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CHARACTERIZATION OF GLIAL-RESTRICTED PRECURSORS FROM RHESUS MONKEY EMBRYONIC STEM CELLS

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Abstract

Glial-restricted precursor (GRP) cells, the earliest glial progenitors for both astrocytes and oligodendrocytes, have been derived from embryos and embryonic stem cells (ESC) in rodents. However, knowledge regarding the equivalent cell type in primates is limited due to restrictions imposed by ethics and resources. Here we report successful derivation and characterization of primate GRP cells from rhesus monkey ESC. The purified monkey GRP cells were A_2B_5-positive and FGF2-dependent for survival and proliferation. The differentiation assays indicated that they were tri-potential *in vitro* and bi-potential *in vivo*. These newly purified GRP cells will help to facilitate understanding of the molecular mechanism of glial development in primates as well as provide a source of therapeutic donor cells for use in neuroregenerative medicine.

Keywords

• Differentiation • Glial-restricted precursor (GRP) • Rhesus monkey • Embryonic stem cell (ESC)

Introduction

Neurons and glia originate from neuroepithelial stem/progenitor cells in the developing central nervous system [1]. The precise ancestor of both astrocytes and oligodendrocytes was unknown until tripotential glial-restricted precursor (GRP) cells were isolated from the developing central nervous system and embryonic stem cells (ESC) in rodents [2-4]. These GRP cells are capable of differentiating into astrocytes and oligodendrocytes both *in vitro* and *in vivo* [5, 6]. They can even promote functional recovery after spinal cord injury [7-10]. The equivalent cells in primates are less well characterized largely due to restrictions imposed by ethics and resources. Still less is understood about human GRP cells of which the only example so far isolated are A_2B_5-positive glial precursors derived from cryopreserved human fetal brain progenitors or gliomas [11, 12].

ESC provide a good model by which to study cell differentiation because of their ability to differentiate into all derivatives of the three embryonic germ layers that constitute the body [13-15]. Multiple types of neural lineage cells have been derived from ESC [16-18]. Unfortunately, the GRP cells had only been successfully derived from mouse ESC [4]. Thus, differentiation of rhesus ESC into GRP cells provides an alternative and superior method to study primate GRP because the rhesus monkey is more closely related both genetically and physiologically to humans [19].

In this study, successful derivation and characterization of GRP cells from rhesus monkey embryonic stem cells (rESC) was demonstrated. The results showed that rhesus A_2B_5-positive GRP cells are capable of differentiating into both oligodendrocytes and astrocytes *in vitro* and *in vivo*.

Experimental procedures

A diagram illustrating the procedures of derivation, purification and differentiation of GRP cells from rESC is shown in Supplemental Figure 1.

ES cell culture, embryoid body (EB) formation and differentiation

The procedures for culture of rESC R366.4 and EB making were described previously [18]. To induce glial precursor (GP) differentiation

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from EB, the 2- or 3-day EB (from rESC) were transferred to another tissue culture dish in suspension culture for an additional 2 or 3 days. Next, the 5-day EB were plated onto 0.1% gelatin-coated culture dishes in rESC differentiation medium. After 24 h of culture, to allow cell attachment and surface spreading, the medium was replaced and replenished every two days with ITSFn medium containing Dulbecco's modified Eagle's medium (DMEM/F12, 1:1, Invitrogen, Waltham, MA, USA) supplemented with 1x ITS supplement (Invitrogen), and fibronectin (5 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 4-9 days. N2 medium consisting of DMEM/F12, laminin (1 µg/ml, Sigma-Aldrich), basic fibroblast growth factor (bFGF, 10 ng/ml, Sigma-Aldrich) supplemented with 1X N2 supplement (Invitrogen) was then added for an additional 6-11 days.

GRP cell culture

After culture in N2 medium for 6-11 days, migrating fibroblast-like GP (passage 0, P0) at the periphery were mechanically dissociated or digested by collagenase IV (1 mg/ml, Invitrogen) in order to obtain GRP cells.

In order to facilitate the expansion of GRP cells, the cells were digested every 5 days by 0.05% trypsin (Sigma-Aldrich) or collagenase IV (1 mg/ml, Invitrogen) and replated onto 0.002% poly-ornithine (PLO) treated dishes with N2 medium. The medium was changed every 2 days and bFGF was added daily. GRP cells were stored in 90% newborn calf serum plus 10% dimethyl sulfoxide (DMSO) in liquid nitrogen and successfully thawed and recovered at a later date.

Proliferation test of GRP cells

To test the proliferation capability of GRP cells, the cells were incubated in either N2 medium containing bromodeoxyuridine (brdU, 10 µM, Sigma-Aldrich) for 48h or in N3 Medium (i.e. N2 medium without bFGF) containing BrdU supplemented with platelet-derived growth factor-AA (PDGF-AA, 10 ng/ml, PeproTech, Rocky Hill, NJ, USA) for 48 h. The cells were then fixed by methanol and immunocytochemistry was performed according to the recommended procedure for the product. In order to further distinguish mitogens for GRP cells, GRP cells freshly digested with 0.05% trypsin were seeded onto 0.002% PLO-coated dishes at a density of 4 x 10^4 cells/well for 4-well dishes (Nunc, Roskilde, Denmark) with a plating efficacy of over 98% in N3 medium supplemented with bFGF (10 ng/ml) or PDGF-AA at 10 ng/ml, 30 ng/ml, and 50 ng/ml, respectively. After culturing for 99 h, single cells were harvested by trypsin digestion and counted with a hemocytometer (Sigma-Aldrich).

GRP differentiation *in vitro* and *in vivo*

In order to demonstrate differentiation of GRP cells *in vitro*, cells at a density of 2.5 x 10^3 cells/cm^2 were plated onto 0.002% PLO-coated dishes and cultured in the appropriate media (Table 1) for one or two weeks. The cells were then fixed by 4% paraformaldehyde (PFA, Sigma-Aldrich) and immunocytochemistry was performed.

To undertake the differentiation assay *in vivo*, 1.5-2.0 µl of 1.0 x 10^8/ml viable GRP cells (P5-7) transfected by the green fluorescent protein (GFP) reporter gene (Invitrogen) or labeled with the red fluorescence marker PHK-26 (Sigma-Aldrich) were injected symmetrically into lateral ventricles (LV) of Sprague Dawley (SD) rats (280-330 g, n = 13) by using the following stereotaxic coordinates: anterior-posterior -0.80 mm, medial-lateral ± 1.5 mm, and dorsal-ventral -3.6 mm. Cell suspensions were delivered at 0.5 µl per min using a 5- or 10-µl injection needle. The needle was left in place for another 3 min before being slowly removed. Grafted rats were immunosuppressed by daily intraperitoneal injection of cyclopamine A (10 mg/kg, Sigma-Aldrich) for a week after cell transplantation. Experimental protocols met the guidelines of the Institute's Internal Research Committee.

Recipient SD rats were euthanized 1, 2, 4, or 6 weeks later and 10 µm coronal sections of the brain were cut on a cryostat to detect the differentiation of transplanted GRP *in vivo* according to standard tissue processing protocol and immunofluorescence, as previously described [5].

Immunocytochemistry

For immunocytochemistry, cells were rinsed three times in phosphate-buffered saline (pH = 7.4) and fixed in 4% PFA for 15-20 min. After treatment with 0.4% Triton-X 100 for 10 min, the cells were blocked with 5-10% goat serum for half an hour at room temperature (20°C). Primary antibodies (Table 2) were used at room temperature for 1 h or at 4°C overnight. Secondary antibodies conjugated with flourescein isothiocyanate, FITC (Santa Cruz, Santa Cruz, CA, USA or Jackson ImmunoResearch, West Grove, PA, USA) or with PE/Texas Red (Santa Cruz / Jackson ImmunoResearch) were incubated with primary antibodies at room temperature for 1 h. Cell nuclei were stained with Hoechst 33258 or propidium iodide (PI, Sigma-Aldrich). The cells were examined using a confocal laser scanning system (LSM 510 META; Carl Zeiss, Jena, Germany).

RNA preparation and gene expression analysis

Total RNA was extracted from cells using a TRIzol RNA (Invitrogen) isolation kit according to the manufacturer's instructions. The potential

Table 1. Cytokines and growth factors used to induce differentiation of GRP cells.

Cytokines/growth factors	Concentration	Promoted cellular phenotypes
BMP4[1]; bFGF[2]	10 ng/ml; 10 ng/ml	Type I astrocytes
CNTF[3]; bFGF[2]	10 ng/ml; 10 ng/ml	Type II astrocytes
LIF[4]; bFGF[2]	1000 U/ml; 10 ng/ml	Type II astrocytes
bFGF[2]; PDGF-AB[3]; TH[2]	10 ng/ml; 10 ng/ml; 10 ng/ml	Oligodendrocytes
RA[2]; NT-3[3]	1 mM; 10 ng/ml	Neurons

Abbreviations: BMP4, bone morphogenetic protein 4; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; PDGF-AB, platelet-derived growth factor-AB; TH, thyroid hormone; RA, retinoic acid; NT-3, neurotrophin-3.
[1] from R&D Systems, Minneapolis, MN, USA; [2] from Sigma-Aldrich, St. Louis, MO, USA; [3] from PeproTech, Rocky Hill, NJ, USA; [4] from Chemicon Temecula, CA, USA.

contaminating genomic DNA was eliminated by DNase I digestion and cDNA was synthesized from 1 μg total RNA using AMV enzyme in a 20 μl reverse transcription (RT) system; 1 μl of RT product was used as a template for each 25 μl polymerase chain reaction (PCR) amplification. PCR included denaturation for 3 min at 94°C followed by repeated cycles of: 94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, and extension at 72°C for 5 min. PCR primers and reaction conditions used are shown in Table 3. PCR products were electrophoresed through a 2.0% agarose gel (Invitrogen) and stained with 0.1 μg/ml of ethidium bromide.

Statistical analysis

The results are presented as means ± standard deviation (SD). Statistical analysis was performed using the one-way analysis of variance (ANOVA) with statistical significance defined as $P < 0.05$.

Results

Direct differentiation of rESC into early glial progenitors (GP)

Using an efficient and reproducible system based on previously described EB differentiation method ([18], Fig. 1B), three distinct neuroectoderm populations including: pseudostratified neural rosettes (NR, Fig. 1C), neuronal precursors, and peripheral bipolar fibroblast-like cells (Fig. 1D-F) were differentiated from the undifferentiated rESC (Oct4$^+$, Fig. 1A). Similar results were obtained in the work by Kuo *et al*. [20]. Characterization of the bipolar fibroblast-like cells showed that they expressed vimentin (Fig. 1E), a marker of ectoderm and radial glia. Further testing also indicated they were PDGFRα$^+$ by immunostaining (Fig. 1F). Interestingly, some fibroblast-like cells started to express the glial progenitor marker A_2B_5 (Fig. 1F), implying these cells are early GP.

Purification and characterization of GRP cells

As PDGFRα and A_2B_5 are typically expressed in GRP cells [3, 6], the early vimentin$^+$/PDGFRα$^+$ GP cells (Fig. 1E, F) were chosen for further expansion. After screening for two passages,

Table 2. Primary antibodies used for immunofluorescence.

Antigen	Species/Type	Dilution	Source
Oct-4	Rabbit IgG	1:100	Santa Cruz
Nestin	Mouse IgG_1	1:100	Chemicon
Vimentin	Mouse IgG_1	1:200	DAKO
PSA-NCAM	Mouse IgM	1:200	Chemicon
Tuj1	Mouse IgG_1	1:150	Chemicon
MAP2	Rabbit IgG	1:600	Chemicon
A_2B_5	Mouse IgM	1:200	Chemicon
PDGFRα	Rabbit IgG	1:200	Santa Cruz
O4	Mouse IgM	1:200	Chemicon
MBP	Rabbit IgG	1:100	DAKO
CD44	Mouse IgG_1	1:40	DAKO
GFAP	Rabbit IgG	1:600	Chemicon
BrdU	Mouse IgG_1	1:20	Chemicon
Ki67	Rabbit IgG	1:100	Santa Cruz

Abbreviations: GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated-protein 2; MBP, myelin basic protein; PDGFRα, platelet-derived growth factor receptor α. Source not described elsewhere in the text: DAKO, Agilent Technologies, Santa Clara, CA, USA.

Table 3. PCR primers and condition for gene analysis.

Genes	Primer sequences	Annealing temp (°C)	Products length (bp)	Accession No. or reference
BLBP	f5'GGCTTTCTGTGCTACCTG 3' r5'CTCCGTGTTCTTGAATGTGC3'	51	178	XM_001108559
FGFR3	f5'GGGGCGAGTGGCAGAAGTGT3' r5'AGCGGAAGCGGACGGTGTTG3'	62	431	XM_001100919
Nestin	f5'CGCTGGAGGAGCAGAATGAG 3' r5'CCAGCGACGTCTCCATGTGT 3'	55	531	NM_006617 XR_020684
OCT-4	f5'GACAACAATGAGAACCTTCA3' r5'CACATCCTTCTCTAGCCCAA3'	54	185	NM_002701 NM_013633
Pax6	f5'CATGCAGAACAGTCACAGCGG3' r5'CCCATCTGTTGCTTTTCGCTA3'	60	414	NM_000280 NM_013627
Pax7	f5'TGCCGCTACCAGGAGACCG3' r5'CCAGGATGCCGTCGAT3'	54	310	AF254422
PDGFRα	f5'CCAAGCCTGACCACGCTAC3' r5'TCATCCAGACCACCCTCCC3'	54	280	XR_010784
PDGFRβ	f5'CTCACCATCATCTCCCTTATC3' r5'GTCTCCGTAGCGGCAGTA3'	55	435	XM_001107595
Sox2	f5'AGCATGATGCAGGAGCAG3' r5'GGAGTGGGAGGAAGAGGT3'	56	270	NM_003106 NM_011443
Sox9	f5'GGTGCTCAAAGGCTACGACTG3' r5'TGCCCGTTCTTCACCGACT3'	59	321	NM_001032868
Sox10	f5'GCTGCTGAACGAAAGTGAC3' r5'TGTAGTGGGCCTGGATGG3'	57	194	XM_525590
Vimentin	f5'GCCAGGCAAAGCAGGAGT3' r5'GTGGGTGTCAACCAGAGG3'	52	383	XM_001093658 X56397
GAPDH	f5'TGAAGGTCGGAGTCAACGGA3' r5'TGGTGCAGGAGGCATTGCTG3'	57	449	NM_002046

nearly all cells had glial progenitor morphology, as described previously [3, 21], being bipolar (or tripolar) (Fig. 2B, C) and forming gliospheres (Fig. 2A). They expressed glial progenitor markers A_2B_5, PDGFRα (Fig. 2B), and vimentin (Fig. 2C) but not astroglial (CD44, GFAP) or oligodendroglial (O4, myelin basic protein, MBP) markers. Neither NSC markers nor neuronal markers (Fig. 2D, E) were expressed in these purified cells.

The proliferation competence assay was performed to determine their mitogen by using BrdU and Ki67. The results revealed that the purified GP were proliferative (Fig. 2H), and numbers doubled over two-fold in the bFGF treatment group. In contrast, the cells in treatment groups with various concentrations (10, 20, 30 ng/ml) of PDGF-AA (Fig. 2G, I) did not divide and over half of them died. These results suggest that purified GP survive and proliferate only under bFGF but not PDGF-AA stimulation in precisely the same manner as do mouse GRP cells [6, 22]. These rhesus GRP cells were capable of self-renewal in adherent culture, without differentiation while maintaining a normal karyotype (42, XY) (Fig. 2F) for at least 10 passages.

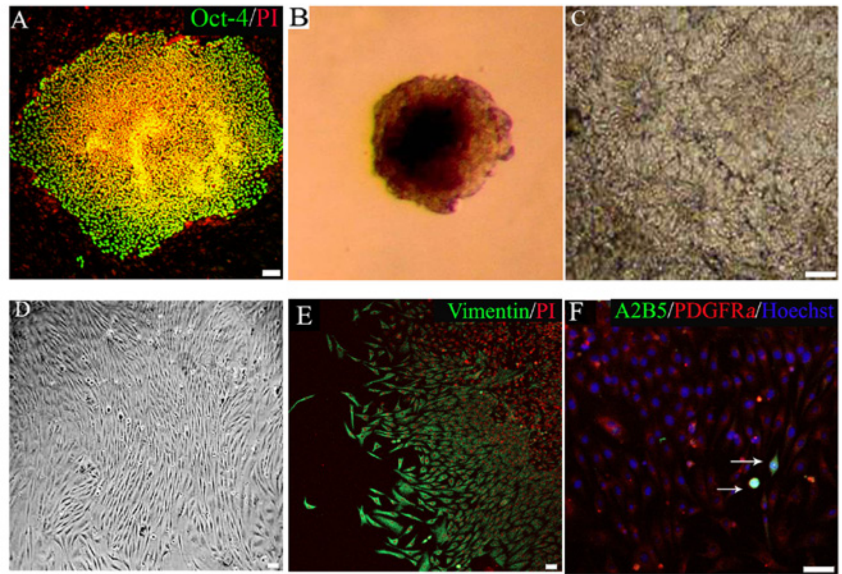

Figure 1. Direct differentiation of rhesus monkey embryonic stem cells (rESC) into early glial progenitors (GP). Undifferentiated rESC (Oct-4$^+$, A) were differentiated into three distinct populations: neural rosettes (C), neuronal precursors and early GP (D-F) via embryoid body (EB). B, EB on day 5; D, phase contrast of migrating glial progenitor-like cells. Migrating bipolar cells at the periphery were stained with vimentin (E), PDGFRα (F) and A_2B_5 (arrow in F). Nuclei were stained by propidium iodide, PI (A, E) or Hoechst stain (F). Magnification: 100x (B). Scale bars: 50 µm (A, C-F).

Differentiation of GRP cells *in vitro* and *in vivo*

When the isolated GRP cells were induced to differentiate under appropriate conditions *in vitro* for one or two weeks (Table 1), three types of glial cells were generated: $A_2B_5^+$/GFAP$^+$ type II astrocytes (Fig. 3A) bearing projections in the presence of CNTF/bFGF or LIF/bFGF; flat $A_2B_5^-$/GFAP$^+$ type I astrocytes (Fig. 3B) with BMP4/bFGF, expressing FGFR3 by RT-PCR (data not shown); and MBP$^+$ oligodendryocytes (Fig. 3C) under stimulation with bFGF/PDGF-AB/TH. None of the cells was labeled by neuronal lineage markers (MAP2 and Tuj1) in the presence of retinoic acid, a potent inducer of neuronal differentiation (Fig. 3D). Therefore, the purified GRP cells from rESC were tripotential *in vitro*.

To further investigate their differentiation ability *in vivo*, PHK-26 or GFP-labeled GRP cells were transplanted into the LV of SD rats. The results showed that transplanted GRP cells successfully migrated 1083.8 ± 84.4 µm away from the injection site and integrated into the ventricular/subventricular zone of host rat brains (Fig. 3E-H). Furthermore, 49.0 ± 13.3% of the transplanted GRP cells turned into oligodendrocytes (MBP$^+$) (Fig. 3F, G) and 47.6 ± 6.4% of them into astrocytes (GFAP$^+$) (Fig. 3E). Neurons (MAP2$^+$ or Tuj1$^+$) were not detected (Fig. 3H). Additionally, no tumor was observed among all the host brain sections. Hence, rhesus GRP cells were bipotential *in vivo*.

Discussion

To the best of our knowledge, the present study represents the first report of a successful attempt at the derivation and characterization of rhesus monkey GRP cells from rESC. The rhesus GRP cells have similar features to mouse GRP cells in aspects of morphology, gene expression profile and differentiation potential (Figs. 2 and 3). Both of these cells are A_2B_5 immunoreactive, and rely on FGF2 to self-renew as opposed to PDGF, which is the survival and proliferation factor of oligodendrocyte precursors (OP) [6]. Differentiation of the purified monkey GRP cells *in vitro* verified their tripotential differentiation capability (Fig. 3). Transplantation in SD rats also showed their astrogenesis and oligodendrogenesis *in vivo* (Fig. 3), fulfilling a major criterion used to distinguish GRP cells from OP [5, 6]. Recently, much progress has been made regarding the application of mouse GRP cells or their derivatives in cell replacement therapies [5-10]. Most strikingly, Hu *et al.* showed that GRP cells could promote functional recovery after spinal cord injury [9]. Our purified monkey GRP cells are worthy of undergoing further testing in applications such as curing spinal cord injury in primate models.

Using the multi-step EB differentiation system, we observed three different neural progenitor populations, as already described by Kuo *et al.* [20]. We further purified and characterized GRP cells from peripheral migratory fibroblast-like cells (Fig. 1D-F, which in Kuo's study were digested together with all the other neural progenitor cells) to track further lineage differentiation potential. The expression of radial glia (RG) marker vimentin [23] and A_2B_5, a glial progenitor marker in mice and humans [3, 4, 6,11, 12], indicates that they

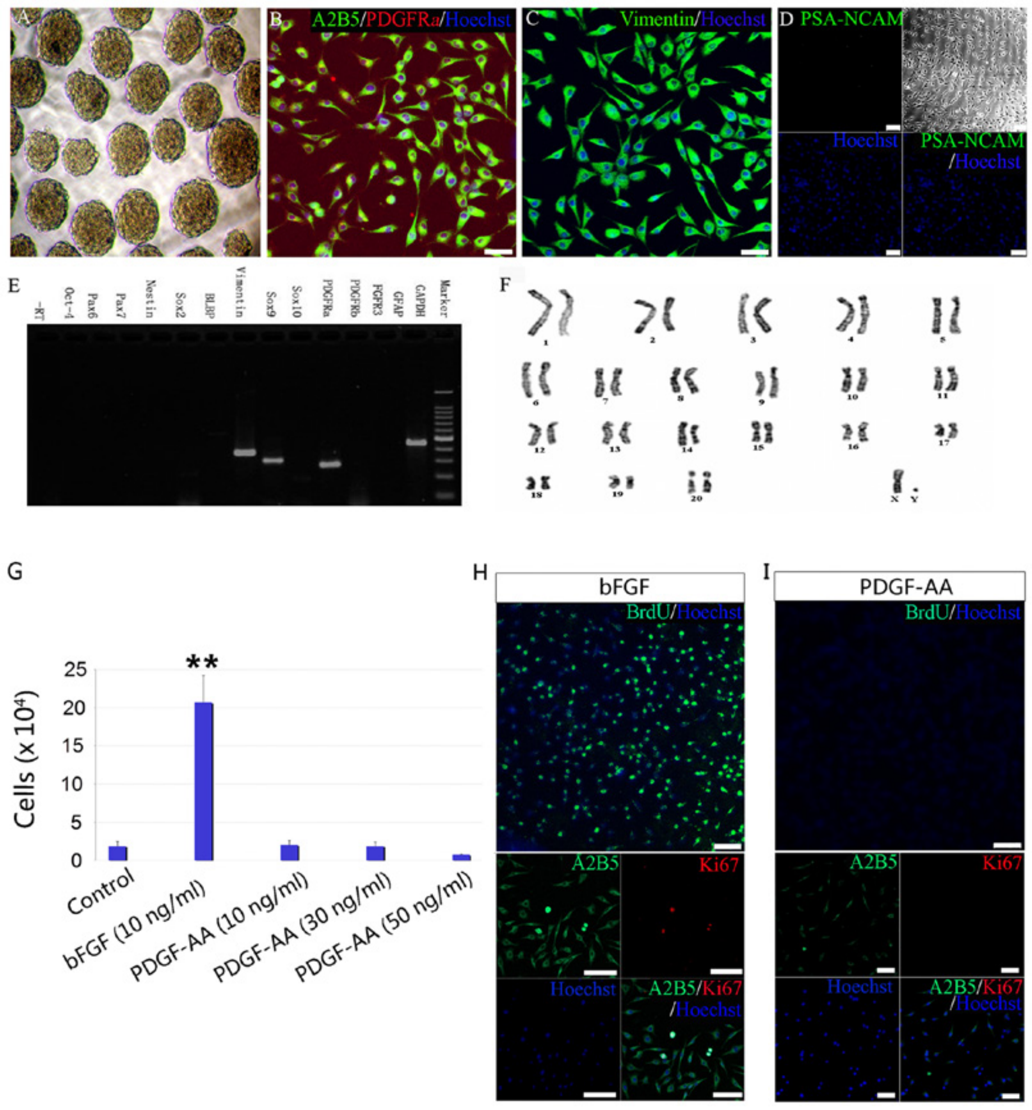

Figure 2. Characterization of glial-restricted precursor (GRP) cells. GRP cells formed gliospheres in suspension culture (A), expressed A_2B_5/PDGFRα (B), vimentin (C) but not PSA-NCAM (D), and displayed proliferative capability (BrdU$^+$, Ki-67$^+$, H) under bFGF stimulation but not PDGF-AA (I). RT-PCR test showed they also expressed glial lineage transcription factor Sox9 (E). F, GRP cells had normal karyotype (42, XY) of rhesus monkey by G-band examination. G, Proliferation test showed that bFGF but not PDGF-AA was the survival factor and mitogen for GRP (n ≥ 4 in 3 independent experiments, ** represents $P < 0.01$). Nuclei were stained by Hoechst stain. Magnification: 100x (A). Scale bars: 50 μm (B-D, H-I).

were not daughter cells of neural crest stem (NCS) cells, which could exist at the periphery of neural rosettes when ESC were induced to neural lineage differentiation [24]. The absence of Sox10 (Fig. 2E), a marker typically expressed by NCS cells further confirms their non-NCS identity [25].

In rodents, there are several controversial hypotheses of gliogenesis. Glial cells may originate from GRP, motor neuron-oligodendrocyte precursor or RG cells [26-28]. In primates the situation is even less clear. Our *in vitro* differentiation model supports the concept that both astrocytes and oligodendrocytes are the progeny of GPR cells. Thus, these newly purified GRP cells are valuable in determining the mechanism of glial lineage cell fate. Interestingly, the GRP cells also provide a good model to study cell migration *in vitro* [29].

In summary, GRP cells were successfully differentiated and characterized from rhesus ESC. This work has the potential to facilitate a better understanding of the molecular mechanism of glial development in primates as well as to provide a donor source for cell replacement therapy in neurodegenerative diseases.

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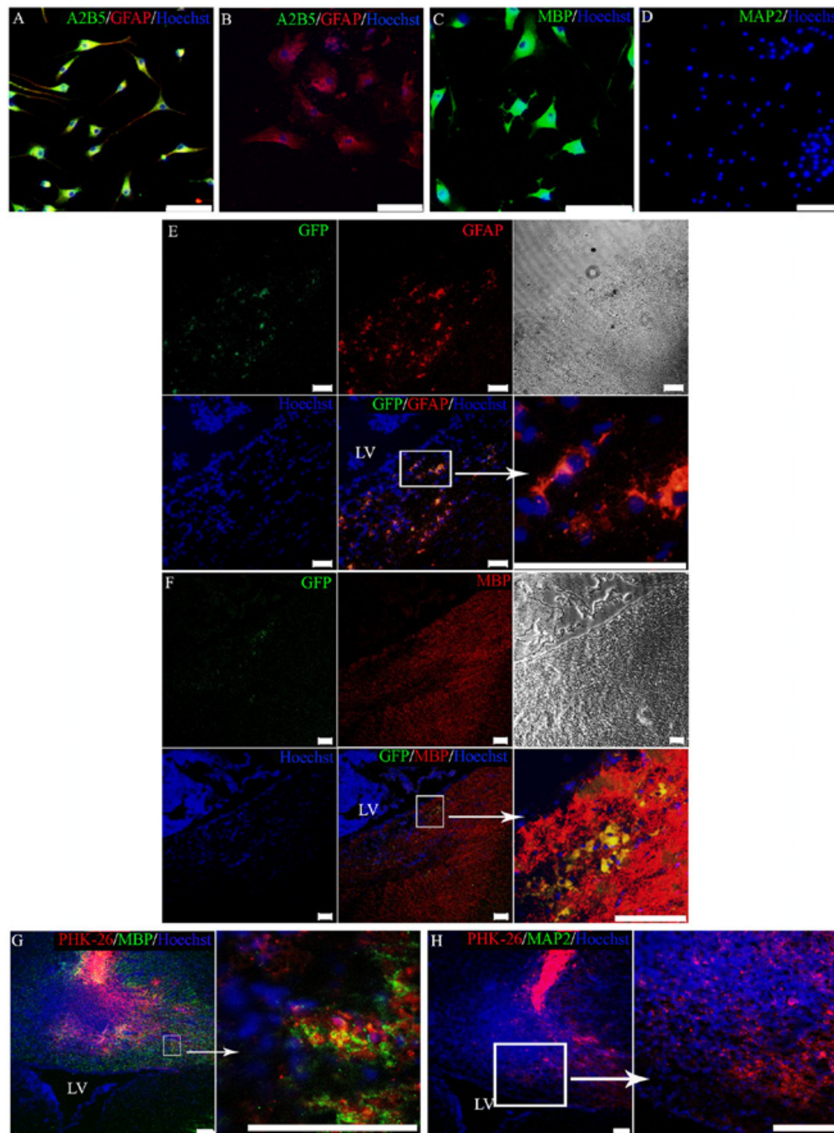

Figure 3. Differentiation of GRP cells *in vitro* (A-D) and *in vivo* (E-H). Under appropriate differentiation conditions *in vitro* (Table 1), GRP cells differentiated into type II astrocytes ($A_2B_5^+$/GFAP$^+$, A), type I astrocytes ($A_2B_5^-$/GFAP$^+$, B) and oligodendrocytes (MBP$^+$, C), but not neurons (MAP2$^-$, D). In 2 weeks, transplanted green fluorescent protein, GFP (E, F) or PHK-26 labeled (G, H) GRP cells migrated and integrated into host Sprague Dawley (SD) rats, differentiating into oligodendrocytes (MBP$^+$, F, G) and astrocytes (GFAP$^+$, E). Neurons were not detected (MAP2$^-$, H). Nuclei were stained by Hoechst stain. Scale bar: 100 µm.

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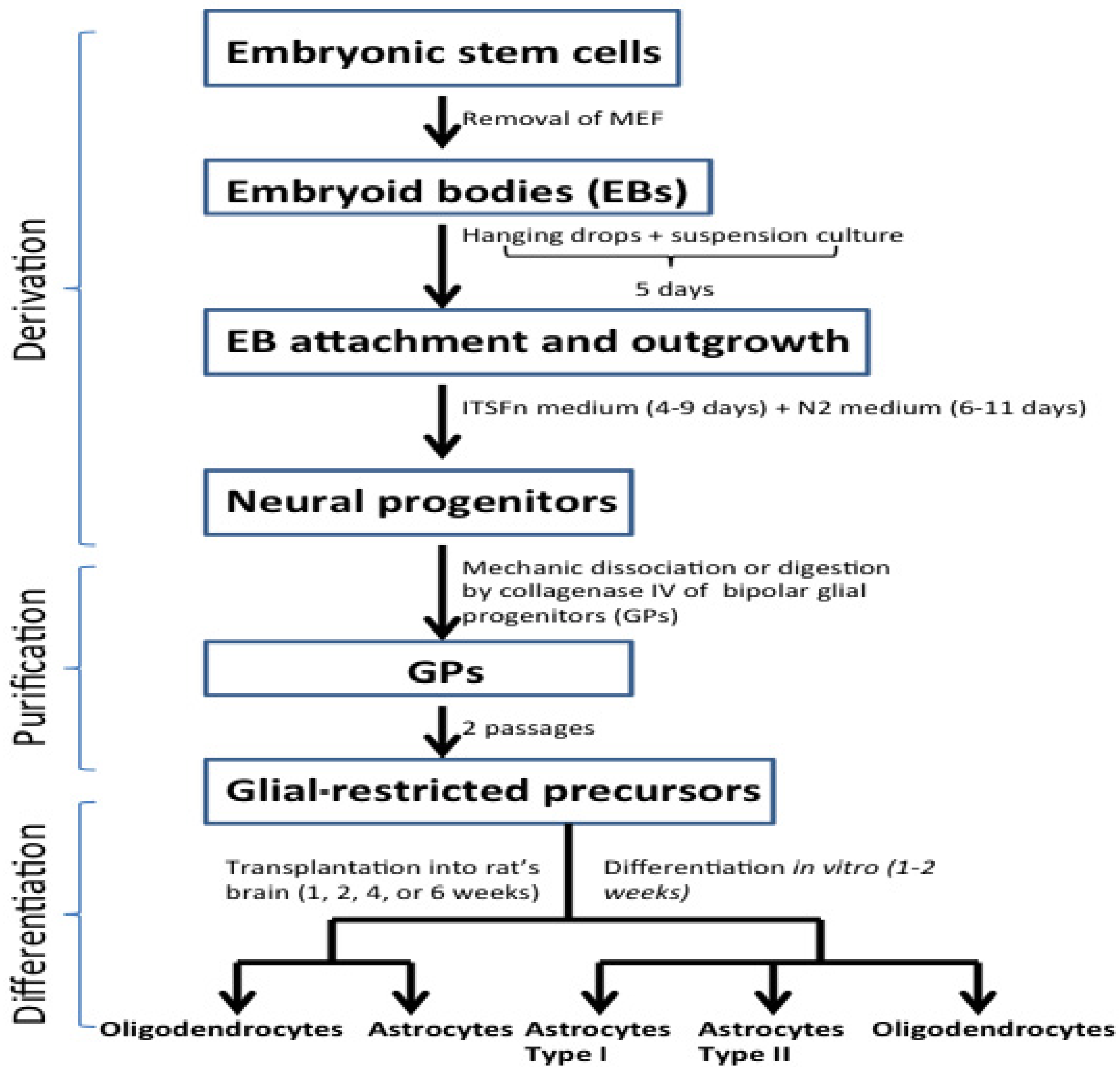

Supplemental Figure 1. Schematic diagram illustrating the procedure of derivation, purification, and differentiation glial-restricted precursor (GRP) cells from rhesus monkey embryonic stem cells. Abbreviation: MEF, mouse embryonic fibroblasts. See text for details.