Elmer, Waltham, MA) at 37°C. After 1 hour, an equal volume of 0.2 N HCl was added to stop the reaction. ³H₂O was separated from unmetabolized [5-³H]glucose by evaporative diffusion of ³H₂O in a sealed equilibration chamber. Samples were then analyzed with a scintillation counter, and readings were normalized by cell number.

Flow cytometry. Cells were plated at 3.12×10^4 cells/cm² and cultured for 3 days, and treated as indicated in the Figure legends (growth factors added daily, media was not changed so that viable and nonviable cells could be analyzed). Adherent cells were passaged into suspension and combined with floating viable and nonviable cells and analyzed for viability based on propidium iodide exclusion. Flow cytometry and data analysis were performed by Jeanette Beers using a FACScaliber system and CELLQuest-Pro software (BD Biosciences, San Jose, CA).

Western blotting. Total cell lysates were prepared in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 1 mM PMSF, and phosphatase inhibitors) and were normalized for protein concentration. Protein was loaded on 4-12% polyacrylamide gels and transferred to nitrocellulose membranes (both Invitrogen, Carlsbad, CA). Primary antibodies include PDGFR α , PDGF β (Santa Cruz Biotech, Santa Cruz, CA), phospho-tyrosine742-PDGFR α (Abcam, Cambridge, MA), phospho-serine-473-Akt, Akt, phospho-threonine-202/tyrosine-204-Erk1/2 (Cell Signaling, Danvers, MA), and tubulin (Sigma, St. Louis MO). Blots were probed with HRP-conjugated secondary antibodies (1:10,000, Jackson Immunoresearch, West Grove, PA) followed by chemiluminescent reagent (1:2, Pierce, Rockford, IL).

Pharmacologic treatments. Unless otherwise stated, we used the following concentrations: LY294002 (10 μ M), PD98059 (50 μ M), rapamycin (1 μ M) (Calbiochem, San Diego, CA). For the effects of kinase inhibitors on activation of signaling pathways

following PDGF-AA treatment, cells were pre-incubated for 1 h with inhibitor prior to PDGF-AA administration. Control cultures were treated with DMSO vehicle.

Statistical Analysis. In all experiments, mean \pm s.d. or s.e.m is presented as stated.

Asterisks identify experimental groups significantly different from control groups by the Student's T-test. P-values < 0.05 were considered significant.

Results

FGF2-responsive CNS stem cells express PDGFR α

Murine E13.5 cortical cells were treated with fibroblast growth factor-2 (FGF2) in order to select for and expand CNS stem cells (15). As CNS stem cells in primary culture proliferated with continued FGF2 administration, increased amounts of PDGFR α , but not a related receptor, PDGFR β , were detected by western blotting (Fig. 1A).

Immunocytochemical analysis using an antibody against PDGFR α confirmed that the receptor is expressed by virtually all FGF2-expanded CNS stem cells (Fig 1B).

PDGF-AA stimulation activates Akt, Erk, and mTOR in a sustained manner and maintains expression of neural precursor markers

We next examined signaling induced by PDGF-AA. We found, by western blotting, that PDGF-AA stimulated phosphorylation of PDGFR α , with maximal activation at 5 min (Fig 1C).

Receptor tyrosine kinases, such as insulin, FGF and PDGF receptors, are known to activate several downstream second messenger signaling pathways. In CNS stem cells, PDGF stimulation rapidly activated PI3K/Akt and Ras/MEK/Erk pathways with sustained kinetics: maximal activation occurred at 5 min yet phospho-Akt and Erk levels remained above baseline even 12h and 24h after addition of the ligand (Fig 1C). While we did not observe rapid activation, elevated phospho-mTOR was observed at 24h

following PDGF treatment (Fig. 2A). These data demonstrate that PDGF-AA activates key downstream survival, proliferation, and growth pathways in CNS stem cells in a sustained fashion over FGF2 treatment alone.

Interestingly, co-stimulation with FGF2 and PDGF-AA or switching from FGF2 to PDGF-AA induced a rapid morphological change in CNS stem cells—in 12h, cells became phase-bright and bipolar. Nevertheless, PDGF-treated cells continued to divide and maintained expression of several neural progenitor markers shared by FGF2-responsive CNS stem cells including nestin, Sox2, A2B5, PDGFR α , NG2, and Olig2 (Fig. 1D-G).

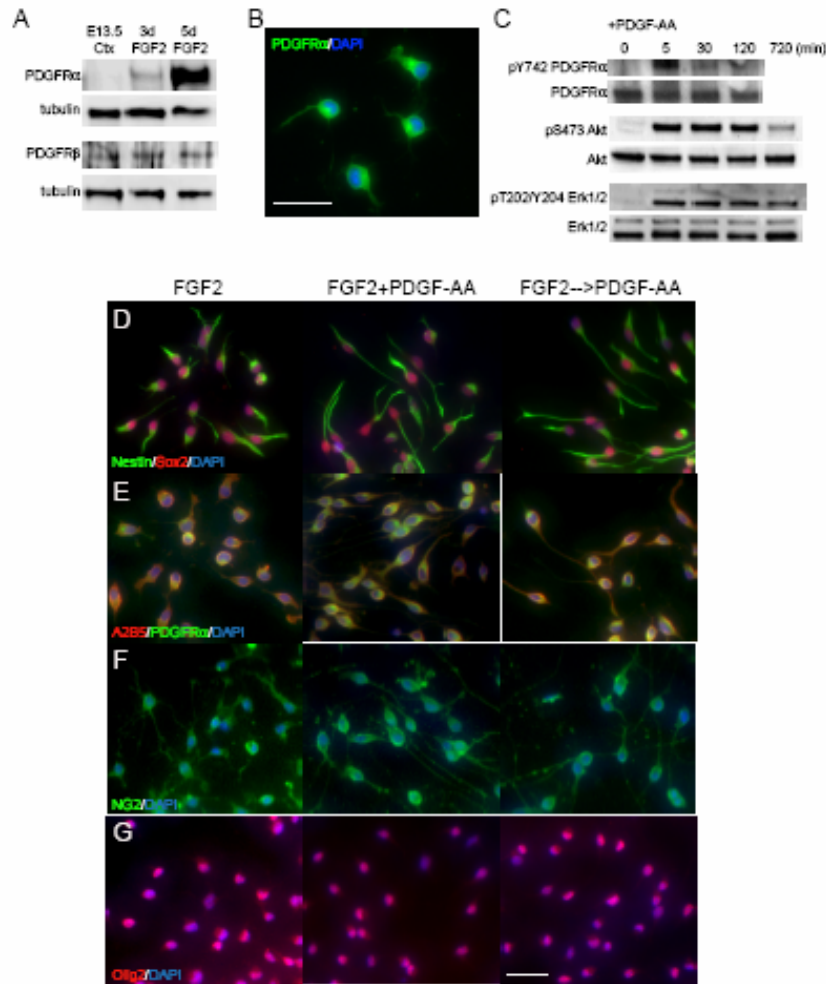


Figure 1. PDGF-AA treatment activates PI3K/Akt and MEK/Erk pathways but maintains expression of precursor markers in embryonic PDGFR α + CNS stem cells. (A) Western blot (WB) analysis of PDGFRs α and β in E13.5 lateral cortex, and after 3d and 5d treatment with FGF2. (B) FGF2-treated CNS stem cells express PDGFR α (PDGFR α , green). Scale bar = 10 μ m. (C) PDGF-AA causes phosphorylation of PDGFR α and rapid and sustained activation of MEK/Erk and PI3K/Akt pathways. (D,E,F,G) Passage 1 CNS stem cells with treated with FGF2 alone, FGF2 + PDGF-AA, or switched from FGF2 to PDGF-AA alone for 3d. PDGF-AA treated cells maintained expression of several precursor markers, including (D) nestin (green), Sox2 (red); (E) A2B5 (red), PDGFR α (green); (F) NG2 (green); (G) Olig2 (red). All nuclei (B, D-G) were stained with DAPI (blue). Scale bar = 20 μ m.

PDGF-AA increases nestin+ cell number, viability, and proliferation

The activation of Akt, Erk, and mTOR pathways in CNS stem cells following PDGF stimulation prompted us to examine whether progenitor cell number, proliferation, and viability may be enhanced. To this end, we cultured CNS stem cells (passage 1) and primary E13.5 cortical cells at low density (5,000 cells/cm²), and assessed cell number 4d later. PDGF-AA treatment increased nestin-positive cell number in FGF2-expanded CNS stem cells and in primary E13.5 cortical cells by two to three-fold ($316 \pm 18.6\%$ and $233 \pm 37.9\%$ of FGF2-treated controls, respectively; $p < 0.02$; Fig 2A, 3B). The increases in cell number correlated with increased Erk1/2, Akt, and mTOR phosphorylation (at 24 h) in PDGF-treated cultures versus control cultures in FGF2 alone (Fig 2A). The PDGF-AA mediated increase in cell number was completely suppressed by pretreatment with the PI3K, MEK, and mTOR inhibitors, LY294002 (10 μ M), PD98059 (50 μ M), rapamycin (1 μ M), respectively ($62.5 \pm 21.1\%$, $65.9 \pm 13.0\%$, $127 \pm 37.2\%$, respectively, vs. $316 \pm 18.6\%$ FGF2+PDGF-AA, $p < 0.03$, Fig 2A). Western blotting confirmed inhibition of these three pathways (Fig 2A). These data show that the Akt, Erk1/2, and mTOR pathways are required for this effect.

To assess whether the PDGF-AA induced increase in cell number was due to changes in proliferation, cells were pulsed with BrdU (10 μ g/ml) for increasing periods of time, and the proportion of cells undergoing S-phase during each pulse was analyzed by immunocytochemistry. At each pulse length, a consistent increase in BrdU incorporation was observed among PDGF-AA treated cells compared to control cells (e.g. for a 12h

pulse, $71.1 \pm 4.15\%$ for FGF2 vs. $86.2 \pm 1.51\%$ for FGF2 + PDGF-AA; $p < 0.015$ at all pulse lengths; $n = 3$ for each pulse; Fig 2B). However, we found the slopes during the linear and plateau phases of the BrdU incorporation curve to be similar between both groups indicating that PDGF-AA does not change cell cycle length (data not shown). A modest but significant increase in cell viability was observed by PDGF-AA treatment, as measured by propidium iodide exclusion ($61.3 \pm 4.62\%$ in controls to $69.5 \pm 0.955\%$, $*p < 0.05$; $n = 3$; Fig 2F). These data demonstrate that PDGF treatment, through relatively modest changes in replication and survival rates, results in significant increases in progenitor cell number and correlates with enhanced levels of basal Akt, Erk, and mTOR phosphorylation.

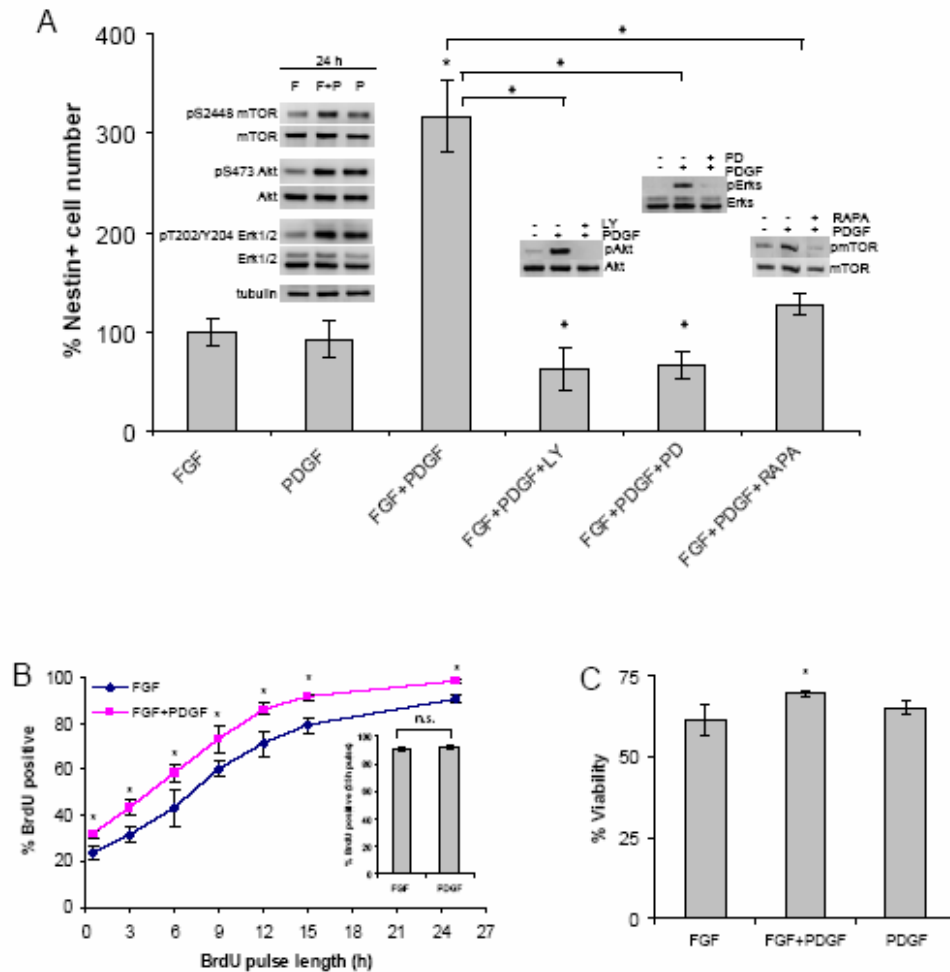


Figure 2. PDGF-AA increases nestin+ cell number, viability, and proliferation; and activates mTOR PI3K/Akt, MEK/Erk pathways. (A) CNS stem cells were plated at low density (5,000 cells/cm²) and grown for 4d ± FGF2, PDGF-AA, inhibitors of PI3K (LY, LY294002, 10µM), MEK (PD, PD98059, 50µM) and mTOR (RAPA, rapamycin, 1µM). Cell number (mean ± s.d., n = 4, *p < 0.03) is expressed as percent of control (FGF2). WB analyses confirm inhibition of mTOR PI3K/Akt, MEK/Erk pathways. (B) CNS stem cells treated with FGF2 alone vs. FGF2 and PDGF-AA were pulsed with 10µM BrdU for increasing time periods, fixed, and labeled with anti-BrdU antibody (mean ± s.d., n = 3, *p < 0.015). To compare S-phase entry of control cells with cells switched from FGF2 to PDGF-AA, cells were pulsed for 24h, and labeled with anti-BrdU antibody (mean ± s.d., n = 3 per time point, n.s. = non-significant). (C) CNS stem cells were grown for 3 d ± FGF2, PDGF-AA, and viability was analyzed based on propidium-iodide exclusion using FACS (n = 3, *p < 0.05).

PDGF-AA maintains cell number, viability, and proliferation in the absence of FGF2 and insulin

CNS stem cells are cultured *in vitro* with high concentrations of insulin (25 $\mu\text{g/ml}$), a growth factor known to activate several downstream intracellular signaling cascades, including PI3K/Akt, MEK/Erk, and mTOR pathways and to promote cell survival and proliferation (49). To assess potential effects of PDGF on CNS stem cells that insulin may mask, we cultured cells in the absence of insulin. In our low density plating assay, in the presence of FGF2, 4 d insulin withdrawal resulted in a significantly diminished cell number ($9.88 \pm 2.30\%$ of control, $p < 0.0002$, Fig 3A). However, PDGF-AA treatment rescued cell number (FGF2+PDGF: $93 \pm 14.85\%$; PDGF only: $46.7 \pm 7.31\%$; Fig 3A).

Interestingly, cultures in PDGF-AA alone were partially rescued, and showed a five-fold increase in cell number over control cultures maintained in FGF2-containing, insulin-free conditions and approximately half that of FGF2 and insulin-treated cultures ($46.7 \pm 7.31\%$ vs. $9.88 \pm 2.30\%$ vs. $100 \pm 14.1\%$, $p < 0.001$, Fig. 3A). Similarly, in primary E13.5 cortical cultures, no difference was observed between nestin+ cell number maintained in FGF2 and PDGF-AA minus insulin and control, FGF2 and insulin-containing cultures ($100 \pm 35.3\%$ in FGF2+insulin vs. $58.1 \pm 22.2\%$ in FGF2+PDGF-AA minus insulin, $p = 0.09$)

To examine survival, FGF2-treated CNS stem cells were treated with or without insulin, and either co-stimulated with or switched from FGF2 to PDGF-AA for 3d. While fresh FGF2 and PDGF-AA were added daily, media was not changed from plating so that dead or dying cells in suspension would not be excluded from viability analyses. At 3 d, media supernatant containing viable and nonviable cells in suspension was combined with the HBSS-passaged, adherent progenitors. The cell suspensions were incubated with propidium iodide and analyzed by FACS sorting. Insulin withdrawal severely compromised the survival of FGF2-treated cells, resulting in a drop in viability (from $61.88 \pm 7.13\%$ to $7.13 \pm 4.94\%$, $p < 0.001$, Fig 3C). Addition of PDGF-AA (in the presence of FGF2) effectively prevented insulin withdrawal-induced reduction in viability, and even PDGF-AA alone (without FGF2 and insulin) showed high viability (FGF2+PDGF-AA minus insulin, and PDGF-AA minus FGF2 and insulin: $60.2 \pm 6.02\%$, and $38.0 \pm 6.01\%$, respectively; Fig 3C).

We then analyzed the insulin masking effect on BrdU incorporation after a 3hr pulse. Similar to the effect on viability, insulin withdrawal, in the presence of FGF2, caused a marked reduction in BrdU incorporation ($31.6 \pm 1.00\%$ in insulin vs. $10.2 \pm 0.623\%$ without insulin, $p < 0.00001$, Fig 3D). Co-stimulation with PDGF-AA and FGF2 or switching to PDGF-AA in FGF2 and insulin-free conditions largely abrogated the decrease in proliferation (FGF2+PDGF-AA minus insulin and PDGF-AA minus FGF2 and insulin: $37.8 \pm 1.09\%$ and $26.6 \pm 1.31\%$, respectively; Fig. 3D). In fact, even in the absence of insulin, PDGF-AA addition actually increased the proportion of cells synthesizing DNA above control cultures ($p < 0.003$, Fig 3D).

Consistent with these results, the levels of p-mTOR, p-Akt, and p-Erk1/2 pathways 24 h following insulin withdrawal significantly decreased (Fig 3A). When cultures were co-stimulated with PDGF-AA or switched to PDGF-AA alone following insulin withdrawal, increased activation of these pathways was evident.

PDGF-AA promotes glucose metabolism

Insulin is a well known factor that stimulates glucose metabolism through several pathways, including through PI3K/Akt activation (50, 51) . Our results indicate that PDGF-AA is able to maintain an elevated level of phospho-Akt and high viability and proliferation without insulin, an effect strikingly distinct from FGF2. Therefore, to assess whether PDGF-AA alters glucose metabolism in CNS stem cells, we measured glycolytic rate in the presence and absence of insulin and PDGF-AA. As predicted, 24h after insulin withdrawal, glycolytic rate largely dropped ($100 \pm 21.4\%$ vs. $29.3 \pm 10.1\%$; $p < 0.0001$, Fig 3E). Co-stimulation with PDGF-AA, however, abrogated the decrease in glycolytic rate (FGF2+insulin: $100 \pm 21.4\%$ vs. FGF2+PDGF-AA minus insulin $80.9 \pm 20.3\%$; $p = 0.105$, Fig 3E). Switching to PDGF-AA alone showed much higher glycolytic rate than culture in FGF2 alone ($56.3 \pm 8.81\%$; $p < 0.01$, Fig 3E). Forty-eight hours after insulin withdrawal, the same pattern remained, and there was little difference in relative glycolytic rates among corresponding treatments at the earlier 24h timepoint (Fig 3E).

We next employed pharmacology to determine which pathways were required for the PDGF-mediated increase in glycolytic rate. Pretreatment with PD98059 and rapamycin nearly halved the glycolytic rate, while pretreatment with LY294002 reduced glycolytic rate to a level below cultures treated with FGF2 minus insulin (FGF2+PDGF-AA minus insulin: $80.9 \pm 20.3\%$; FGF2+PDGF-AA+rapamycin minus insulin $44.4 \pm 3.25\%$; FGF2+PDGF-AA+PD98059 minus insulin $42.2 \pm 10.8\%$; FGF2+PDGF-AA+LY294002 minus insulin $11.2 \pm 3.25\%$. FGF2+PDGF-AA minus insulin vs. each inhibitor-treated group, $p < 0.015$, Fig 3E). These data demonstrate that PI3K/Akt, MEK/Erk, and mTOR pathways mediate the PDGF-induced increase in glycolytic rate, and that PI3K/Akt is a particularly important regulator of glycolytic rate in general.

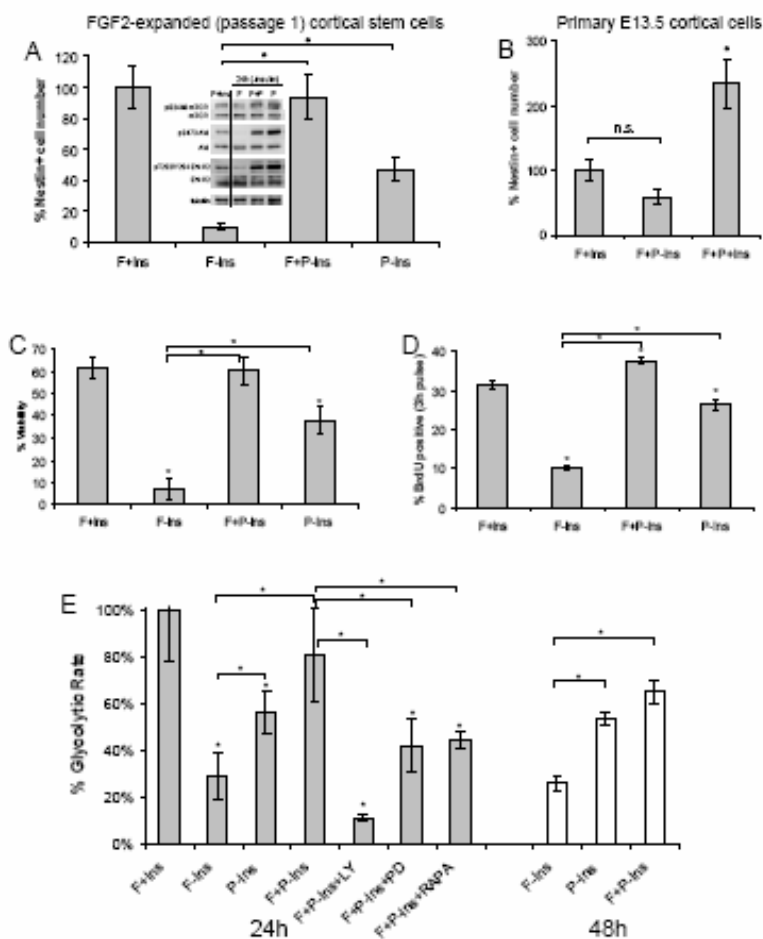


Figure 3. PDGF-AA rescues nestin+ cell number, viability, and proliferation in the absence of FGF2 and insulin and increases glycolytic rate. (A) CNS stem cells were plated at low density and grown for 4 d \pm FGF2 (F), PDGF-AA (P), and insulin (Ins). Cell number (mean \pm s.d., $n = 4$, $*p < 0.001$) is expressed as percent of control (FGF2). (B) Freshly dissected E13.5 primary cortical cells were plated at low density and grown for 4 d \pm FGF2, PDGF-AA, and insulin (mean \pm s.e.m., n.s. = non-significant [$p = 0.09$], $*p < 0.02$). (C) CNS stem cells were grown for 3 d \pm FGF2, PDGF-AA, and insulin; viability was analyzed based on propidium-iodide exclusion using FACS (mean \pm s.d., $n = 3$, $*p < 0.003$). (D) CNS stem cells were pulsed with BrdU for 3 h, fixed and then labeled with an anti-BrdU antibody (mean \pm s.d., $n = 3$, $*p < 0.003$). (E) CNS stem cells were cultured for 24 or 48 h \pm F, P, Ins, LY, PD, or RAPA and glycolytic rate was measured. Glycolytic rate (mean \pm s.d., $n = 4$, $*p < 0.015$) is expressed as percent of control (F+Ins).

PDGF-AA promotes oligodendroglial differentiation from CNS stem cells

PDGF-AA treatment of CNS stem cells resulted in a rapid morphological change: by 24h, the majority of cells became phase-bright, and extended long bipolar processes. However, in the presence of PDGF-AA, all cells expressed neural progenitor markers including nestin and Sox2, and remained in the cell cycle, with virtually all cells undergoing S-phase in 24h (Figs 1D-G, 2B). We found that, in order to induce exit from the cell-cycle and terminal differentiation, PDGF-AA had to be withdrawn from the culture medium. Given the morphological change and rapid activation of the PI3K/Akt and MEK/Erk pathways and their known involvement in CNS cell differentiation, we wondered if a transient exposure to PDGF-AA would be sufficient to alter differentiation. First-passage CNS stem cells were exposed to PDGF-AA for 12 h, and were switched to differentiation medium (Neurobasal + B27 supplement without PDGF-AA or FGF2) for 4-5 d. By 3-4 d, PDGF-AA treated cultures showed obvious oligodendroglial differentiation, with the majority of cells extending numerous fine processes. At 4-5d, PDGF-AA treated cultures were visibly enriched for oligodendrocytes. Immunocytochemistry with the oligodendrocyte-specific antibody, O4, confirmed that PDGF-AA treatment, either by co-stimulation with FGF2 or switching to PDGF-AA alone, increased the proportion of oligodendrocytes by 2.3 and 2.8 fold, respectively (FGF2-only control: $21.4 \pm 2.81\%$; FGF2+PDGF-AA: $49.3 \pm 4.25\%$; FGF2 \rightarrow PDGF-AA: $60.3 \pm 0.918\%$. Control vs. each PDGF-AA treated group, $p < 0.00003$). These data demonstrate that transient exposure of CNS stem cells to PDGF-AA, for a duration roughly less than the length of a cell-cycle, induces robust oligodendroglial differentiation from CNS stem cells.

An interesting feature of PDGF-AA treatment of CNS stem cells was that a short exposure to the cytokine had a robust, long-term effect on oligodendroglial differentiation. This feature allowed us to use pharmacologic inhibitors for a short period of time and minimize toxicity to cells, especially during the 4-5 d period of differentiation. To assess which PDGF-AA activated pathways, if any, were required to induce oligodendroglial differentiation from CNS stem cells, we pretreated CNS stem cells with LY294002 and PD98059 for 1h, and then added PDGF-AA in the presence of inhibitor for 12 h. After 12-13 h, the inhibitor and exogenous PDGF-AA (and FGF2) were washed out by replacing media with differentiation media. The PI3K/Akt and MEK/Erk pathways were specifically chosen because they are rapidly activated by PDGF-AA by 5 min and the elevated activity persists for 12h. Transient blockade of the PI3K/Akt pathway partially inhibited PDGF-AA mediated oligodendroglial differentiation, while the pro-oligodendroglial effect of PDGF-AA was completely inhibited with blockade of the MEK/Erk pathway (FGF2+PDGF-AA+LY294002: $38.5 \pm 4.71\%$ [vs. FGF2 only, $p < 0.002$; vs. FGF2+PDGF-AA, $p < 0.025$]; FGF2+PDGF-AA+PD98059: $26.0 \pm 5.38\%$ [vs. FGF2 only, $p = 0.200$; vs. FGF2+PDGF-AA, $p < 0.0015$]). Thus, while both the PI3K/Akt and MEK/Erk pathways are required for PDGF-AA mediated oligodendroglial differentiation, the MEK/Erk pathway appears more important for this effect.

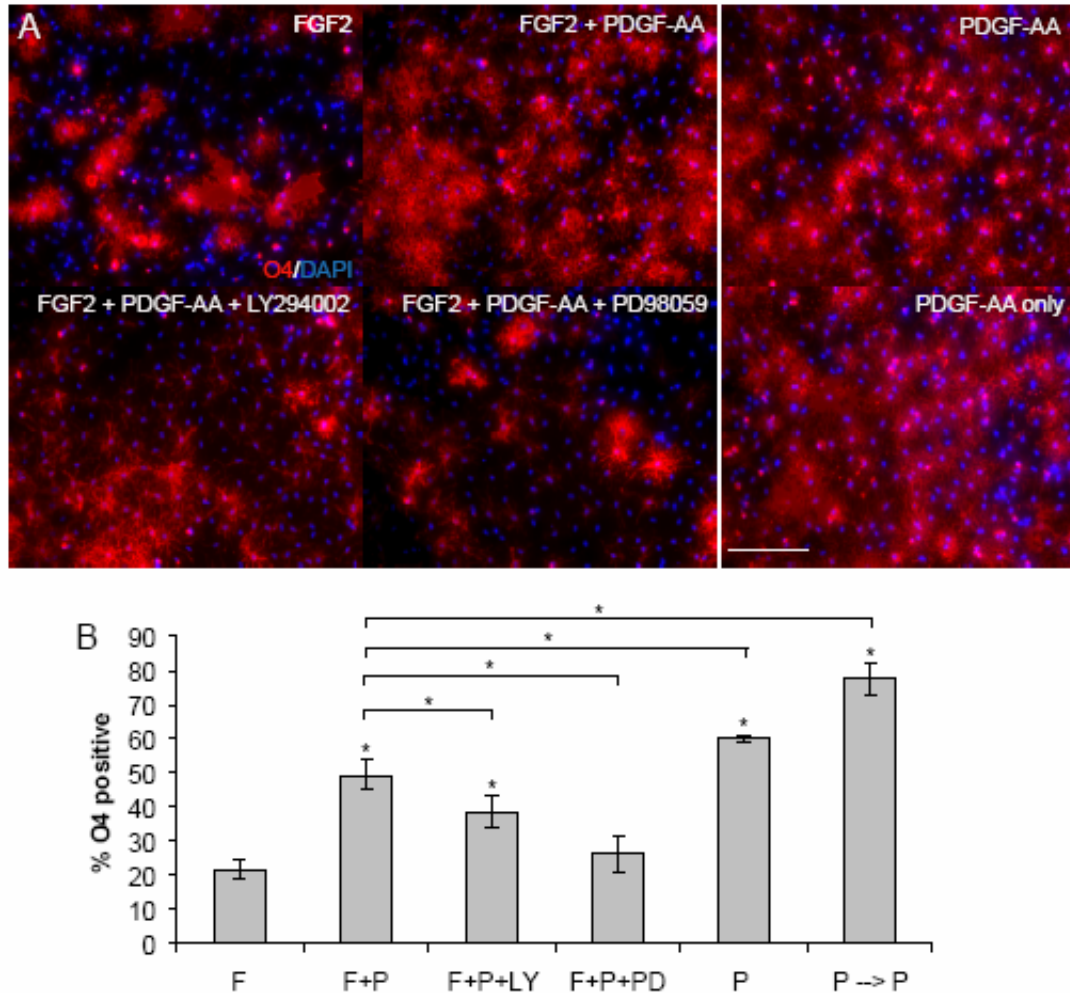


Figure 4. PDGF-AA promotes oligodendroglialogenesis from CNS stem cells. (A) CNS stem cells were plated at 25,000 cells/cm² and pulsed for 12 h ± FGF2, PDGF-AA, LY, and/or PD (cultures were pretreated with inhibitors for 1h prior to PDGF-AA pulse). Cells in “PDGF-AA only” group were obtained by plating primary E13.5 cortical cells in PDGF-AA alone (no exogenous FGF2 was added) for 5-7 d, passaging, and plating at same density in (A). This enriched population of oligodendrocyte progenitors was treated with PDGF-AA for an additional 12 h. After cytokine and/or inhibitor treatment, cells were then switched to serum-free differentiation medium, cultured for 4-5 d, fixed, and labeled with anti-O4 antibody. Scale bar = 20 μm. (B) Percentage of O4+ oligodendrocytes of total cells (mean ± s.d., n = 4 [n = 3 for inhibitor-treated groups], *p < 0.025).

Transient exposure to PDGF-AA is associated with a delay in cell cycle exit during differentiation

When CNS stem cells were exposed to PDGF-AA for only 12 h immediately prior to differentiation, it was clear, 4 d later, that this short pulse affected not only oligodendroglialogenesis, but also total cell number. In fact, differentiated cultures co-stimulated with a 12 h pulse of PDGF-AA 4 d earlier had an approximate 2-fold increase in total cell number, but cell numbers in cultures switched from FGF2 to PDGF-AA, were not significantly different from FGF2-only control cultures (FGF2-only control: 499 ± 92.4 cells/field, FGF2+PDGF-AA: 956 ± 92.3 , $p < 0.005$; FGF2→PDGF-AA: 732 ± 143 , $p = 0.0826$; Fig 5A). To account for the difference in cell number, we tracked proliferation and apoptosis rates. To monitor proliferation during differentiation, we pulsed parallel cultures with BrdU 2 h before fixation, every day for 4 d, and stained with a monoclonal anti-BrdU antibody to score cells undergoing S-phase. We assayed apoptosis, each day of differentiation, with a polyclonal antibody against caspase-3. Analysis of these parameters revealed that the differences in cell number were predominately due to delayed exit from the cell cycle among cells transiently exposed to PDGF-AA. In fact, 24h to 48h after withdrawal of PDGF-AA, treated cells exhibited a 2.23 to 3.55-fold increase in BrdU incorporation versus control cultures (e.g. at 24 h, FGF2-only control: $11.8 \pm 3.87\%$, FGF2+PDGF-AA: $42.1 \pm 3.60\%$, $p < 0.00075$; FGF2→PDGF-AA: $26.5 \pm 2.98\%$, $p < 0.0075$). By day 4 of differentiation, however, BrdU incorporation in all cultures dropped to well below 1%.

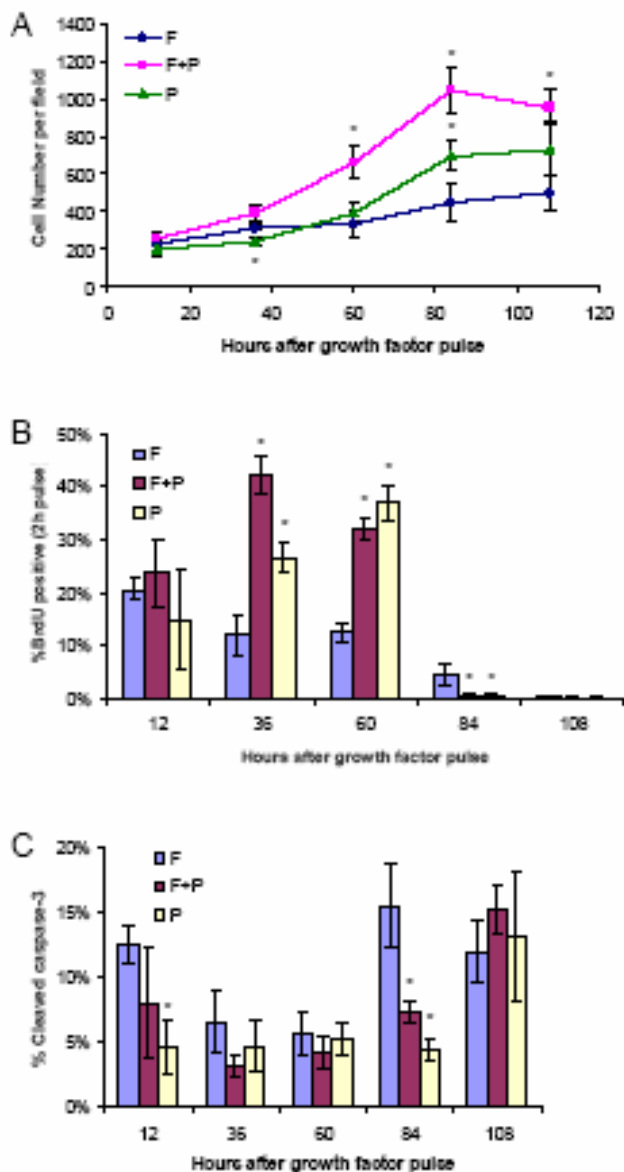


Figure 5. Transient exposure to PDGF-AA is associated with a delay in cell cycle exit during differentiation. (A) CNS stem cells were treated as in Fig 4A. Twelve, 36, 60, 84, and 108 hours after growth factor pulse, differentiating cells were fixed (BrdU was added 2 h before fixation as indicated), and cell number, (B) S-phase, and (C) apoptosis was measured by labeling with DAPI, anti-BrdU, and anti-cleaved caspase-3 antibodies (mean \pm s.d., $n = 3$ for each timepoint, * $p < 0.04$).

PDGF-AA enables direct derivation of oligodendrocyte progenitors from the embryonic cortex

We found that primary cells from E12.5 – E15.5 lateral cortex directly with PDGF-AA alone (no FGF2 or insulin) were selected for embryonic lineage oligodendrocyte progenitors. Two to three days after PDGF-AA treatment of primary embryonic cortical cells, small foci of bipolar, phase-bright cells were visible. By 5-7d of treatment, these bipolar cells were 70-80% confluent, requiring passage. In the presence of PDGF-AA, these cells continued to divide, and could be passaged up to 4-5 times without FGF2 and insulin. Withdrawal of PDGF-AA induced oligodendroglial differentiation (addition of insulin is required for survival of differentiating cells): as high as $77.4 \pm 4.67\%$ were O4+ oligodendrocytes (“PDGF-AA only,” Fig 5A).

Discussion

Clonal analysis shows that multipotent cells can be isolated from the CNS that give rise to neurons and glia (15). The proportion of differentiated cells can be regulated by single factors and the differentiation to astrocytes by activation of the Jak/STAT pathway has been widely studied as a model of fate choice (52-56). A simple method to generate oligodendrocytes from CNS stem cells might also contribute to our understanding differentiation pathways. Here we show that PDGF-AA, through PDGFR α , leads to a rapid increase in the proportion of oligodendrocyte progenitors. The growth factor stimulates rapid and long-lasting activation of Akt and Erk1/2 (57) but phosphorylation of Tyr705 on STAT3 was not observed (data not shown). The use of pharmacological inhibitors suggests that the Erk pathway is required for differentiation but there may also be a role for Akt activation in oligodendrocyte differentiation. The generation of large numbers of oligodendrocyte progenitors by a brief treatment with PDGF-AA can now be used to analyze the molecular mechanisms controlling the specification of this fate.

The PI3K/Akt pathway is a central mediator of glucose metabolism (50, 58, 59). Furthermore, glycolytic rate is highly correlated to cell viability and proliferation (24). Given that PDGF-AA treatment induced sustained Akt phosphorylation, increased survival and proliferation, especially in insulin-free culture, we investigated whether PDGF regulates glucose metabolism in CNS stem cells. PDGF-AA was as effective as insulin in increasing glycolytic rate and over 2.75-fold as effective as FGF2. Along with PI3K/Akt, we looked for other signaling mediators necessary for PDGF-AA regulation of

glycolytic rate. The rapamycin-sensitive, mTOR complex 1, is downstream of Akt and mediates several nutrient transport and metabolic functions (60). Akt can activate mTOR, either by direct phosphorylation, or indirectly through inactivation of tuberin, and an activated mTOR mutant has been shown to maintain glycolytic rate in hematopoietic cells even in the absence of growth factor/Akt signaling (25). We found that rapamycin partially blocked the PDGF-AA increase of glycolytic rate. Additionally, we show that the MEK/Erk pathway is required for the PDGF-AA mediated increase in glycolytic rate in CNS stem cells. Indeed, MEK/Erk pathway has been shown to promote glycolysis in the setting of hypoxia and serum exposure by increasing expression of specific glycolytic enzymes (61, 62).

We have shown that PDGFR α is expressed by virtually all CNS stem cells *in vitro*, and its specific ligand, PDGF-AA, can induce robust oligodendroglialogenesis (2.5-3 fold over control cultures; 50-60% of total cells) via a single 12 h pulse in serum-free culture conditions. The short nature of the cytokine exposure permitted us to interrogate intracellular signaling through the use of several pharmacologic inhibitors and had the added advantage of minimizing exposure to these inhibitors during the subsequent 4-5d differentiation period. While inhibition of PI3K/Akt pathway only partially blocked the pro-oligodendroglial action of PDGF-AA, MEK/Erk blockade almost completely blocked this effect, when compared to control cultures. Thus the MEK/Erk pathway appears particularly important for PDGF-AA mediated oligodendroglialogenesis from CNS stem cells.

We have previously reported that continuous PDGF-AA treatment of rat embryonic and adult CNS stem cells promotes continued proliferation of a neuronal-type precursor, based on expression of the antigen, MAP2 (15). However, there are also reports of MAP2 expression in the oligodendrocyte lineage (63, 64). Also, given the potent mitogenic effect of PDGF-AA as observed by rapidly increasing cell number and BrdU incorporation, continued expression of nestin and Sox2, immature morphology, we wondered if withdrawal of the growth factor was required to allow cell cycle exit and terminal differentiation. After withdrawal of FGF2 and PDGF-AA but in continued presence of N2 medium, we observed some degree of differentiation but this was accompanied by compromised viability and incomplete maturation. We used another chemically defined, widely used serum-free medium, Neurobasal plus B-27 supplement, to promote survival and differentiation of the cells. In this medium, a dramatic increase in survival and maturation of differentiating cells was observed. By 3-5 d, it was obvious that extensive oligodendroglial differentiation was taking place. Others have reported that short pulses of PDGF-AA enhances neuronal differentiation (65, 66), however these groups used non-purified, E12-E14 primary cortical progenitors, without prior expansion with FGF2; many of these primary cells are committed neuronal progenitors. In contrast, FGF2-expanded embryonic neural cells are relatively homogenous and highly enriched for multipotent stem cells (15), and thus were used as our starting population for signal transduction and differentiation assays.

The enhanced proliferation of PDGF-AA treated cells may imply that the cytokine exerts, in part, a selective effect to promote oligodendroglialogenesis. However it remains possible

that the brief pulse of PDGF-AA may exert an instructive change in fate, and that, after PDGF-AA withdrawal, newly specified oligodendrocyte progenitors undergo a higher number of divisions than do neuronal and astrocyte-committed progenitors as they exit the cell cycle and differentiate. Furthermore, a potential instructive component in the action of PDGF-AA on CNS stem cells remains difficult to rule out as virtually all cells express PDGFR α , the cytokine pulse is brief, and that, in response to PDGF-AA, several signaling pathways are rapidly activated and most cells undergo extensive morphologic changes in less than 24 h. Therefore, there is evidence for both selective and instructive components in the action PDGF-AA to promote oligodendroglioneogenesis from CNS stem cells.

Our ability to generate mitotic oligodendrocyte progenitors from CNS stem cells and directly from mid- to late-gestation primary cortical cells, in serum-free conditions, may be particularly advantageous for cell transplantation, as well as for *in vitro* differentiation studies. While others have reported derivation of enriched oligodendrocyte progenitors from neonatal rodent neural stem cells, termed “oligospheres,” the method involves a lengthy selection procedure, including phased administration of B104 neuroblastoma conditioned medium over a period of days to weeks (67, 68). Similarly, the isolation of highly purified oligodendrocyte progenitors from the neonatal and postnatal CNS—well known for 25 years—requires extensive, multistep selection protocols (3, 69). In contrast, we describe a rapid, serum-free method, based on exposure to a single factor, to derive oligodendrocyte progenitors directly from CNS stem cells or embryonic cortex without inducing terminal differentiation. These oligodendrocyte progenitors continue to divide,

do not terminally differentiate, can be passaged 4-5 times in the absence of exogenous FGF2 and insulin, enabling the generation of tens of million progenitor cells from a single embryo (approximately one million oligodendrocyte progenitors can be generated from a single E13.5 embryo at first passage, after 5-7 d *in vitro* cells). When PDGF-AA is withdrawn, cells differentiate into approximately 60-80% O4+ oligodendrocytes.

Taken together, our study links particular signaling pathways downstream of PDGFR α to CNS stem cell growth, survival, metabolism, and fate. With the recent evidence for elimination for embryonic-lineage oligodendrocyte progenitors (2), it will be interesting to compare fetal oligodendrocyte progenitors with those that are derived postnatally, as well as embryonic CNS stem cell-derived oligodendrocyte progenitors with those isolated directly from the embryonic cortex. If human oligodendrocyte progenitors can similarly be generated, this method presented here may be used to derive large numbers of these progenitors for cell therapy for CNS injury or demyelinating disease.

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