

Application Note

Derivation of oligodendrocyte progenitor cells from a human neural stem cell line

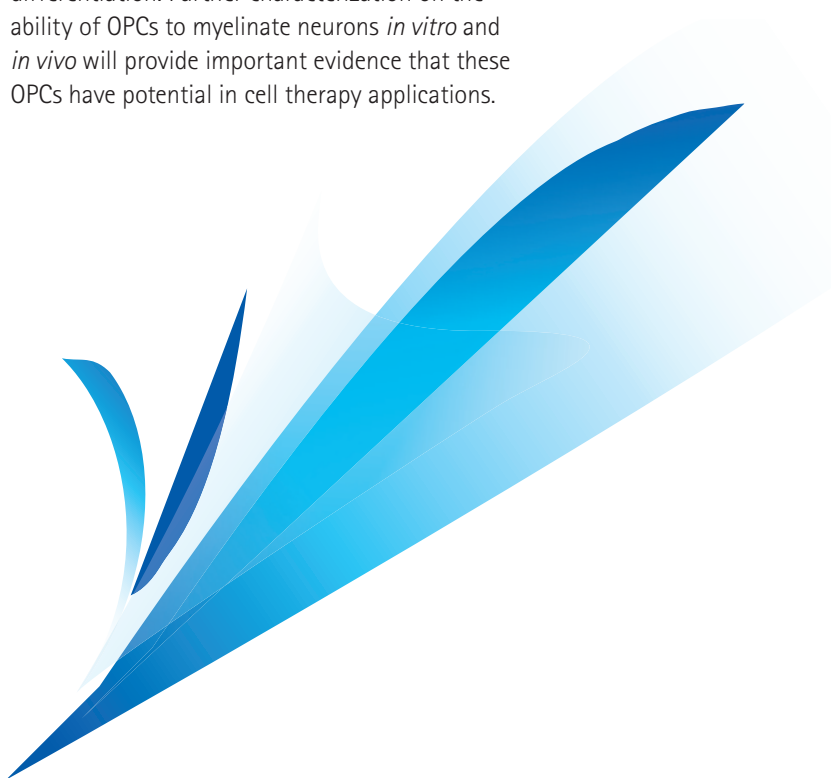
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Introduction

Human neural stem cells (hNSCs) have been intensely studied for their therapeutic potential in neurodegenerative diseases and spinal injuries. Depending on the microenvironment and growth stimuli, these cells can undergo neurogenesis and replace damaged cells. In the central nervous system, neurogenesis alone is not sufficient to repair neuronal damage¹. Functional neurons need to be protected by a myelin sheath, which is synthesized by oligodendrocytes, in order to successfully deliver a long-distance action potential. Understanding oligodendrocyte development is a necessary milestone towards developing clinical and pharmaceutical treatments for neurodegenerative pathologies.

In general, hNSCs generate low and inconsistent numbers of oligodendrocytes in *in vitro* culture. To develop a model system for cultured oligodendrocytes, three neural stem cell lines were tested for their ability to differentiate into oligodendrocyte progenitor cells (OPCs). Two of the three cell lines responded to a regime that included small molecules and growth factors, PDGF, NT3, thyroid hormone (T3) and FGF2. Both cell lines exhibited a 30 to 50% increase in the OPC population as measured by immunofluorescent staining. These

OPCs could be further differentiated to mature oligodendrocytes under mitogen-free conditions. OPCs derived from one of the cell lines were further characterized for changes in proliferation rate, gene expression as well as protein expression. Our data showed that these cells maintained stable OPC characteristics for at least three passages and expressed multiple oligodendrocyte markers upon differentiation. Further characterization on the ability of OPCs to myelinate neurons *in vitro* and *in vivo* will provide important evidence that these OPCs have potential in cell therapy applications.



Material and Methods

Cell line and culture condition

Two human immortalized neural stem cell lines, ReNcell[®] VM (passage 16 to 20) and ReNcell CX (passage 10 to 12) were used along with a human embryonic stem cell-derived neural progenitor cell line (hESC-NPC) (passage 3 to 5). ReNcell CX and VM cells were cultured as a monolayer using ReNcell maintenance medium freshly supplemented with 20 ng/mL FGF-2. ENStem[™]-A expansion medium freshly supplemented with 20 ng/mL FGF-2 and 2 mM glutamine was used to culture the hESC-NPC. ReNcell lines were cultured on 20 µg/mL laminin-coated flasks while hESC-NPC cells were cultured on 20 µg/mL ECL Cell Attachment Matrix (EHS Mouse Tumor) coated flasks. All cells were maintained in a 5% CO₂, 37 °C tissue culture incubator, enzymatically passaged with Accutase[®] solution and seeded at 10⁵/cm² density until they reached 70–80% confluency.

Oligodendrocyte progenitor cell enrichment

Human neural stem cells were harvested and resuspended in their respective growth media at 5 × 10⁵ cells/mL on ultra-low adhesion plates to form neurospheres. The neurospheres were treated with retinoic acid before being switched to oligodendrocyte enrichment medium (OEM: DMEM/F12 with 1X NEAA, 2 mM glutamine, N21 medium supplement, a cocktail of growth factors (thyroid hormone (T3, Sigma Cat. No. T5516); recombinant human PDGF-AA, recombinant human neurotrophin-3, and recombinant human basic FGF). Neurospheres continued to be cultured in OEM for several passages before being plated on the ECL Cell Attachment Matrix-coated flask for expansion. Seeding density for enriched OPC cultures was 1–2 × 10⁴ cells/cm².

Oligodendrocyte progenitor characterization

10⁴ cells/cm² OPCs were plated on 0.1% poly-L-ornithine and 10 µg/mL laminin-coated Millicell[®] EZ SLIDES or T25 flasks and cultured overnight before switching to growth factor-free medium. Oligodendrocytes were allowed to mature for 2 to 3 weeks before being fixed with 2% paraformaldehyde or lysed for RNA extraction.

Proliferating OPCs were characterized with antibodies specific to early oligodendrocyte markers, including anti-Sox10 (1:200), anti-NG2 (1:200), anti-Olig-2 (1:100), and anti-O4 (1:50) while two-week differentiated oligodendrocytes were characterized with antibodies to intermediate

to late oligodendrocyte markers, including anti-GalC (1:200), anti-PLP-1/DM20 (1:100), anti-MBP (1:25), anti-MoG (1:25), anti-CNPase (1:100), and anti-RIP (1:25). Astrocytes were labeled with anti-GFAP (1:250) and neurons were labeled with anti-βIII tubulin (1:100) or anti-MAP2 (1:200). Immunofluorescent staining was performed by blocking cells with 5% normal goat serum and 5% BSA with or without 0.1% TX-100 in phosphate-buffered saline (PBS) for 2 hours at room temperature (RT) followed by incubating with primary antibodies at indicated concentrations overnight at 4 °C.

Following the primary antibody incubation, cells were washed 4 times with blocking solution and incubated with the appropriate fluorescent conjugated goat anti-mouse or anti-rabbit antibodies for 1 hour at RT. 1:5000 of Hoechst 33342 dye was also added in secondary antibody solution to stain nuclei. After incubation, cells were washed 4 times with 1X PBS and mounted for microscope observation. Images were taken either by a Leica[®] DMI-6000 epifluorescent microscope equipped with Hamamatsu ORCA[™] CCD camera or with a Leica DMI-4000 microscope equipped with confocal scanning.

RT-PCR assay

Cells from one T25 flask were lysed and total RNA was extracted using The Absolutely RNA[®] Miniprep Kit, (Stratagene Cat. No. 400800). 2–5 µg of total RNA was used to synthesize cDNA using the SuperScript[®] III Reverse Transcriptase (Life Technologies Cat. No. 18080-051). Primers against GAPDH (NM_002046.3); NKX2.2 (NM_002509.2); Olig-2 (NM_005806.2); PLP-1 (NM_000533.3 and splice variant NM_001128834.1); MBP (NM_001025081.1 and splice variants NM_001025090.1, NM_001025092.1, NM_001025101.1); β-tubulin (NM001069.2); GFAP (NM_002055.3); b-actin (NM_001101.3) and PDGF-α (NM_006206.4) were used to validate the level of expression of each target. Quantitative two-color real-time PCR, using the Amplifluor[®] Human/Mouse Oct4 primer set was performed to measure transcription of the pluripotency gene, Oct4 in oligodendrocyte progenitor cells. As a housekeeping gene control, GAPDH transcription was measured using the Amplifluor Human/Mouse GAPDH primer set.

Discussion and Results

The combination of small molecules and growth factor cocktail increased enrichment of the OPC population from human NSC lines

Three neural progenitor cell lines were tested for OPC enrichment. Among them, ES-derived NPC line (HuESC-NPC) and the immortalized human fetal NSC line (ReNcell VM) contained a significant number of A2B5- and GalC-positive cells in OEM culture. In contrast, the immortalized human cortical NSC line (ReNcell CX cell line) did not demonstrate a significant difference in marker expression between the parental NSC cell line and the directed differentiation conditions (data not shown). Because the ReNcell CX cell line is a clonal line, this may limit its ability to differentiate into an oligodendrocyte-specific lineage. It is also possible that NSCs derived from different parts of the brain have different capacities to differentiate into oligodendrocytes.

Figure 1 shows the quantitative and representative images of human NSCs cultured in original medium (parental) or in oligodendrocyte enrichment medium (OEM). HuESC-NPC (Figure 1 A and B) displayed moderate increases in the O2A progenitor marker, A2B5, and a 3-fold increase in the intermediate oligodendrocyte marker, GalC in OEM culture. In contrast, ReNcell VM cells (Figure 1C and D) cultured in OEM demonstrated more than 6-fold increases in A2B5 expression and less than 2-fold increase in the GalC expression. This illustrates that, even when subjected to the same enrichment procedure, each NSC line responds differently to small molecules and growth factors. We hypothesized that the immortalization of the ReNcell VM line may have altered its developmental progression. As a result, OPCs from HuESC-NPC cells were selected for further characterization.

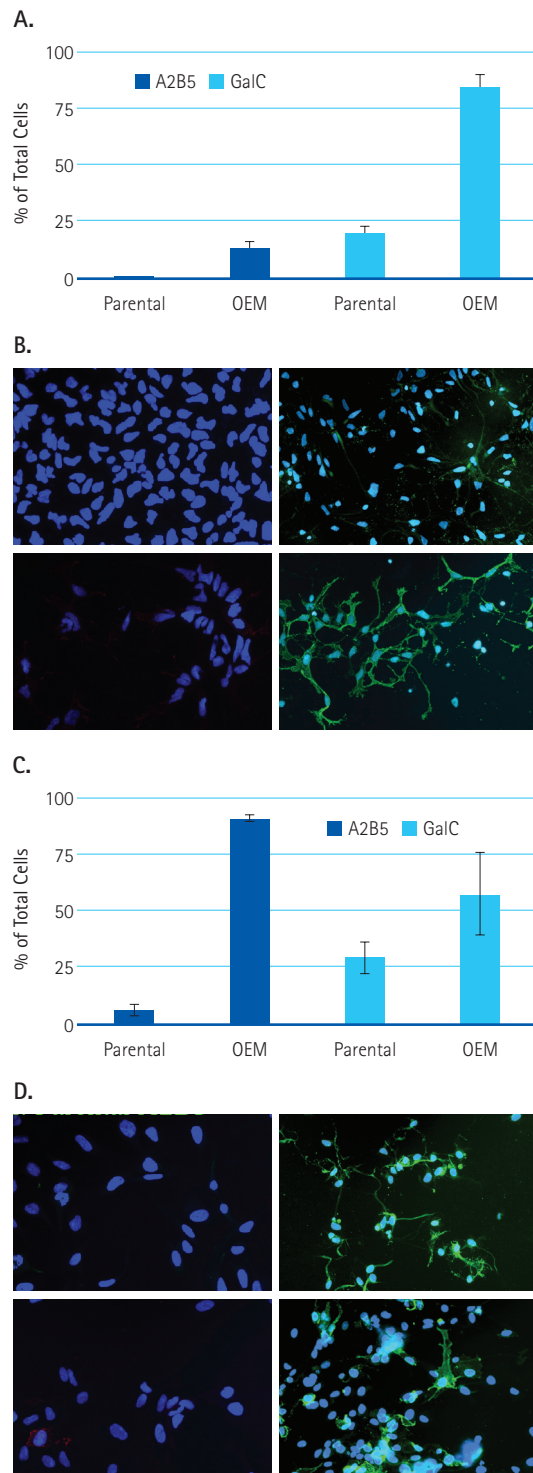


Figure 1. Derived OPC from Human Neural Stem Cell lines. Quantitative measurement of A2B5- or GalC-positive cells in (A) HuESC-NPC or (C) ReNcell VM-derived cultures in standard medium (parental) or oligodendrocyte-enrichment medium (OEM). Data was calculated based on three randomly selected fields and presented as mean with standard error. (B) and (D) show representative images for (A) and (C). Images were taken with 20X objective. A2B5-Parental (B, D, upper left); A2B5-OEM (B, D, upper right); Gal C-Parental (B, D, bottom left); Gal C-OEM (B, D, bottom right).

Growth characterization

To examine whether OPCs could be stably maintained in oligodendrocyte enrichment medium, we monitored cell proliferation rate by measuring doubling time. Cells doubled every 48 hours for at least 7 weeks in culture (Figure 2A) and the bipolar morphology suggested that these cells were in the neural progenitor/oligodendrocyte progenitor stage of differentiation (Figure 2B).

Protein and gene expression

We next examined the OPC cells for protein expression by immunofluorescent staining and gene expression by RT-PCR between passage 3 and passage 5. We observed high numbers of cells expressing Olig-2 (92%, Figure 3A), Sox10 (81%, Figure 3 B), O4 (82%, Figure 3C), and NG2 (64%, Figure 3D). After 2 weeks of spontaneous differentiation, cells turned into 50 to 70% of MAP2- or β III-tubulin-positive neurons (Figure 3J, K) and less than 5% of GFAP-positive astrocytes. 30 to 50% of cells showed robust expression of intermediate to late oligodendrocyte markers (Figure 3E to I).

Similar results were obtained using RT-PCR to show that the enriched OPC culture was capable of differentiating into MBP-positive cells after 20 days of differentiation (Figure 4B).

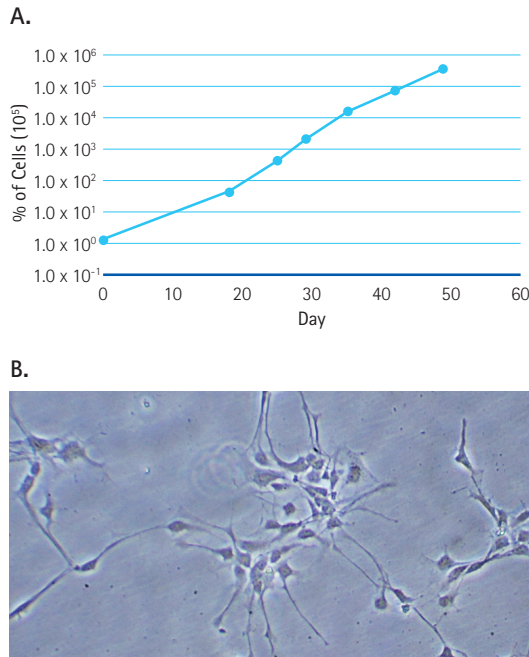


Figure 2. Human NSC derived Oligodendrocyte Progenitor Cells Double Every 48 hours. (A) Proliferation as measured by counting cells. (B) Bright field images of OPC seeding at $2 \times 10^4/\text{cm}^2$.

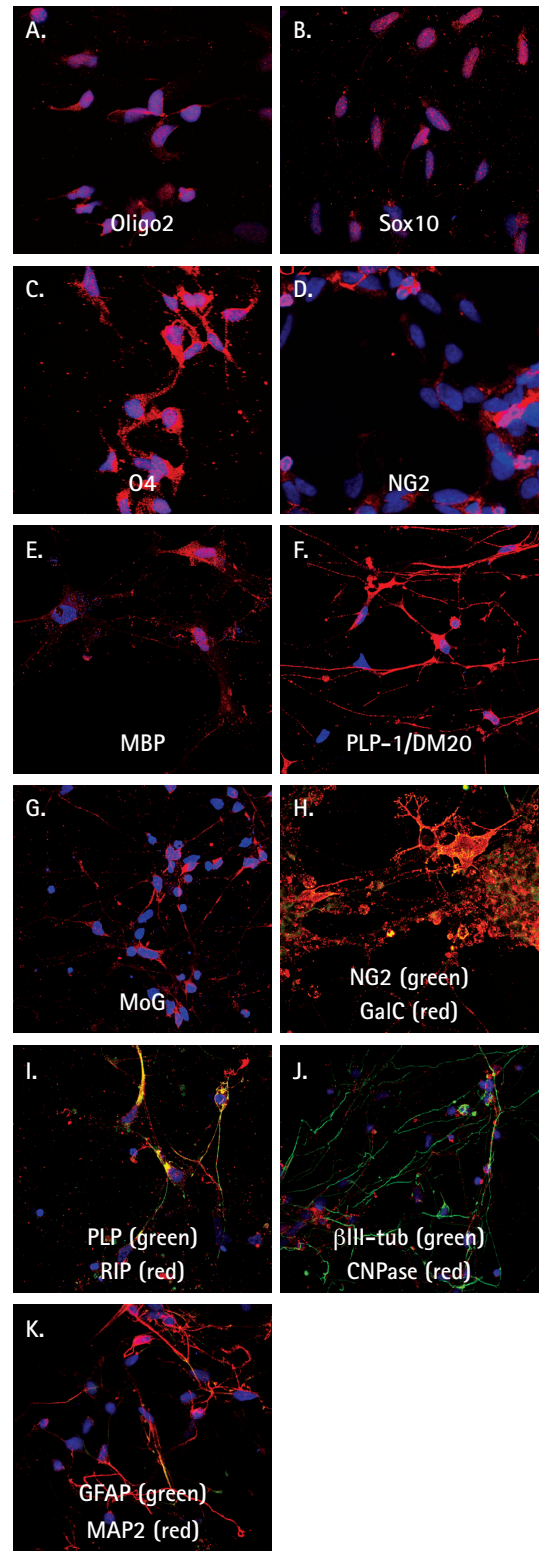


Figure 3. Immunofluorescent staining for Human NSC-derived Oligodendrocyte Progenitor Cells. Immunofluorescent staining and confocal microscope analysis of oligodendrocyte marker expression.

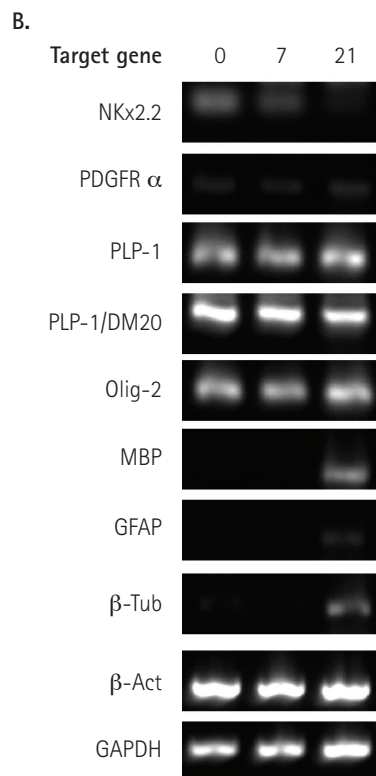
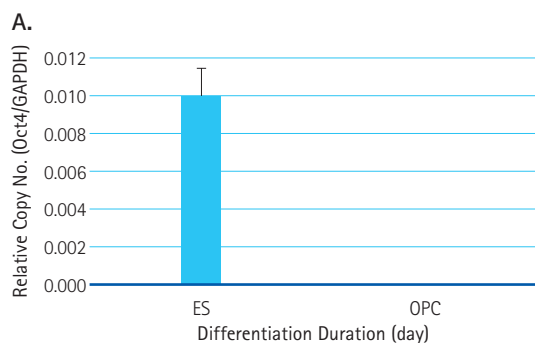


Figure 4. RT-PCR Analysis of human ESCs, OPCs and Differentiated Oligodendrocytes. RT-PCR analysis of 0, 7 and 20 days differentiated cultures. 50 ng total cDNA was used for RT-PCR. For housekeeping genes, β -actin and GAPDH, PCR reactions were set at 30 cycles. For astrocyte marker (GFAP) and neuronal marker (β III-tubulin), PCR reactions were set at 35 cycles. For early/intermediate oligodendrocyte markers, PLP/DM20, Olig-2 and PDGFR α , PCR reactions were set at 35 cycles. For the late oligodendrocyte marker, MBP, the PCR reaction was set at 40 cycles.

Conclusion

We have developed a stable cell line that can maintain a high percentage of oligodendrocyte progenitors in culture and that could potentially yield functional, myelin-producing oligodendrocytes upon spontaneous differentiation. This convenient, reliable source of oligodendrocytes may help to advance research into neuroregenerative therapies for diseases such as multiple sclerosis.

References

- (1) Galvan V, Bredesen DE. Neurogenesis in the adult brain: implications for Alzheimer's disease. *CNS Neurol Disord Drug Targets*. 2007 Oct;6(5):303-10.

Ordering Information

Description	Qty	Catalogue No.
Human Oligodendrocyte Differentiation Kit		SCR600
Includes:		
• 500,000 viable Oligodendrocyte Progenitors (OPCs)		
• 100 mL expansion medium (sufficient for 3 passages)	100 mL	SCM107
• 100 mL differentiation medium (sufficient for 4 weeks of culture)	100 mL	SCM106
Human Oligodendrocyte Characterization Kit		SCR601
• Contains 6 antibodies against early and late markers of oligodendrocyte differentiation		

Related Products

Description	Catalogue No.
ReNcell VM Human Neural Progenitor Cell Line	SCC008
ReNcell CX Human Neural Progenitor Cell Line	SCC007
ENStem Human Neural Progenitor Expansion Kit	SCR055
ReNcell NSC Maintenance Medium	SCM005
Fibroblast Growth Factor basic, human recombinant	GF003
ENStem-A Neural Expansion Medium	SCM004
Laminin, mouse purified	CC095
ECL Cell Attachment Matrix	08-110
Accutase Solution	SCR005
N21 Medium Supplement (50X)	SCM081
Platelet Derived Growth Factor-AA, recombinant human	GF142
Neurotrophin 3	GF031
Fibroblast Growth Factor basic, human recombinant	GF003
Millicell EZ SLIDE 4-well glass, sterile	PEZGS0416
Sox10 Antibody, rabbit polyclonal	AB5727
NG2 Chondroitin Sulfate Proteoglycan Antibody	AB5320
Olig-2 Antibody	AB9610
O4 Antibody, clone 81 (also referred to in the literature as mAB O4)	MAB345
Galactocerebroside Antibody, clone mGalC	MAB342
Myelin Oligodendrocyte Glycoprotein (MOG) Antibody	MAB5680
Myelin Proteolipid Protein (PLP-1) Antibody	MAB388
MBP Antibody, clone SkB3	05-675
CNPase Antibody, clone 11-5B	MAB326
RIP Antibody (Anti-Oligodendrocytes, clone NS-1)	MAB1580
GFAP Antibody	AB5804
bIII tubulin Antibody	AB15708
MAP2 Antibody, clone AP20	MAB3418
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