Enrichment of Oligodendrocyte Progenitors from Differentiated Neural Precursors by Clonal Sphere Preparations

Daisuke Umebayashi,[1,2] Brenda Coles,[1] and Derek van der Kooy[1]

Remyelination is the goal of potential cell transplantation therapies for demyelinating diseases and other central nervous system injuries. Transplantation of oligodendrocyte precursor cells (OPCs) can result in remyelination in the central nervous system, and induced pluripotent stem cells (iPSCs) are envisioned to be an autograft cell source of transplantation therapy for many cell types. However, it remains time-consuming and difficult to generate OPCs from iPSCs. Clonal sphere preparations are reliable cell culture methods for purifying select populations of proliferating cells. To make clonal neurospheres from human embryonic stem cell (ESC)/iPSC colonies, we have found that a monolayer differentiation phase helps to increase the numbers of neural precursor cells. Indeed, we have compared a direct isolation of neural stem cells from human ESC/iPSC colonies (protocol 1) with monolayer neural differentiation, followed by clonal neural stem cell sphere preparations (protocol 2). The two-step method combining monolayer neuralization, followed by clonal sphere preparations, is more useful than direct sphere preparations in generating mature human oligodendrocytes. The initial monolayer culture stage appears to bias cells toward the oligodendrocyte lineage. This method of deriving oligodendrocyte lineage spheres from iPSCs represents a novel strategy for generating OPCs.

Introduction

R EMYELINATION AFTER CELL TRANSPLANTATION is a potential therapy for demyelinating diseases such as multiple sclerosis [1] and other central nervous system injuries [2–5]. Enhanced remyelination has helped to restore neurological function in demyelinating diseases and after other central nervous system injuries [5–8]. Remyelination is performed by oligodendrocytes in the central nervous system and by Schwann cells in the peripheral nervous system [9]. Oligodendrocyte precursor cells (OPCs) are known to be a good cell source for transplantation therapy for remyelination in the central nervous system [10,11].

With respect to cell transplantation therapy, an important breakthrough was the generation of induced pluripotent stem cells (iPSCs) [12]. This advance may enable humans to receive autograft cell transplantation therapy, given that heterologous immune rejection will be avoided with autologous iPSC transplantation, although of course this will not abrogate the autoimmune attack underlying some demyelinating diseases. This approach also may overcome the ethical issues associated with the use of human embryonic stem cells (ESCs) [13]. However, many other problems and potential risks remain for iPSC transplantation therapy, such as oncogenesis due to transplanted immature or undiffer-

entiated cells, as well as the optimal differentiation of iPSCs to the specific cell types needed.

Clonal sphere preparations (such as neurospheres for neural stem cells) are reliable cell culture methods for purifying select populations of proliferating cells [14]. However, some of the characteristics and behaviors of pluripotent cell-derived neurospheres appear different from those of brain-derived neurospheres, with less differentiation bias to glial cells in pluripotent cell-derived neurospheres, less responsiveness to epidermal growth factor (EGF) even as secondary or tertiary spheres, and lower differentiation speed compared with primary brain cultured neurospheres [15–18]. The ability to differentiate to oligodendrocytes is particularly poor in human pluripotent cell-derived neurospheres, perhaps because human pluripotent stem cells are not in the naïve state of mouse pluripotent stem cells [19]. To further remove undifferentiated cells and achieve more specific remyelinating cell fates, it may be useful to derive more oligodendrocyte lineage-specific cell sources (such as OPCs) from human pluripotent cells.

Although recent reports indicate the successful differentiation of human ESC/iPSCs to OPCs, these protocols are time-consuming and reproducibility is still low, owing perhaps partially to variations in the lines of iPSCs [11,20–23].

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[1]Department of Molecular Genetics, University of Toronto, Toronto, Canada.
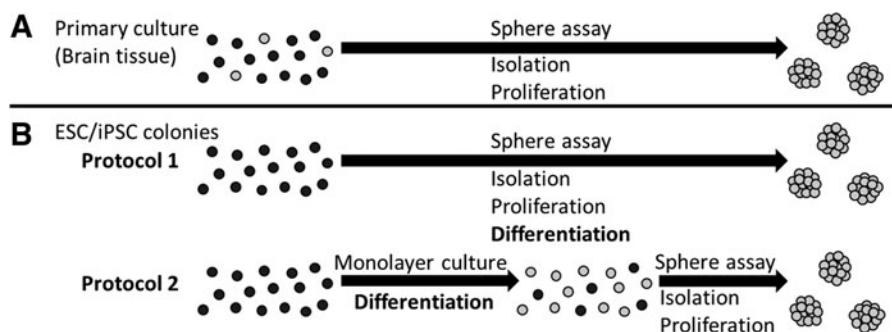[2]Department of Neurosurgery, Nagoya University School of Medicine, Nagoya, Japan.

FIG. 1. Schematic diagrams outlining protocols for clonal sphere formation from tissues and ESC/iPSC colonies. **(A)** Use of neurosphere cultures to isolate and expand neural stem cells from mouse brain. **(B)** Direct isolation of neurospheres from human ESC/iPSC colonies (protocol 1). An alternative protocol (2) delays the formation of neurospheres from ESC/iPSC colonies until after monolayer differentiation. *Gray circle* = neural stem cell; *black circle* = other cells. ESC, embryonic stem cell; iPSC, induced pluripotent stem cell.

The target of these protocols is the induction of OPCs, imitating the differentiation of spinal motor neuron progenitors to oligodendrocytes during development, employing platelet-derived growth factor-AA (PDGF) [22]. However, other shorter protocols rely on spontaneous differentiation [22] similar to the differentiation of oligodendrocytes directly from brain-derived neural stem cells by the depletion of growth factors from the culture media [23]. Most current protocols succeed in deriving OPC populations effectively; however, long differentiation times from OPCs to mature oligodendrocytes result in low efficiency in vitro [11,20].

The advantage of clonal sphere preparations is the isolation and proliferation of specific cell precursors. For example, clonal sphere preparations permit the purification and proliferation of neural stem cells from brain tissue (Fig. 1A). To make clonal neurospheres from human ESC/iPSC colonies, we have found that monolayer neuralizing differentiation may help to increase the numbers of neural stem cells in ESC/iPSC colonies. Indeed, we have compared a direct isolation of neural stem cells from human ESC/iPSC colonies (protocol 1; Fig. 1B) with monolayer neural differentiation first, followed by clonal neural stem cell sphere preparations (protocol 2; Fig. 1B).

Using these protocols, we now report the selection of specific populations of neural progenitor types in the early phases of pluripotent cell culture, without the contamination of heterogeneous non-neural populations observed during differentiation through embryoid bodies. Furthermore, we compared several types of oligodendrocyte differentiation conditions for differentiation of our progenitor cells. The characteristics and differentiation fates of clonal spheres cultured in different growth factor-containing media are outlined. Finally, we compared the efficiency of these two protocols after employing conditions to maximize the final differentiation of mature oligodendrocytes.

Materials and Methods

Culture of human pluripotent stem cells

For differentiation protocol 1 (Fig. 2A), human ESCs (H9) [24] and human iPSCs (FR, ZAN11, RTT-Δ3–4 #37) were propagated and maintained on mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder layers. Each undifferentiated pluripotent cell line was cultured and passaged appropriately according to each provider's protocol [25,26]. FR was provided by A. Nagy (Samuel Lunenfeld Research Institute). ZAN11 was provided by P. W. Zandstra (Institute of Biomaterials and Biomedicals and Biomaterials and Biomedical Engineering). RTT-Δ3–4 #37 was provided by J. Ellis (The hospital for Sick Children).

H9, FR, and ZAN11 cells were cultured in media containing 80% DMEM/F12 and 20% knockout serum replacement, 3 mM NaHCO$_3$, 0.25 mM sodium pyruvate, 2 mM Glutamax, 1% nonessential amino acids, 0.05 mM 2-mercaptoethanol (all from Life Technologies), and 10 ng/mL basic fibroblast growth factor (FGF2; Sigma). Confluent colonies were disaggregated using 0.1% type IV collagenase (Life Technologies) and passaged 1:3–4 for H9 and ZAN11, 1:10–15 for FR every 4–7 days.

The RTT-Δ3–4 #37 line was cultured on inactivated MEF feeder layers in media containing 85% knockout DMEM and 15% knockout serum replacement, 2 mM Glutamax, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol (all from Life Technologies), and 10 ng/mL FGF2 (Sigma) or cultured on growth factor-reduced Matrigel (BD Bioscience) as a feeder-free culture in mTeSR1 (StemCell Technologies). Confluent colonies were disaggregated using 0.1% type IV collagenase (Life Technologies) in feeder-dependent culture or using 2 mg/mL dispase solution (BD Bioscience) and passaged, 1:2 or 1:4 every 7 days.

For differentiation protocol 2, human ESCs (H9) [24] and human cell lines (NCRM1, CCRM5R) were propagated and maintained on a feeder-free system that utilized BD Matrigel™ hESC-qualified matrix (BD Bioscience) and TeSR™-E8™ (StemCell Technologies). Each undifferentiated pluripotent cell line was cultured and passaged appropriately according to the WiCell feeder-independent pluripotent stem cell protocol using E8 medium (WiCell Research Institute, Wisconsin). NCRM1 derived from activated CD3$^+$ T cells enriched from umbilical cord blood was provided by Lonza through the Center for the Commercialization of Regenerative Medicine (CCRM, Toronto, Canada). CCRM5R derived from adult dermal fibroblast cells was provided by CCRM. Confluent colonies were disaggregated using Gentle Cell Dissociation Reagent (StemCell Technologies) and passaged, 1:3–4 every 4–7 days.
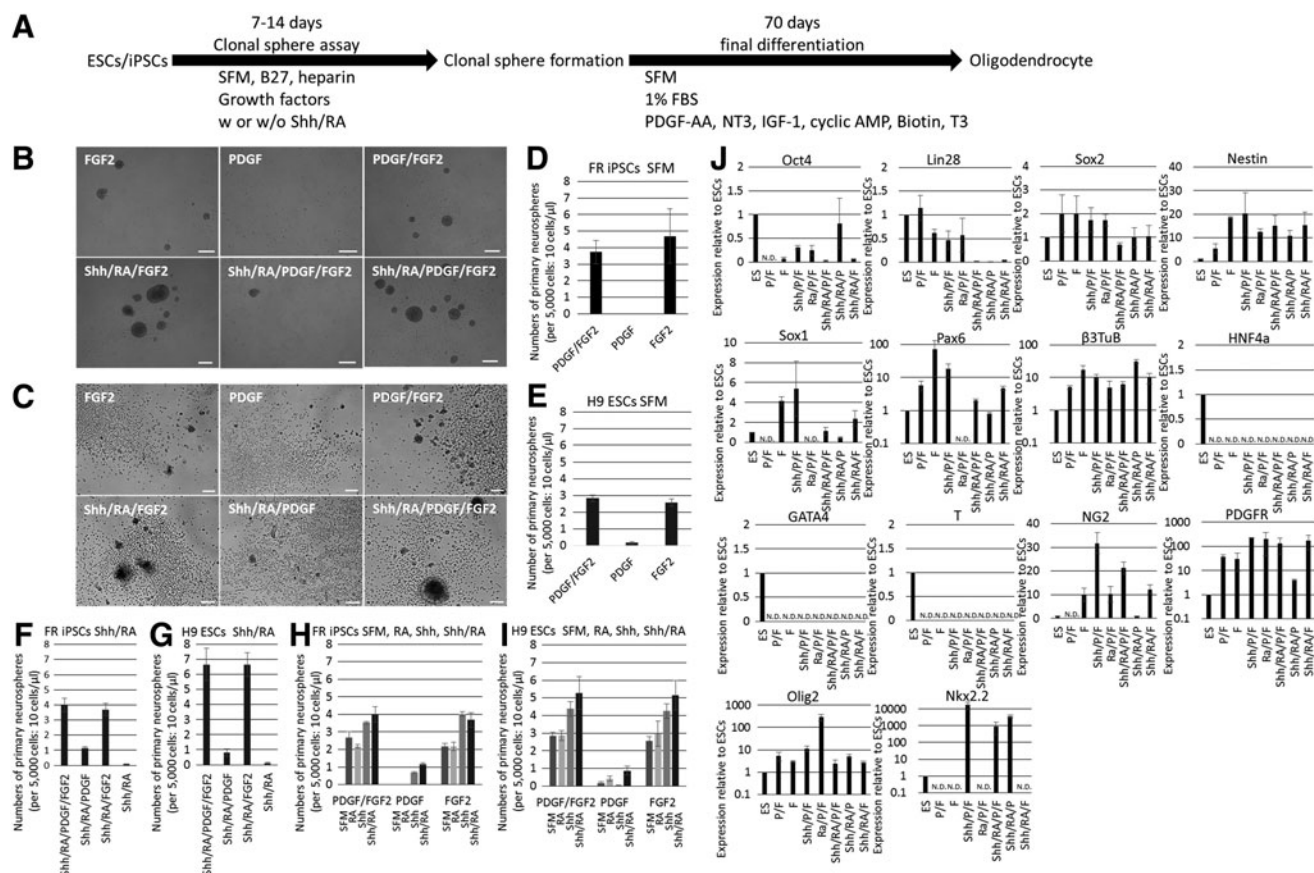
FIG. 2. **(A)** A schematic diagram of protocol 1 for direct neurosphere formation from human iPSCs and differentiation. **(B, C)** Neurosphere formation from human FR iPSCs **(B)** and H9 ESCs **(C)** in SFM with the addition of the noted growth factors. Scale bar = 100 μm. **(D, E)** Numbers of neurospheres in SFM with the noted growth factors. FR iPSCs ($n=12$ separate experiments) **(D)** and H9 ESCs ($n=9$ separate experiments) **(E)**. PDGF alone did not induce neurosphere formation in either FR iPS or H9 lines. There are significant differences between PDGF and PDGF+FGF2 and between PDGF and FGF2 in both **(D)** and **(E)** ($P<0.05$). **(F, G)** Neurosphere formation in Shh/RA combined with the noted growth factors. FR iPSCs ($n=3$ separate experiments) **(F)**. H9 ESCs ($n=3$ separate experiments) **(G)**. The combination of Shh and RA with no other growth factors did not induce neurosphere formation. The combination of Shh and RA allows some induction of neurospheres in PDGF. **(H, I)** Effects of Shh, RA, and Shh/RA combinations with other noted growth factors. FR iPSCs **(H)** ($n=5$ separate experiments). H9 ESCs **(I)** ($n=4-9$ separate experiments). RA did not show a significant increase in spheres compared with SFM in any conditions for both FR iPSCs and H9 cells. Shh is more potent in generating clonal spheres than RA ($P<0.05$) in both of the FGF2 and PDGF+FGF2 conditions in FR iPSCs, but not H9 ESCs. Only the additions of the Shh plus RA combination increased the numbers of neurospheres effectively ($P<0.05$). **(J)** Gene expression of cell-type-specific markers in spheres grown under the noted growth factor conditions ($n=3$ separate experiments, FR iPSC). The pluripotency marker of *Oct4* was decreased after sphere generation, except under the Shh/RA+PDGF conditions. *Lin28*, which is highly expressed in human ESCs and iPSCs, decreased the most in spheres grown under Shh/RA conditions. The neural marker, *Nestin*, is increased in spheres compared with the starting human pluripotent cells. The neural marker of Pax6 also increased in spheres compared with the staring pluripotent cells, except when Shh and RA were added. Endodermal and mesodermal markers (*HNF4a*, *Gata4*, and *T*) decreased in spheres compared with the starting pluripotent cells. NG2 was increased by the combination of Shh, PDGF, and FGF2 condition, including the combination of Shh/RA+PDGA+FGF2. RA alone increased *Olig2* expression, but neither Shh alone nor Shh and RA in combination did this. Nkx2.2 expression was increased by Shh+PDGF, including the combinations of Shh+PDGF+FGF2, Shh/RA+PDGF+FGF2, and Shh/RA+PDGF. These results indicated that the Shh/RA+PDGF+FGF2 condition induced OPC characters. Data are presented as the means ± SEMs. F, FGF2; FGF, FGF2; FGF2, basic fibroblast growth factor; ND, not detected; OPC, oligodendrocyte precursor cell; P, PDGF-AA; PDGF, PDGF-AA; RA, retinoic acid; SFM, serum-free media; Shh, sonic hedgehog; Shh/RA, combination of Shh and RA.

In both protocols, spontaneously differentiating colonies were removed manually before every passage.

Differentiation protocol 1

Neurosphere formation. Clonal neurospheres (Figs. 1B, protocols 1 and 2A) were derived directly from pluripotent stem cells as previously described [27]. Briefly, undifferentiated pluripotent stem cell colonies on MEF cell layers were incubated in Accutase (StemPro) for dissociation for 10 min at 37°C. Detached cells and aggregates were triturated gently a few times to dissociate them to single cells, filtered through a 40-μm cell strainer, and then incubated in the appropriate ES/iPSC media (see above) for 30 min at

37°C to separate undifferentiated pluripotent cells from MEF cells.

Undifferentiated pluripotent cells were plated on ultralow attachment surface 24-well plates (Corning) at a density of 10 cells/μL in the defined serum-free media (SFM) for the growth of clonal neurospheres [28]. The defined SFM contained a 1:1 mixture of DMEM/F12 (Life Technologies) with 0.6% D-glucose, 5 mM HEPES (Life Technologies), 3 mM NaHCO$_3$ (Life Technologies), 2 mM glutamine, 25 μg/mL insulin, 100 μg/mL transferrin, 20 nM progesterone, 60 μM putrescine, and 30 nM sodium selenite (all Sigma). Growth factors that were added singly or in combination included 2 μg/mL heparin, 1× B27 (Life Technologies), 10 ng/mL FGF2, 100 ng/mL recombinant PDGF-AA (Peprotech), 0.1 μM of retinoic acid (RA; Sigma), and 1 μM of purmorphamine (Calbiochem).

Refeeding was performed every week with an equal volume of plating media. For secondary sphere formation, primary spheres were incubated in Accutase for dissociation for 20 min at 37°C and gently triturated a few times to dissociate them to single cells, filtered through a 40-μm cell strainer, and then plated in the same media as used for primary sphere formation, along with 10 μM Rho kinase inhibitor Y-27632 (Calbiochem) [29]. Spheres that had smooth spherical shapes and were over 50 μm in diameter were counted.

Differentiation of sphere colonies. Approximately 100 μm diameter clonal spheres (one sphere per well) were plated on Matrigel (BD Bioscience)-coated 48-well plates in defined SFM with 1% fetal bovine serum (FBS). For oligodendrocyte differentiation, 10 ng/mL PDGF-AA (Peprotech), 10 ng/mL recombinant human NT3 (R&D Systems), 10 ng/mL recombinant human IGF-1 (R&D Systems), 1 μM cyclic AMP (Sigma), 100 ng/mL biotin (Sigma), and 60 ng/mL T3 (Sigma) were added to the media. Refeeding was performed every 5 days.

Differentiation protocol 2

Induction of neural cells. Neural cells were derived in monolayer cultures from pluripotent stem cells using PSC Neural Induction Medium (Life Technologies) and propagated using the Neural Expansion Medium containing 49% Neurobasal Medium, 49% advanced DMEM/F12, and 2% Neural Induction Supplement (all Life Technologies) according to the manual (MAN0008031) provided by Life Technologies Corporation (Figs. 1B, protocols 2 and 4A); 0.1 μM of RA was added to the Neural Expansion Medium after neural induction. After 5 days, 1 μM of purmorphamine and 0.1 μM of RA [sonic hedgehog (Shh)/RA treatment] were added to the medium containing DMEM/F12 (Invitrogen) with N-2 supplement (Life Technologies), 10 ng/mL FGF2, and 20 ng/mL EGF (Sigma).

Monolayer oligodendrocyte induction (control for spheres from protocol 2). After 6 days of Shh/RA treatment, the medium was changed to DMEM/F12 with N-2 supplement, 10 ng/mL FGF2, 20 ng/mL EGF, and 10 ng/mL PDGF-AA for another 4 days. Finally, cells were differentiated as a monolayer in media containing the defined SFM with 1× B27, 10 ng/mL recombinant human NT3, and 60 ng/mL T3 for 70 days. Cotreatment of 50 ng/mL transforming growth factor-β (TGFβ) and 50 ng/mL activin B (ActB) (all Peprotech) was started at day 4 of this 70-day differentiation. Refeeding was performed every 5 days.

Neurosphere formation. After DMEM/F12 with N-2 supplement, 10 ng/mL FGF2, 20 ng/mL EGF, and 10 ng/mL PDGF-AA for 1 day, clonal neurospheres were derived from each monolayer of cells. Briefly, neural cells after Shh/RA treatment were incubated in Accutase (StemPro) for dissociation for 15 min at 37°C. Detached cells and aggregates were gently triturated a few times to dissociate to single cells, filtrated with a 40-μm cell strainer, and then plated on ultralow attachment surface 24-well plates (Corning) at a density of 10 cells/μL in the defined SFM for the growth of clonal neurospheres, as defined in differentiation protocol 1. Growth factors that were added singly or in combination included 2 μg/mL heparin, 1× B27, 10 ng/mL FGF2, and 100 ng/mL PDGF-AA.

Refeeding was performed every week with an equal volume of plating media. For secondary sphere formation, primary spheres were incubated in Accutase for dissociation for 20 min at 37°C and gently triturated a few times to dissociate the spheres to single cells, filtrated through a 40-μm cell strainer, and then plated in the same media as used for primary sphere formation with growth factors, along with 10 μM Rho kinase inhibitor Y-27632. Spheres that had smooth spherical shapes and were over 50 μm in diameter were counted.

Differentiation of sphere colonies. Approximately 100 μm diameter clonal spheres (one sphere per well) were plated on 20 μg/mL poly-L-ornithine (Sigma) and 10 μg/mL laminin (Sigma)-coated 48-well plates in defined differentiation media. For the attachment and spread of sphere colonies, the same growth factors as used for the sphere preparations were added on the first day. The differentiation media contained the defined SFM with 1× B27, 10 ng/mL recombinant human NT3, and 60 ng/mL T3 for 70 days, with or without cotreatment of 50 ng/mL TGFβ and 50 ng/mL ActB, which was started at day 4 of differentiation. Refeeding was performed every 5 days.

RNA extraction and quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from samples using an RNeasy extraction kit (Qiagen) with DNase treatment. cDNA was prepared using SuperScript III RNase H-Reverse Transcriptase (Life Technologies) and 0.2 ng random hexamer primer (6-mer; Fermentas). Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed on a 7900HT Fast Real-time PCR System (Applied Biosystems) using TaqMan gene expression assays (Table 1) with TaqMan Universal PCR master mix (both Applied Biosystems). All samples were run in triplicate. Undifferentiated colonies of each cell line were used as controls.

The resulting values were normalized to the endogenous control (*GAPDH* and *HPTR1*), which was consistent across all samples. Results are presented as the relative expression to that of the control using the comparative Ct method (ΔΔCt). Control was indicated as a standard value of 1; other values are shown as ratios of that value.

Immunocytochemistry

Differentiated cells were rinsed with PBS, followed by 4% paraformaldehyde, for 10 min at room temperature. Cells

TABLE 1. TAQMAN GENE EXPRESSION ASSAYS

Gene	Identification number
GAPDH	Hs99999905_m1
HPRT1	Hs02800695_m1
OCT4	Hs01895061_u1
LIN28	Hs00702808_s1
SOX2	Hs00602736_s1
NES	Hs00707120_s1
SOX1	Hs00534426_s1
PAX6	Hs00240871_m1
TUBB3	Hs00801390_s1
FOXA2	Hs00232764_m1
HNF4A	Hs00766846_s1
GATA4	Hs00171403_m1
T	Hs00610080_m1
NG2	Hs00361541_g1
PDGFR	Hs00998018_m1
OLIG2	Hs00300164_s1
GFAP	Hs00909233 _m1
GalC	Hs01012300_m1
PLP	Hs00166914_m1
CNPase	Hs00263981_m1
MBP	Hs00921945_m1
NKX2.2	Hs00159616_m1

were permeabilized with 0.3% Triton X-100 for 10 min and preblocked with 5% normal goat serum for 1 h at room temperature. Primary antibodies, rabbit anti-beta III tubulin (β3TUB, 1:400; Sigma), rabbit anti-glial fibrillary acidic protein (GFAP, 1:400; Sigma), mouse anti-Nestin (NES, 1:400, Millipore;1:50, Molecular Probes), mouse anti-Pax6 (1:50; Molecular Probes), mouse anti-Sox1 (1:50; Molecular Probes), mouse anti-Sox2 (1:50; Molecular Probes), mouse anti-O4 (1:100, Millipore; 1:500, R&D systems), and rat myelin basic protein (MBP, 1:500; abcam), were reacted for 3 h at room temperature or overnight at 4°C. Samples then were incubated with Alexa Fluor secondary antibodies conjugated with Alexa488, Alexa568, or Alexa647 (Life Technologies) for 1 h at room temperature. Nuclei were stained with Hoechst dye 33258 (Sigma). Permeabilization was omitted for surface antigens. Cell staining was examined under a fluorescence microscope (Axio Observer. D1; Carl Zeiss) with AxioVision 4.8 software (Carl Zeiss).

Statistics

Data are represented as means ± SEMs. One-way analysis of variances, followed by Tukey's post hoc tests, was used for multiple comparisons. $P < 0.05$ was considered as a significant difference.

Results

Differentiation Protocol 1

Most current methods employed to generate NSCs pass through the heterogeneous populations that are present in embryoid bodies [11,20,21]. However, clonal NSC spheres can be derived directly at low cell density [28] from human pluripotent stem cells (Figs. 1B, protocols 1 and 2A) [27]. This protocol includes simultaneously the isolation, proliferation, and initial differentiation phases within the sphere preparations, that is, direct derivation of neurospheres from

human pluripotent stem cell colonies. The following results showed that self-renewing neurospheres generated in the combination of Shh, RA, PDGF, and FGF2 conditions demonstrated more oligodendrocyte lineage-specific characteristics than those shown by primary FGF2 neurospheres. Furthermore, these clonal spheres differentiated into mature oligodendrocyte progeny in vitro.

PDGF alone does not induce the formation of neurospheres from human ESCs/iPSCs

PDGF-responsive oligodendrocyte precursors that form clonal spheres in PDGF alone can be isolated from mouse and human brain tissues [30]. These PDGF-responsive oligodendrocyte precursors differentiate into large numbers of postmitotic oligodendrocytes. We applied these same growth factor conditions to human pluripotent cells in our previously reported clonal neurosphere-forming assay [27].

We were able to generate neurospheres in some growth conditions, such as PDGF/FGF2, FGF2, Shh/RA/PDGF/ FGF2, Shh/RA/PDGF, and Shh/RA/FGF2 (Fig. 2B, C). However, neurospheres in PDGF alone were never or rarely generated from human pluripotent cell lines (FR iPS: 0.00 ± 0.00; H9: 0.13 ± 0.07 spheres per 5,000 cells; ZAN11: 0.05 ± 0.03; RTT-Δ3–4 #37: 0.09 ± 0.03 spheres per 5,000 cells) (Fig. 2D, E, and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd).

The number of spheres was very low, similar to those grown without growth factors [27,30,31] in our negative control cultures (Fig. 2F,G). In brain tissue, some OPCs are present [32], and these PDGF-responsive oligodendrocyte precursor spheres arise in PDGF alone, perhaps due to the isolation of selective OPC precursors to proliferate as clonal spheres. On the other hand, there may be no oligodendrocyte precursors present, yet, among early differentiating ESC/IPSC colonies. Indeed, PDGF receptor (PDGFRA) gene expression was not detected in pluripotent cell colonies (Supplementary Fig. S2).

These findings also suggest that FGF2 is necessary for generating spheres in these primary human pluripotent cell assays. One human iPS cell line (ZAN11) generated fewer spheres in FGF2 or in PDGF/FGF2 than that in the two other iPSC lines (FGF2 condition: 0.91 ± 0.13; PDGF/FGF2 condition: 0.70 ± 0.16 spheres per 5,000 cells) (Supplementary Fig. S1). Therefore, we stopped using this cell line further because of its inefficiency in our clonal sphere preparations.

The combination of Shh and RA increases numbers of neurospheres arising in FGF2 and/or PDGF

At embryonic stages of neural tube formation, positional information appears to determine differentiation potential. Oligodendrocyte precursors are derived from ventral neural progenitors, which also give rise to motor neurons [20,33,34]. Shh and RA are crucial to the fates of these progenitor cells. These factors appear to instruct the dorsal–ventral and anterior–posterior axes of the developing neural tube in a concentration-dependent manner. High Shh and low RA induce ventral positional fates, which are the origin of OPCs [35]. On the other hand, a higher concentration of RA induces a shift of neural progenitors to dorsal fates. Low Shh and high RA induce the formation of dorsal fates and

disturb ventralization in the neural tube. Following the work of others, we attempted to use these factors to induce sphere-forming oligodendrocyte lineage neural progenitors from human ESC/iPSCs [11,20,21] and to evaluate the numbers of spheres and their sizes (Fig. 2D–I).

The optimal concentrations of Shh and RA were chosen based on previous reports, which included dose-dependent results [20,35,36]. Only the Shh/RA condition significantly increased sphere formation in all SFM growth factor conditions in both the FR iPSCs (PDGF+FGF2 condition: 2.68 ± 0.34 vs. 4.00 ± 0.46, $P < 0.05$; PDGF conditions: 0.00 ± 0.00 vs. 1.15 ± 0.08, $P < 0.05$; FGF2 condition: 2.17 ± 0.19 vs. 3.69 ± 0.40, $P < 0.05$) and H9 ESCs (PDGF/FGF2 condition: 2.84 ± 0.21 vs. 5.28 ± 0.94, $P < 0.05$; PDGF conditions: 0.13 ± 0.07 vs. 0.64 ± 0.25, $P < 0.05$; FGF2 condition: 2.57 ± 0.22 vs. 5.15 ± 0.83, $P < 0.05$) (Fig. 2H, I).

In addition, the neurospheres generated in the Shh/RA conditions were larger than in conditions omitting these two factors (Fig. 2B, C, and Supplementary Fig. S3), suggesting that Shh/RA also increased the proliferation of the neural progenitor cells that constitute the majority of the cells in each sphere generated from an NSC.

Addition of Shh and RA to FGF2 generated spheres expressing oligodendrocyte lineage-specific genes

Gene expression of cell-type-specific markers was compared among spheres generated in various growth factor conditions (Fig. 2J). The pluripotent marker, Oct4, was decreased in spheres generated in all conditions compared with the starting pluripotent cells, except in the Shh/RA/PDGF condition. These results suggested that the sphere arising in the Shh/RA/PDGF condition still may be pluripotent and thus we no longer pursued this specific growth factor combination after the qPCR experiments, although other gene expression in the spheres generated in this condition hinted that this condition also was sufficient for oligodendrogenesis.

On the other hand, Lin28, which is highly expressed in human ESCs and iPSCs, decreased more in spheres grown in Shh/RA (Shh/RA/PDGF/FGF2, Shh/RA/PDGF, and Shh/RA/FGF2) than spheres grown in other growth factor conditions. This finding indicates that the spheres in the Shh/RA (Shh/RA/PDGF/FGF2, Shh/RA/PDGF, and Shh/RA/FGF2) conditions are not able to maintain pluripotency. Constitutive *LIN28* expression caused a complete block of gliogenesis and an increase in neurogenesis [37]. The remaining *LIN28* expression may indicate the presence of some early neural progenitors, with neurons differentiating first perhaps, followed by the differentiation of glial cells, recapitulating in vivo development [37]. Thus, a decrease of *LIN28* may also imply a more gliogenic fate.

The expression of the neural markers, Nestin and Pax6, was increased in spheres derived in all conditions compared with the starting human pluripotent cells, except in the RA/P/F and Shh/RA/P conditions with respect to Pax6 gene expression. However, adding Shh and RA did not increase the expression of these two neural markers, which suggests that Shh and RA are not diminishing the neural fates of the spheres. Expression of endodermal and mesodermal markers (*HNF4a*, *Gata4*, T) decreased in the spheres in all conditions compared with the starting pluripotent cells.

NG2, PDGFR, Olig2, and *NKx2.2* are well-characterized, early oligodendrocyte lineage-specific gene markers in the central nervous system [34]. *NG2* expression in spheres was increased by the combination of Shh, RA, PDGF, and FGF2 (Shh/PDGF/FGF2 compared with PDGF/FGF2, $P < 0.05$; Shh/RA/PDGF/FGF2 compared with SFM/PDGF/FGF2, $P < 0.05$; Tukey's post hoc tests). However, Shh or Shh/RA without PDGF did not increase *NG2* expression. The expression of *NG2* was increased only when both Shh and PDGF were added, except in the Shh/RA/P condition.

Similarly, *Nkx2.2* expression in spheres was detected only when Shh/PDGF was added (ie, the Shh/PDGF/FGF2, Shh/RA/PDGF/FGF2, and Shh/RA/PDGF conditions only). *NKx2.2* expression in spheres generated in the Shh/RA/FGF2 conditions was low compared with that in spheres generated in the Shh/RA/PDGF and Shh/RA/PDGF/FGF2 conditions, suggesting that *NKX2.2* expression may have been repressed by FGF2, but then rescued by PDGF addition. PDGFRA was expressed to a greater extent in spheres grown in Shh/PDGF/FGF2, RA/PDGF/FGF2, Shh/RA/PDGF/FGF2, and Shh/RA+FGF2 than in spheres grown without FGF2 (Shh/RA+PDGF alone).

The spheres grown in all of the above conditions that included FGF2 showed self-renewal given that they were passageable in the same growth factor conditions in which they were originally grown (the dissociation of the primary spheres gave rise to clonal secondary spheres) (Supplementary Fig. S4). These results indicate that the Shh/RA/PDGF/FGF2 condition induced the most oligodendrocyte lineage-specific gene expression characteristics in the spheres derived from pluripotent cells.

The glial and neural differentiation potentials of spheres derived directly from human pluripotent cells in different growth factor conditions

To confirm the transcriptional differentiation potentials of spheres grown in each growth factor condition, we immunostained the cells from individual spheres plated and differentiated in 1% FBS (Fig. 3A, B). Spheres grown in Shh/RA/FGF2 and Shh/RA/PDGF/FGF2 differentiated into more GFAP-positive astrocytes in 1% FBS than did spheres grown in SFM conditions without Shh/RA (FGF2 vs. Shh/RA/FGF2, $P < 0.05$; PDGF/FGF2 vs. Shh/RA/PDGF/FGF2, $P < 0.05$; Tukey's post hoc tests), as well as more β3 tubulin neurons (FGF2 vs. Shh/RA/FGF2, $P < 0.05$; PDGF/FGF2 vs. Shh/RA/PDGF/FGF2, $P = 0.06$; Tukey's post hoc tests) (Fig. 3C). In contrast, the percentages of Nestin-positive precursor cells were decreased in spheres grown in Shh/RA conditions compared with spheres grown in the SFM conditions without Shh/RA (FGF2 vs. Shh/RA/FGF2, $P < 0.05$; PDGF/FGF2 vs. Shh/RA/PDGF/FGF2, $P < 0.05$; Tukey's post hoc tests) (Fig. 3C and Supplementary Fig. S5). These results indicate that the presence of Shh/RA generated spheres that differentiated into more astrocytes and neurons.

If the primary spheres were passaged before differentiation, the percentages of β3 tubulin-positive neurons were decreased in the secondary spheres ($P < 0.05$ in all conditions), whereas the percentages of GFAP-positive astrocytes tended to increase compared with the differentiation of the primary spheres (PDGF/FGF2, not significant; FGF2, $P < 0.1$; Shh/RA/PDGF/FGF2, $P < 0.1$; Shh/RA/PDGF/FGF2,
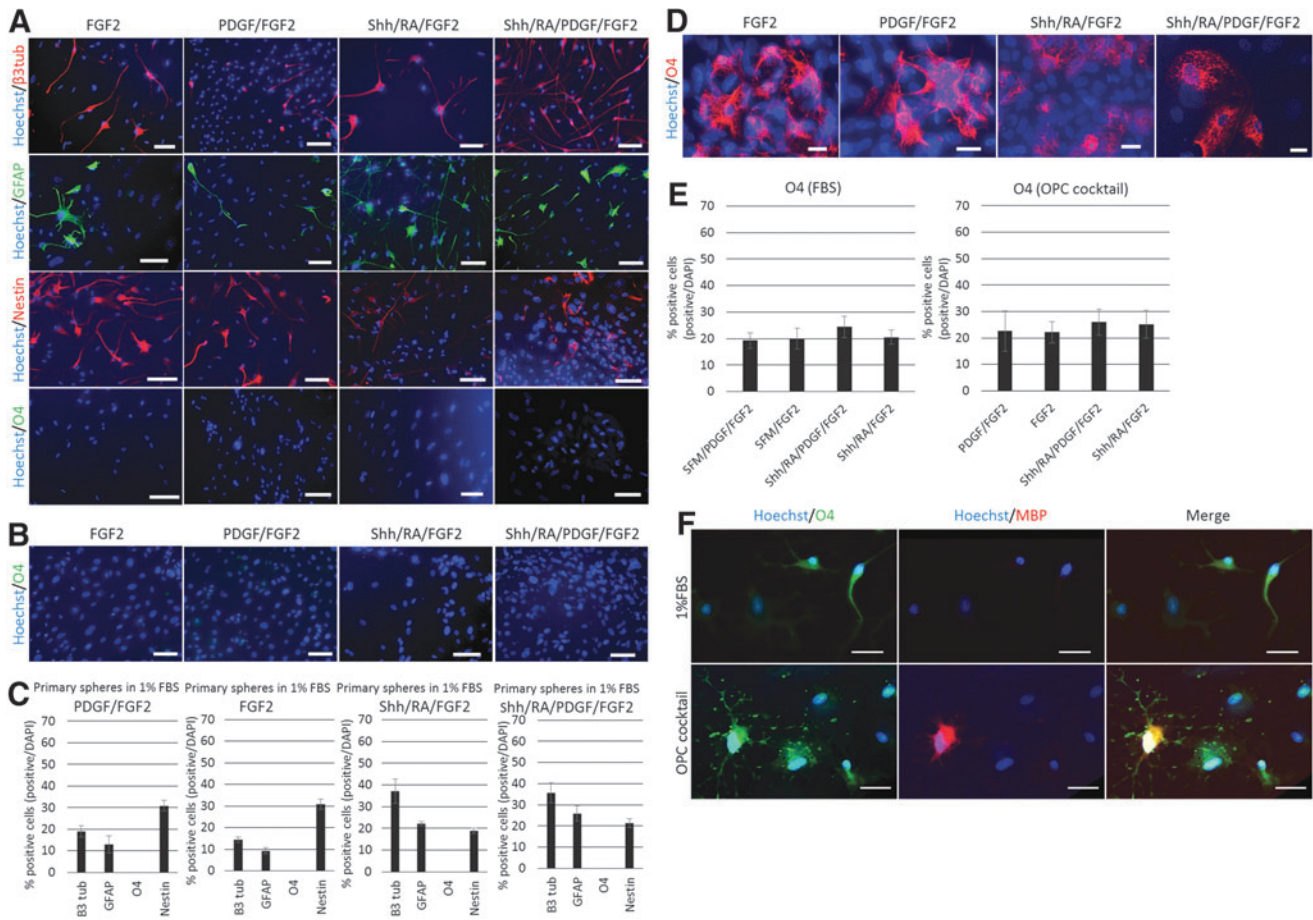
FIG. 3. The immunocytochemical differentiation potentials of FR iPSC-derived spheres. Neurospheres were allowed to differentiate for 8 weeks. **(A, B)** The proportions of cells positive for each cell-type-specific marker after differentiation in 1% FBS. O4 differentiation was not observed in FR iPSC-derived primary spheres in 1% FBS **(A)**, even with the addition of the OPC cocktail **(B)**. Scale bar = 100 μm. **(C)** The proportions of cells positive for each cell-type-specific marker by immunocytochemistry (n = 3 separate experiments). **(D)** The immunocytochemical differentiation potentials of oligodendrocytes from secondary FR iPSC-derived spheres. O4 staining in the noted growth factor conditions by immunocytochemistry. Neurospheres were differentiated in 1% FBS with the OPC cocktail. Scale bar = 20 μm. **(E)** The proportions of O4-positive cells in each condition. Neurospheres were differentiated in 1% FBS (*left panel*) or in 1% FBS with OPC cocktail (*right panel*) (n = 3 separate experiments). **(F)** O4 and MBP double labeling of differentiating oligodendrocytes grown in 1% FBS and differentiated in 1% FBS with the OPC cocktail. Scale bar = 50 μm. Data are presented as the means ± SEMs. Beta3tub, beta3 tubulin; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; PDGF, PDGF-AA.

P < 0.05) (Supplementary Fig. S6). This finding indicates that secondary spheres have more glial fate bias (perhaps including astrocytes and oligodendrocytes) than do primary spheres.

During embryonic development, NSCs produce glial-restricted progenitors, which can differentiate to astrocytes and oligodendrocytes (NG2 and O-2A-like cells [38,39]), followed by differentiation to OPCs [38]. However, iPSC-derived neurospheres that arose from embryoid bodies gave rise to neither astrocytes nor oligodendrocytes [17], although brain-derived neurospheres differentiate to neurons, astrocytes, and oligodendrocytes. Interestingly, the differentiation of GFAP-positive cells as well as neurons was observed from our clonal primary neurospheres grown in FGF alone or in combination with PDGF (Figs. 3A, C and 6A, B), suggesting that these clonal spheres arose from neural stem cells and not glial-restricted progenitors [17,36,39].

However, with respect to the oligodendrocyte lineage, no O4-positive oligodendrocytes were generated in 1% FBS from primary spheres grown in any of the growth factor conditions (Fig. 3A). This observation agrees with reports from Okano's group that no primary neurosphere cells differentiate to oligodendrocytes from pluripotent stem cells [17,39], even in spheres with more oligodendrocytic characteristics by gene expression during long-term culture. In contrast, secondary spheres grown in Shh/RA conditions (Shh/RA/PDGFA/FGF2 and Shh/RA/FGF2) as well as spheres in SFM conditions (PDGF/FGF2 and FGF2) generated O4-positive cells in 1% FBS (Fig. 3D and Supplementary Fig. S7A).

We failed to observe O4-positive cells differentiating from our primary spheres. FBS biases the differentiation of precursor cells to astrocytes [40], although its concentration in our media was very low (only 1%). The presence of FBS
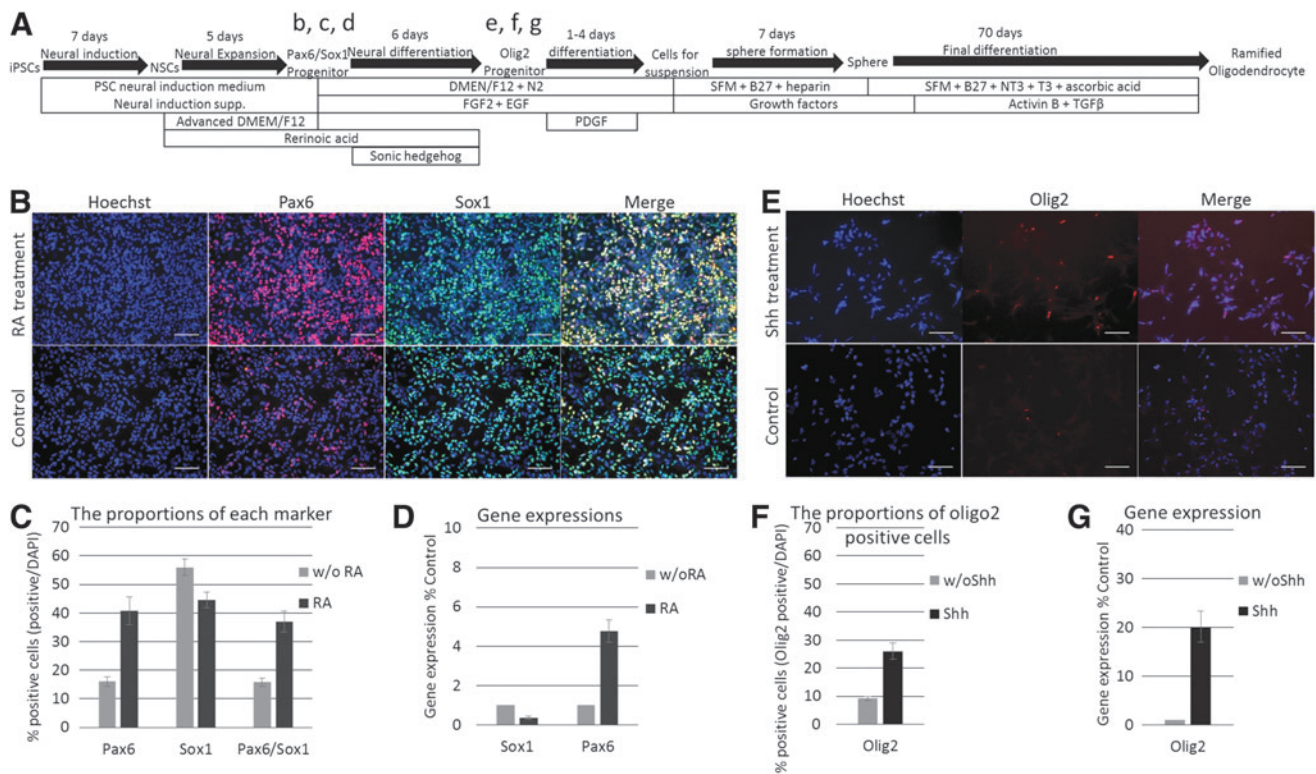
FIG. 4. **(A)** A schematic diagram of protocol 2 combining monolayer neuralizing culture with later neurosphere formation from human iPSCs. *Small letters* b–g above the time line denote when the samples from **(B–G)** were taken. **(B)** The immunocytochemical neural differentiation of NCRM1 iPSC-derived neural stem cells after RA treatment. Scale bar = 100 μm. **(C)** The proportions of cells positive for Pax6 and Sox1 after RA treatment ($n = 3$ separate experiment). RA treatment increased PAX6-positive cells ($P < 0.05$) and Pax6/Sox1 double-positive cells ($P < 0.05$). **(D)** Gene expression of *Pax6* and *Sox1* also was increased in cells grown with RA treatment ($P < 0.05$) ($n = 3$ separate experiments). **(E)** The proportions of cells positive for Olig2 after Shh treatment with or without RA treatment. Scale bar = 100 μm. **(F)** Shh treatment increased Olig2-positive cells ($P < 0.05$) ($n = 3$ separate experiments). **(G)** Gene expression of *Olig2* also was increased in cells grown with Shh treatment ($P < 0.05$) ($n = 3$ separate experiments). These data are presented as the means ± SEMs.

in our differentiation media may bias the differentiation away from oligodendrocytes, even though the oligodendrocyte lineage neural progenitor genes are expressed in the undifferentiated spheres. Additionally, the oligodendrocyte lineage neural progenitors in primary spheres may need more time to mature into the appropriate precursors; hence, we may only get O4-positive cells through sphere passaging.

With respect to the secondary spheres, we had expected that spheres derived in Shh/RA conditions would differentiate into more oligodendrocytes given that these spheres have more oligodendrocyte precursor characteristics by gene expression. However unexpectedly, with respect to the percentages of O4-positive cells, there was no significant difference among secondary spheres grown in any of the growth factor conditions, although spheres derived in each condition showed different transcriptional oligodendrocyte precursor characteristics (Fig. 3E). Our spheres, which have transcriptional oligodendrocyte precursor characteristics, may lose that potential when differentiated in 1% FBS.

These results indicate that the oligodendrocyte precursor fate may be more stable and less likely to be lost in secondary spheres than in primary spheres. However, even in secondary spheres, the advantage of transcriptional oligodendrocyte precursor characteristics may be partially lost during differentiation in 1% FBS.

An OPC cocktail (PDGF, NT3, IGF-1, cyclic AMP, biotin, and T3) helps oligodendrocyte maturation

To encourage oligodendrocyte survival and differentiation, many previous studies have used additional factors, including PDGF, NT3, IGF-1, cyclic AMP, biotin, T3, CNTF, VEGF, and HGF, either singly or in combination [11,20,21,39,41]. The most frequently used of these factors (PDGF, NT3, IGF-1, cyclic AMP, biotin, and T3) were employed during growth and differentiation of spheres grown in each growth factor condition. However, no O4-positive oligodendrocytes were observed during the differentiation of primary spheres in any growth factor condition, even with the addition of the factors listed above (Fig. 3B and Supplementary Fig. S5C).

In contrast, cells in clonal secondary spheres differentiated to O4-positive and more morphologically mature oligodendrocytes, even in 1% FBS, and to the same extent with or without the addition of the oligodendrocyte differentiation factors (OPC cocktail). Nevertheless, these O4-positive oligodendrocytes that differentiated in 1% FBS alone without the additional oligodendrocyte differentiation factors did not mature to MBP-positive cells even after 8 weeks of differentiation (Fig. 3F). Application of the additional oligodendrocyte differentiation factors did allow maturation of the O4-positive cells into MBP-positive and ramified

oligodendrocytes (Fig. 3F), in agreement with reports that observed NT3 for B12 cells and T3 and CNTF for embryonic mouse brain-derived neurospheres [36].

The maturation of the oligodendrocytes may be due to the survival and differentiation effects of PDGF, NT3, IGF-1, cyclic AMP, biotin, and T3 [42], which help to increase the number and induce the mature morphology of MBP-expressing cells that differentiated in 1% FBS alone [36].

Unfortunately, the advantage of employing neurospheres with transcriptional oligodendrocyte precursor characteristics was lost partially during our long-term in vitro differentiation, even when adding an OPC cocktail. We suggest that oligodendrocyte lineage progenitors in spheres may change their fates.

First, even when adding an OPC cocktail, which helps oligodendrocyte lineage survival [42], the advantage of transcriptional oligodendrocyte precursor characteristics did not result in large numbers of mature oligodendrocytes differentiating. Second, OPCs are not oligodendrocyte committed, but rather progenitors that retain the ability to differentiate to astrocytes and oligodendrocytes. Indeed, NG2 cells and O2A cells have this differentiation ability (these cells are often called OPCs). Additionally, we added FBS in our differentiation media, which biases the differentiation of precursor cells to astrocytes [40], as mentioned above.

Differentiation Protocol 2

To more closely replicate the sphere preparations from brain tissue-derived primary neurosphere cultures, ESC/iPSC colonies were differentiated to the neural lineage first, and then the clonal sphere preparations were applied for the isolation and proliferation of specific neural stem cells (Figs. 1B, protocols 2 and 4A). Employing a published neuralizing protocol in monolayer culture [43] (Supplementary Fig. S8A, B), we designed a modified protocol to induce more oligodendrocyte characteristics in neural precursors in the monolayer cultures. However, monolayer cultures invariably include a heterogeneous non-neural cell population, in particular the remaining undifferentiated iPSCs that may result in oncogenesis. To remove the non-neural cells present in these neuralized monolayer cultures, we then performed clonal sphere preparations (Figs. 1B, protocol 2 and 4A).

Addition of Shh and RA generated more oligodendrocyte lineage progenitor cells in the initial monolayer cultures

As mentioned above, Shh and RA are crucial to the fates of neural precursor cells. In development, OPCs are derived from Olig2 progenitors, which are derived from Pax6$^+$/Sox1$^+$ progenitors in response to RA, a caudalizing factor, and Shh, a ventralizing morphogen [44,45]. RA treatment increased the numbers of Pax6-positive cells and Pax6/Sox1 double-positive cells ($P < 0.05$) in the neuralizing monolayer cultures before neurosphere formation (Fig. 4B, C). RA treatment also increased *Pax6* gene expression ($P < 0.05$) (Fig. 4D). The numbers of Sox1 immunopositive cells and *Sox1* gene expression were decreased ($P < 0.05$) in these same monolayer cultures (Fig. 4C, D). Some of the Sox1-positive cells presumably differentiated to Pax6 only positive cells because our monolayer cultures already were Sox1-rich before RA treatment [46,47].

Application of Shh treatment after RA treatment increased Olig2 immunopositive cells and *Olig2* gene expression ($P < 0.05$) (Fig. 4E–G). As a result, these sequential Shh/RA treatments generated more oligodendrocyte lineage progenitor cells.

Large numbers of clonal spheres expressing oligodendrocyte lineage-specific genes were induced by protocol 2

We applied the same growth factor conditions used in protocol 1 to the Shh/RA-treated clonal neural stem cell colonies derived from neuralized monolayer cultures. Spheres in each condition can be derived from the Shh/RA-treated neural precursors at low cell density (Fig. 5A). In contrast to protocol 1, PDGF alone induced the formation of neurospheres, although these were small (Fig. 5A, B). However, large numbers of spheres were induced, even in the PDGF alone condition (Fig. 5C and Supplementary Fig. S9A).

To determine the optimal timing to undertake the clonal sphere preparations, we investigated the numbers of spheres formed each day after the end of Shh/RA treatment. With regard to sphere formation ability, cultures 1 day after the end of Shh/RA treatment produced the largest numbers of clonal spheres. Sphere-forming ability decreased daily (Day 1 vs. 4, $P < 0.05$; Tukey's post hoc tests) (Supplementary Fig. S9B).

The primary spheres passaged effectively, although the small PDGF alone spheres required the addition of FGF2 with the PDGF to generate secondary spheres (Supplementary Fig. S9C). However, the oligodendrocyte precursor characteristics (*NG2, PDGFR,* and *Olig2* gene expression) of these primary spheres disappeared after passaging (Fig. 5D). Furthermore, PDGF alone primary spheres were not large enough to spread well on the plates in our final differentiation condition. Therefore, we tested the condition of PDGF alone spheres cultured in PDGF/FGF2 for one more week.

Gene expression of cell-type-specific markers was compared among spheres generated in the various growth factor conditions (Fig. 5E). The pluripotent markers, *Oct4* and *Lin28,* were decreased in spheres generated in all conditions compared with the starting pluripotent cells. Expression of neural markers (*Nestin, Sox1, Pax6,* and *β3Tub*) was increased in spheres derived in all conditions, especially in the PDGF alone spheres. Significant increases compared with the starting human pluripotent cells ($P < 0.05$; Tukey's post hoc tests) were seen in *Nestin,* within PDGF alone spheres, PDGF spheres cultured in PDGF/FGF2, and in FGF2 alone spheres; in *Sox1,* within PDGF spheres cultured in PDGF/FGF2; and in *β3Tub,* within PDGF spheres cultured in PDGF/FGF2.

Expression of endodermal and mesodermal markers (*Foxa2, Gata4, T*) decreased in the spheres in all conditions compared with the starting pluripotent cells. Gene expression of oligodendrocyte characteristic markers (*PDGFR, Olig2, NG2, PLP, CNPase, GalC*) in spheres grown under the noted growth factor conditions was increased under all conditions compared with the starting pluripotent cells. PDGF/FGF2 spheres also showed significant increases in *PLP* gene expression ($P < 0.05$; Tukey's post hoc tests).
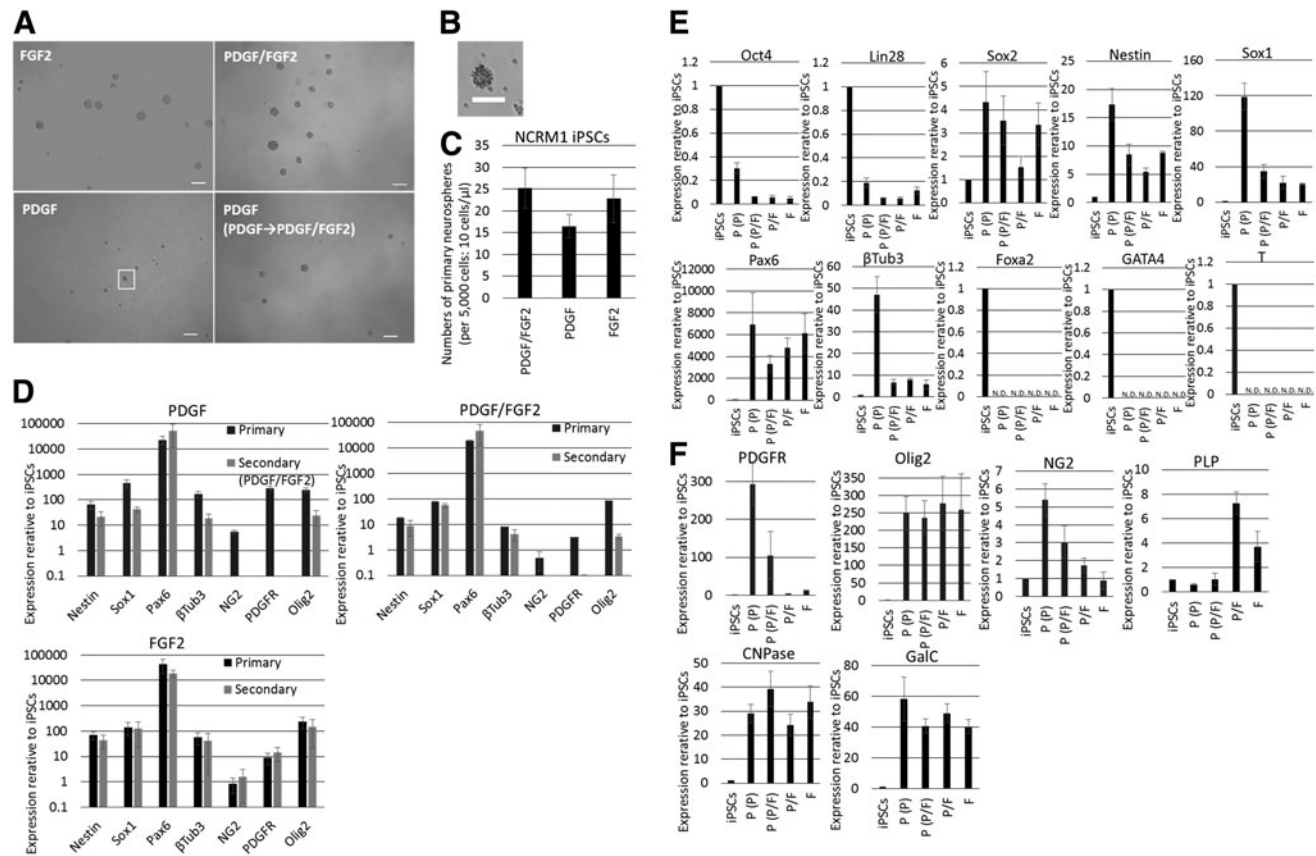
FIG. 5. **(A)** Neurosphere formation from NCRM1 iPSC-derived neural stem cells after Shh/RA treatment using protocol 2 in SFM with the addition of the noted growth factors. Scale bar = 200 μm. **(B)** Higher magnification of the area marked by the *white rectangle* in **(A)** (PDGF). Scale bar = 100 μm. **(C)** The numbers of neurospheres from NCRM1 iPSC-derived neural stem cells after Shh/RA treatment, using protocol 2 in SFM with the addition of the noted growth factors ($n = 3$ separate experiments). All conditions induce clonal neurosphere formation effectively. **(D)** Gene expression of neural and oligodendrocyte markers in primary spheres and secondary spheres. PDGF alone spheres were passaged in the PDGF+FGF2 condition. *NG2* was decreased significantly in secondary PDGF spheres ($P < 0.05$). *PDGFR* and *Olig2* were decreased significantly after passage of both PDGF spheres and PDGF+FGF2 spheres, $n = 2$–3 separate experiments. **(E)** Gene expression of cell-type-specific markers in spheres grown under the noted growth factor conditions. Undifferentiated colonies of the appropriate cell line were used as controls. The resulting values were normalized to *GAPDH*. Data are presented as the means ± SEMs ($n = 3$ separate experiments). The pluripotency markers, *Oct4* and *Lin28,* were decreased after sphere generation. The neural markers (*Nestin, Sox1, Pax6,* and *β3Tub*) are increased in spheres compared with the starting human pluripotent cells. Endodermal and mesodermal markers (*Foxa2, Gata4,* and *T*) decreased in spheres compared with the starting pluripotent cells. **(F)** Gene expression of oligodendrocyte characteristic markers in spheres grown under the noted growth factor conditions. Undifferentiated colonies of the same cell line were used as controls. The resulting values were normalized to *HPRT1*. Data are presented as the means ± SEMs. FGF, FGF2; PDGF, PDGF-AA.

PDGF spheres cultured in PDGF/FGF2 as well as FGF2 alone spheres showed significant increases in *CNPase* gene expression ($P < 0.05$; Tukey's post hoc tests). On the other hand, PDGF alone spheres showed significant increases in each *PDGFR, NG2,* and *CNP* ($P < 0.05$; Tukey's post hoc tests).

These gene expression results from spheres suggest that PDGF alone spheres may have the most oligodendrocyte characteristics and thus be the best candidates for oligodendrocyte differentiation. However, PDGF alone spheres were not able to spread on the plates in our final differentiation condition and thus their differentiation was difficult to determine. One possible reason for this failure is that the PDGF spheres were unable to proliferate more because they had already differentiated.

Clonal sphere preparations enriched for oligodendrocyte precursors that displayed ramified O4-positive processes during final differentiation

To confirm the oligodendrocyte differentiation potentials of spheres grown in each growth factor condition, we immunostained the cells from individual primary spheres plated and differentiated in SFM with B27, NT3, and T3. Because of the risk that our spheres, which have transcriptional oligodendrocyte precursor characteristics, may lose that potential when differentiated in 1% FBS as described in protocol 1, we modified our final differentiation condition to SFM with B27, NT3, and T3 [43].
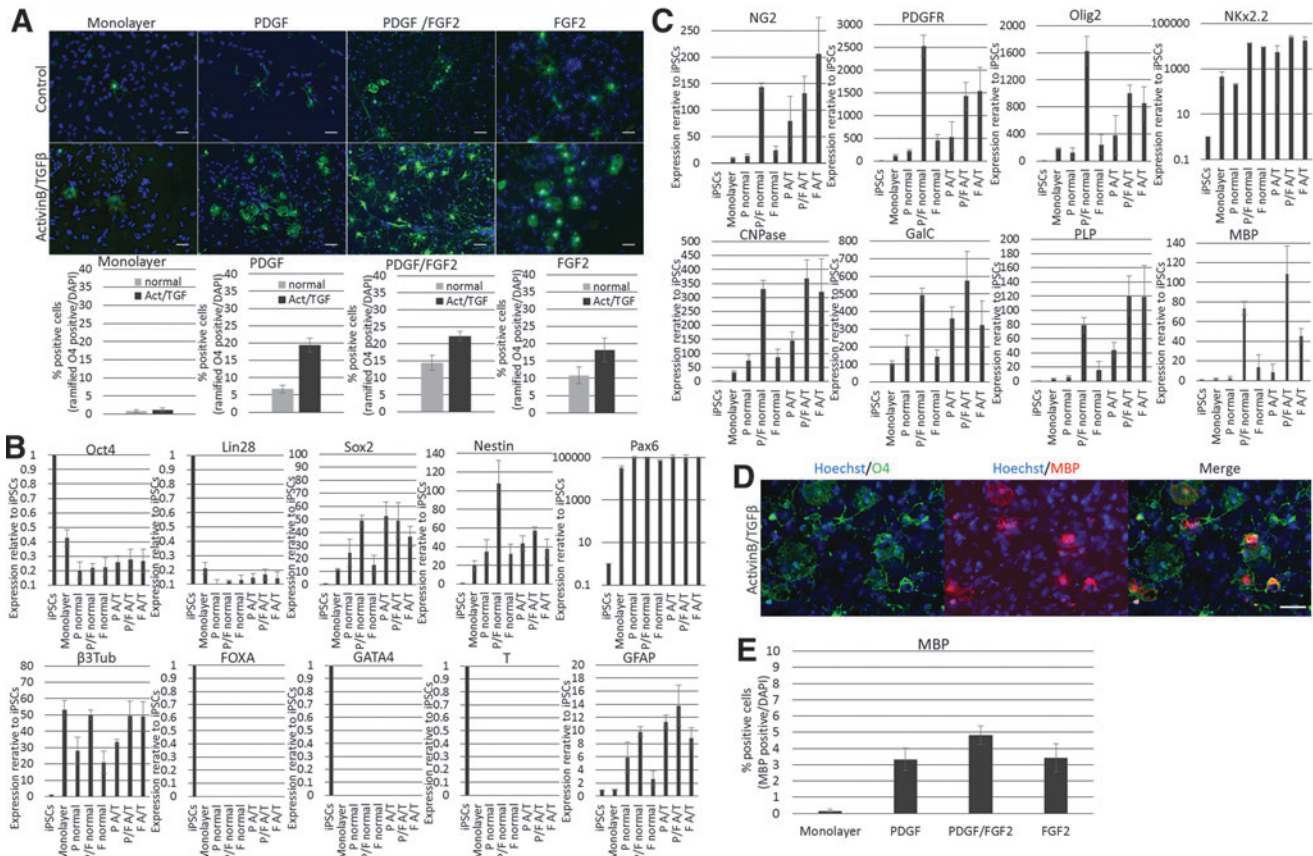
FIG. 6. **(A)** The immunocytochemical differentiation potentials of oligodendrocytes from NCRM1 iPSC-derived spheres. O4 staining in the noted growth factor conditions by immunocytochemistry and the resulting proportions of O4-positive cells in each condition. Scale bar = 50 μm (n = 3–8 separate experiments). **(B)** Gene expression of cell-type-specific markers was compared among spheres generated in various growth factor conditions (n = 3 separate experiments). The pluripotent markers, *Oct4* and *Lin28*, were decreased and endodermal and mesodermal markers (*Foxa2, Gata4, T*) were not detected in all conditions (including the monolayer culture) compared with the starting pluripotent cells. Expression of *Sox2* and other neural markers (*Nestin, Pax6,* and *β3Tub*) was increased in all conditions compared with the starting pluripotent cells. Expression of *GFAP* showed significant increases in PDGF + FGF2 spheres without the ActB and TGFβ treatment, in PDGF + FGF2 spheres with the ActB and TGFβ treatment, and in FGF2 spheres with the ActB and TGFβ treatment compared with the starting pluripotent cells (P < 0.05; Tukey's post hoc tests). Undifferentiated colonies of the parent cell line were used as controls. The resulting values were normalized to *HPRT1*. Data are presented as the means ± SEMs (n = 3 separate experiments). **(C)** Gene expression of oligodendrocyte characteristic markers (*NG2, PDGFR, Olig2, Nkx2.2, PLP, CNPase, GalC, MBP*) in all conditions was increased compared with the starting pluripotent cells (n = 3 separate experiments). Compared with the starting pluripotent cells, PDGF + FGF2 spheres without the ActB and TGFβ treatment showed significant increases in *NG2, PDGFR, Olig2, PLP, CNPase, GalC,* and *MBP* gene expression (P < 0.05; Tukey's post hoc tests). PDGF + FGF2 spheres with the ActB and TGFβ treatment showed significant increases compared with the starting pluripotent cells not only in *NG2, PDGFR, Olig2, PLP, CNPase, GalC,* and *MBP* but also in *Nkx2.2* (P < 0.05; Tukey's post hoc tests). FGF2 spheres with the ActB and TGFβ treatment showed significant increases compared with the starting pluripotent cells in *NG2, PDGFR, Olig2, Nkx2.2, PLP, CNPase, GalC,* and *MBP* (P < 0.05; Tukey's post hoc tests). Undifferentiated colonies of the appropriate cell line were used as controls, except for *MBP* gene expression where monolayer cultures were used as the controls. The resulting values were normalized to *HPRT1*. Data are presented as the means ± SEMs. PDGF, PDGF-AA; F, FGF2; P, PDGF-AA; A/T, activin B and TGFβ treatment. **(D)** Representative figure of O4 and MBP double labeling of differentiating oligodendrocytes grown in protocol 2 with ActB and TGFβ treatment from NCRM1 iPSC spheres derived in PDGF/FGF2. Scale bar = 50 μm. **(E)** The proportions of cells staining for MBP from spheres grown under various growth factor conditions in protocol 2 (ActB and TGFβ treatment of NCRM1 iPSCs) (n = 3 separate experiments). Monolayer cultures versus each of PDGF, PDGF/FGF2, and FGF2 (P < 0.05; Tukey's post hoc tests). Data are presented as the means ± SEMs. ActB, activin B; TGFβ, transforming growth factor-β.

In these differentiation conditions, well-ramified O4-positive oligodendrocytes differentiated with clear oligodendrocyte morphology (Supplementary Fig. S10). In contrast to the final differentiation of primary spheres with protocol 1 (Fig. 3D, F), we saw clear O4-positive oligodendrocyte differentiation from the primary spheres of protocol 2. We passaged our neural precursor cells several times in the monolayer culture phase in protocol 2 to avoid confluence. This monolayer passaging in protocol 2 may have served a similar maturation process to the sphere passaging of protocol 1, allowing the maturing of oligodendrocyte precursors.

After monolayer culture, well-ramified O4-positive oligodendrocytes differentiated from primary spheres grown in

all of the growth factor conditions in the protocols (Fig. 6A). Compared with the control monolayer cultures, subject to the same differentiation conditions as protocol 2, all spheres grown in each growth factor condition in protocol 2 showed more O4-positive cells (Monolayer vs. PDGF/FGF2 sphere, $P < 0.05$; Monolayer vs. FGF2 sphere, $P < 0.05$; Tukey's post hoc tests) (Fig. 6A).

To clarify the final differentiation results and potentially to encourage the differences in oligodendrocyte differentiation among culture conditions, we added a combination treatment of ActB and TGFβ, which promote oligodendrocyte development [48,49]. TGFβ and ActB promote oligodendrocyte generation in the mammalian spinal cord and activate canonical Smad3 and noncanonical MAP kinase signaling, which enhance viability and promote proliferation and differentiation of oligodendrocytes within in vitro OPC cultures [49,50]. When adding a combination of ActB and TGFβ, O4-positive oligodendrocytes were increased after differentiation of spheres generated in all conditions compared with the cultures without the ActB and TGFβ treatment ($P < 0.05$), although these treatments did not increase oligodendrocyte differentiation in the continued monolayer culture (Fig. 6A).

Given that the numbers of O4-positive oligodendrocytes in the continued monolayer culture were always very small after differentiation, the continued monolayer culture might include only small numbers of oligodendrocyte lineage progenitors that could respond to the ActB and TGFβ treatment.

Similar results to those seen here with this iPS cell line (NCRM1) were observed using another human iPS cell line (CCRM5R). When adding the ActB and TGFβ treatments, increased oligodendrocyte differentiation was seen compared with continued monolayer conditions with PDGF/FGF2 spheres ($P < 0.05$) and with FGF2 spheres, ($P < 0.05$) (Tukey's post hoc tests) (Supplementary Fig. S11).

Adding the sphere preparations after the monolayer neuralization protocol greatly increased the numbers of O4-positive cells at final differentiation, especially employing PDGF/FGF spheres. Together, these data suggest that the sphere preparations may enrich an oligodendrocyte lineage population and thus improve the protocol for oligodendrocyte differentiation.

The clonal sphere protocol increases expression of oligodendrocyte lineage-specific genes during final differentiation

To confirm our immunocytochemical differentiation results, we measured gene expression in differentiated cells derived from spheres grown in each of the growth factor conditions after monolayer culture with and without the ActB and TGFβ treatments during final differentiation (Fig. 6B, C). The pluripotent markers, *Oct4* and *Lin28*, again were decreased [and endodermal and mesodermal markers (*Foxa2, Gata4, T*) were not detected] during final differentiation from all sphere growth conditions (and even in continued monolayer culture without a sphere stage) compared with the starting pluripotent cells. On the other hand, the expression of *Sox2* and other neural markers (*Nestin, Pax6,* and *β3Tub*) was increased in all conditions compared with the starting pluripotent cells. The remaining

Sox2 expression indicates the presence of neural precursors even after long-term differentiation culture.

Expression of astrocytic *GFAP* was increased significantly after the differentiation of PDGF/FGF2 spheres both with and without the ActB and TGFβ treatments and of the FGF2 spheres with the ActB and TGFβ treatments compared with the starting pluripotent cells ($P < 0.05$; Tukey's post hoc tests).

Expression of oligodendrocyte markers (*NG2, PDGFR, Olig2, Nkx2.2, PLP, CNPase, GalC, MBP*) was increased during the final differentiation of all protocol 2 conditions compared with the starting pluripotent cells (Fig. 6C). Compared with the starting pluripotent cells, PDGF/FGF2 spheres without the ActB and TGFβ treatments showed significant increases in *NG2, PDGFR, Olig2, PLP, CNPase, GalC,* and *MBP* gene expression during the final differentiation stage ($P < 0.05$; Tukey's post hoc tests). *PDGFR* and *Olig2* expression in PDGF/FGF2 spheres without the ActB and TGFβ treatments was greater than that in PDGF/FGF2 spheres with the ActB and TGFβ treatments during final differentiation. However, PDGF/FGF2 spheres with the ActB and TGFβ treatments showed significant increases compared with the starting pluripotent cells not only in *NG2, PDGFR, Olig2, PLP, CNPase, GalC,* and *MBP* but also in *Nkx2.2* gene expression, whereas PDGF/FGF2 treatment without the ActB and TGFβ did not show significant increases ($P < 0.05$; Tukey's post hoc tests).

In addition, PDGF/FGF2 spheres with the ActB and TGFβ treatments showed stronger gene expression of other mid and late oligodendrocyte-specific genes (*Nkx2.2 PLP, CNPase, GalC, MBP*). *PDGFR* is specifically expressed in immature OPCs, but its expression is rapidly decreased when OPCs undergo terminal differentiation [51,52]. *Olig2* also is a relatively early oligodendrocyte marker.

These results suggest that the ActB and TGFβ treatments promote the final oligodendrocyte maturation from clonal spheres that arose in PDGF/FGF2. Furthermore, spheres grown in FGF2 alone with the ActB and TGFβ treatments showed significantly increased expression compared with the starting pluripotent cells in many oligodendrocyte genes after final differentiation [*NG2, PDGFR, Olig2, Nkx2.2, PLP, CNPase, GalC, MBP* ($P < 0.05$; Tukey's post hoc tests)], although FGF2 spheres without the ActB and TGFβ treatments did not show increases in these oligodendrocyte genes. These data emphasize the oligodendrocyte maturation seen after ActB and TGFβ treatments.

Similar to the immunocytochemical results above, the gene expression analyses again confirm the strong bias to mature oligodendrocyte differentiation in PDGF/FGF2 clonal spheres with the ActB and TGFβ treatments.

MBP-positive mature oligodendrocytes were produced by protocol 2

To confirm the oligodendrocyte maturation potentials of the spheres grown in our growth factor conditions, we double labeled O4 and MBP-differentiating oligodendrocytes grown in protocol 2 (with ActB and TGFβ treatment) from NCRM1 iPSC-derived spheres plated and differentiated in SFM with B27, NT3, and T3.

Cells from the clonal spheres produced by protocol 2 differentiated into O4-positive morphologically mature

oligodendrocytes and could mature to MBP-positive cells in all growth factor conditions (Fig. 6D, E). Compared with the control monolayer cultures subject to the same differentiation conditions as protocol 2, all spheres grown in each growth factor condition in protocol 2 showed more MBP-positive cells (Monolayer vs. each PDGF, PDGF/FGF2, FGF2, $P < 0.05$; Tukey's post hoc tests) (Fig. 6E).

Thus, adding the sphere preparations after the monolayer neuralization protocol greatly increased the numbers of cells that were MBP positive and O4 positive at final differentiation.

The spheres from protocol 2 have more oligodendrocyte differentiation potential than those from protocol 1

Both protocols generated spheres that have oligodendrocytic characteristics and produced differentiated oligodendrocytes. Both protocols (protocol 1 employing the direct differentiation of clonal spheres and protocol 2 using a monolayer neuralization phase before the clonal spheres)

revealed that adding the combination of Shh and RA before the sphere preparations or during sphere growth (in FGF2 and PDGF-AA) induced more oligodendrocyte lineage gene expression. We saw clear differences among spheres generated in various growth factor conditions under protocol 2, although we were unable to see such differences in differentiation under protocol 1, which included FBS (that encouraged astrocyte differentiation at the expense of oligodendrocyte differentiation).

To more directly compare spheres derived by each protocol, we then used the same final differentiation conditions for oligodendrocyte differentiation. Therefore, we differentiated the spheres from both protocols in SFM with B27, NT3, and T3 (along with ActB and TGFβ treatments), which we had used in protocol 2 (Fig. 4A). The variation between iPS cell lines proved to be an additional problem comparing differentiation protocols, in that the cell line variability reduced reproducibility among different experiments. For this reason, we used the most reliable pluripotent cell line, H9 ESCs, for our direct comparison of protocols.
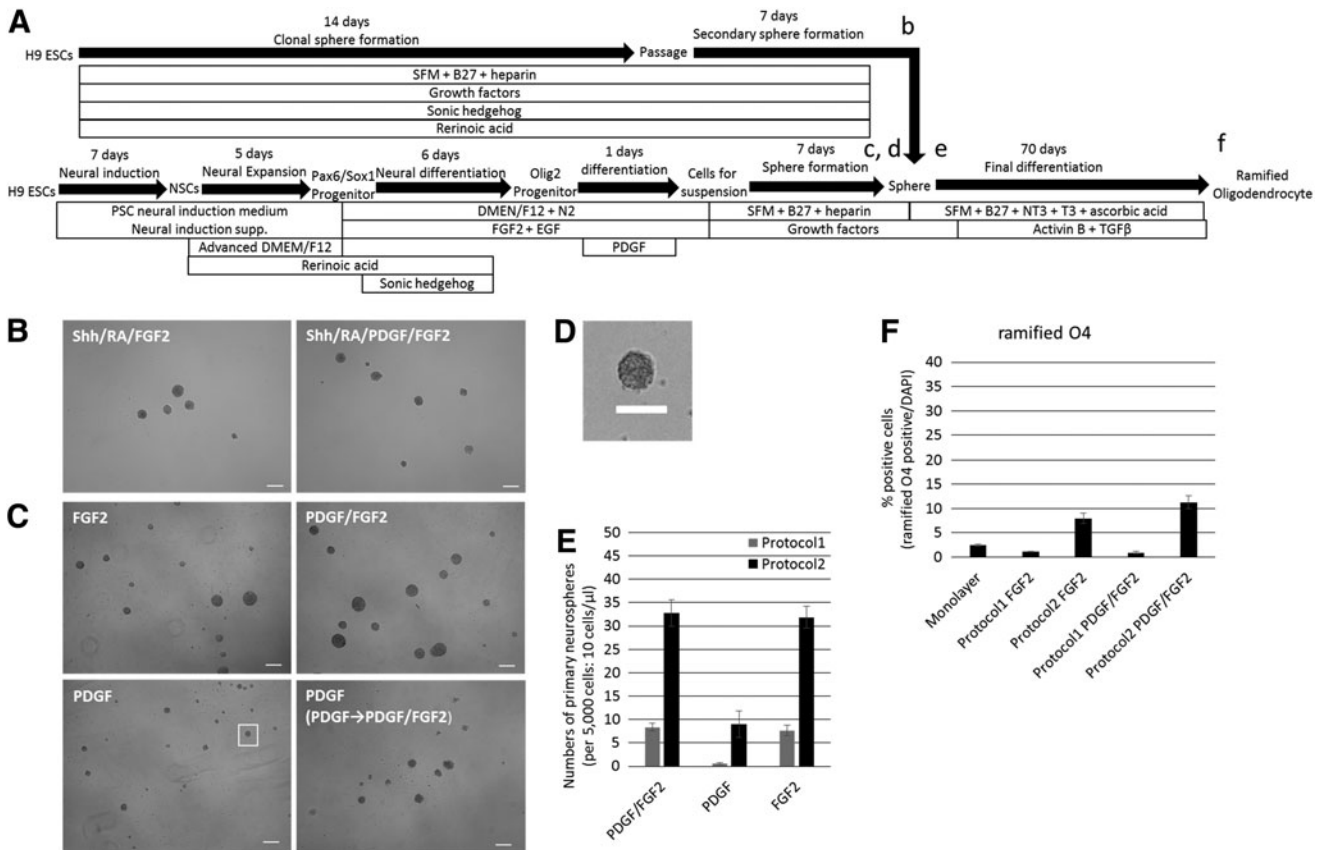

FIG. 7. **(A)** A schematic diagram comparing protocols 1 and 2 in oligodendrocyte derivation. *Small letters* b–f above the time line denoted when the samples from **(B–F)** were taken. **(B, C)** Neurosphere formation from H9 human ESCs. Neurosphere formation using protocol 1 **(B)** and protocol 2 **(C)** in SFM with the addition of the noted growth factors. Scale bar = 200 μm. **(D)** Higher magnification of the area marked by the *white rectangle* in B. Scale bar = 100 μm. **(E)** The numbers of clonal neurospheres formed under each protocol. Protocol 2 significantly increased sphere formation in all growth factor conditions ($P < 0.05$). Data are presented as the means ± SEMs ($n = 3$ separate experiments). **(F)** Both FGF2 spheres and PDGF + FGF2 spheres generated by protocol 2 showed significantly more oligodendrocyte differentiation than spheres generated in the same growth factor condition by protocol 1 (FGF2 spheres with the Shh/RA treatment generated by protocol 1 vs. protocol 2, $P < 0.05$; PDGF + FGF2 spheres with the Shh/RA treatment generated by protocol 1 vs. protocol 2, $P < 0.05$; Tukey's post hoc tests). Data are presented as the means ± SEMs ($n = 3$ separate experiments). FGF, FGF2; PDGF, PDGF-AA.

Under these conditions, optimized to compare the two protocols, we examined FGF2 spheres and PDGF/FGF2 spheres with the Shh/RA treatments of each protocol 1 and protocol 2; thus, we employed the Shh/RA/FGF2 spheres and Shh/RA/PDGF/FGF2 spheres of protocol 1, along with the FGF2 spheres and PDGF/FGF2 spheres, which were derived from the Shh/RA-treated neural precursors of protocol 2. All spheres were derived from H9 ESCs (Fig. 7B–D). The numbers of clonal spheres generated in protocol 2 were much larger than that in protocol 1 under all growth factor conditions (Fig. 7E). Under protocol 1, no primary spheres showed oligodendrocyte differentiation. Therefore, we passaged spheres under protocol 1 before plating in the differentiation conditions.

All spheres under both protocols contained cells that differentiated toward oligodendrocytes after culture in the differentiation conditions mentioned above for 70 days. However, neither FGF2 spheres nor PDGF/FGF2 spheres with the Shh/RA treatment derived by protocol 1 differentiated very well to mature oligodendrocytes. On the other hand, FGF2 spheres and PDGF/FGF2 spheres with the Shh/RA treatment generated from H9 ESCs by protocol 2 showed clear mature oligodendrocyte differentiation, which was reproduced by protocol 2 using the NCRM1 and CCRM5R iPSCs.

Both the FGF2 spheres and the PDGF/FGF2 spheres with the Shh/RA treatment generated by protocol 2 showed significantly more oligodendrocyte differentiation than spheres generated in the same growth factor condition in protocol 1 (FGF2 spheres with the Shh/RA treatment generated by protocol 1 vs. protocol 2, $P < 0.05$; PDGF/FGF2 spheres with the Shh/RA treatment generated by protocol 1 vs. protocol 2, $P < 0.05$; Tukey's post hoc tests) (Fig. 7F). The percentages of O4-positive ramified oligodendrocytes from H9 ESCs were less than those from NCRM1 iPSCs even in protocol 2. This may be due to the cell line variability.

From all of these results, we can conclude that spheres generated by protocol 2 have more oligodendrocyte differentiation potential (at least in this SFM differentiation with B27, NT3, and T3, along with the ActB and TGFβ treatments).

Discussion

In this study, we described the advantages of clonal sphere preparations in purifying human oligodendrocyte precursors from pluripotent cells. Furthermore, we demonstrated the advantage of protocol 2 (monolayer neuralization, followed by sphere formation) over protocol 1 (the immediate formation of spheres) in terms of the increased numbers of spheres and increased oligodendrocyte differentiation.

Protocol 1, which derived clonal neural stem cell colonies directly from human pluripotent cells, revealed that Shh and RA induced oligodendrocyte characteristics in the clonal neurospheres. These spheres were generated relatively quickly (7–14 days) compared with the neural precursors derived from other neural differentiation protocols, which pass through an aggregation stage or an embryoid body stage (20–35 days) [11,20,21,53]. In addition, those protocols that employ an aggregation stage or an embryoid body stage before the formation of neural precursors that do have oligodendrocyte potential [11,20,21,53] were unable to study the

mechanisms by which the neural precursors gain that potential [39].

Furthermore, spheres that arose directly and clonally from human ESC and iPSC colonies at low cell densities prevented heterogeneous cell aggregation and thus should decrease potential oncogenesis by excluding pluripotent cells. The cells in these neurospheres (and in particularly the secondary neurospheres) can not only be differentiated to oligodendrocytes but also neurons and astrocytes, similarly to previous studies using longer-term monolayer cultures [11,20,21,53], indicating that such neurospheres may be a good cell source for cell transplantation therapy.

Protocol 2 overcame the limitations of protocol 1, which were the small numbers of spheres derived from pluripotent cells and the poor oligodendrocyte differentiation of primary spheres. The monolayer differentiation phase of protocol 2 helped to increase the numbers and quality of neural stem cells. As a result, neural precursors after the monolayer differentiation phase responded well to the stimulation of oligodendrocyte differentiation. Therefore, protocol 2 also helped to induce more mature oligodendrocyte precursors and thus the primary spheres of protocol 2 were able to differentiate into well-ramified oligodendrocyte morphologies.

Protocol 2 was based on published initial monolayer culture neuralizing methods [43]. Although monolayer neuralization is simple and allowed the culture of large numbers of neural cells, the cultures remain heterogeneous and contained non-neural cell populations. In particular, the remaining undifferentiated pluripotent cells are a problem because of the potential for oncogenesis after cell transplantation therapy. To eliminate these problems within our monolayer neuralization culture, we applied clonal sphere preparations at low cell density in the next stage of protocol 2.

By employing this combination of monolayer neuralizing culture, followed by clonal sphere preparations, we are able to generate effectively large numbers of pure neural progenitors. Similar to protocol 1, clonal sphere preparations at low cell density in protocol 2 were able to reveal the onset of oligodendrocyte precursor differentiation under the growth of spheres in different combinations of growth factor conditions. Furthermore, the cells in spheres generated by protocol 2 showed more oligodendrocyte differentiation compared with the cells in spheres generated by protocol 1 in vitro, indicating that protocol 2 neurospheres could be a good cell source for remyelination therapy.

OPCs are characterized by PDGFRA and NG2 expression [54]. Our oligodendrocyte characteristic spheres grown in these protocols meet this requirement, but other cells in the spheres also can differentiate to neurons and astrocytes. Thus, these clonal spheres are different from glial-restricted progenitors [38], but resemble PDGF-responsive progenitor spheres grown in PDGF [30,36]. Our results revealed that different numbers of morphologically and biochemically mature oligodendrocytes differentiate depending on the protocols and small molecules added during various differentiation conditions. Differentiation using SFM with B27, NT3, and T3, along with ActB and TGFβ treatments, appears to be the best differentiation condition. In this optimized condition, gene expression revealed that ActB and TGFβ treatments promote the final oligodendrocyte maturation from clonal spheres. In particular, PDGF/FGF2 spheres showed the strongest oligodendrocyte bias in gene expression.

These gene expression findings correspond to the immunocytochemical results. Nevertheless, there remains room to further improve the numbers of biochemically and morphologically mature oligodendrocytes that are differentiating under our protocol 2.

The variation between human ES and iPS cells and among iPS cell lines remains a problem given that it may reduce reproducibility among different experiments. For this reason, we tested one ES and three iPS cell lines in protocol 1 and one ES and two iPS cell lines in protocol 2. However, we found that one iPS cell line derived from activated CD3^{+} T cells enriched from umbilical cord blood showed lower efficiency in generating clonal spheres at low cell densities than the two other iPS cell lines derived from fetal fibroblast cells in protocol 1. Whether variation in differentiation depends on the cell types of the originally reprogrammed cells is controversial. One report has indicated that iPS cell differentiation may depend on the cell type of original cells, given that the iPS cell lines retain some of the epigenetic characteristic of their cells of origin [55]. On the other hand, recently, it was reported that variations in hepatic differentiation were attributable largely to the human donor differences rather than to the types of the original cells [56].

Interestingly, we found that the one iPS cell line derived from activated CD3^{+} T cells enriched from umbilical cord blood did not show lower efficiency in generating clonal spheres at low cell densities in our protocol 2 (as it did in protocol 1) compared with the other iPS cell lines derived from fetal fibroblast cells. The clonal sphere preparation in protocol 2 is performed after the initial neural precursor differentiation, which should reduce the numbers of separate non-neural cell types in the preneurosphere cultures. Thus, this iPS cell line derived from activated CD3^{+} T cells enriched from umbilical cord blood may not be optimal for the direct derivation of clonal neurospheres from iPSC colonies (protocol 1), but it may be useful for protocol 2 involving the monolayer neuralizing culture, followed by clonal neurosphere preparations.

In conclusion, a two-step method combining monolayer neuralization, followed by clonal sphere preparations, is more useful than direct sphere preparations in generating mature human oligodendrocytes. Oligodendrocyte-biased clonal sphere preparations may have some therapeutic advantages in terms of speed, removal of non-neural cells, and producing large numbers of mature oligodendrocytes.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Derek van der Kooy
Department of Molecular Genetics
University of Toronto
Toronto
Ontario M5S 3E1
Canada

E-mail: derek.van.der.kooy@utoronto.ca