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

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 A2B5-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.

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 A2B5-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 A2B5-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...
was performed according to the recommended 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 fluorescein 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 33342 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; bFGF | 10 ng/ml; 10 ng/ml | Type I astrocytes |
| CNTF; bFGF | 10 ng/ml; 10 ng/ml | Type II astrocytes |
| LIF; bFGF | 1000 U/ml; 10 ng/ml | Type II astrocytes |
| bFGF; PDGF-AB; TH | 10 ng/ml; 10 ng/ml; 10 ng/ml | Oligodendrocytes |
| RA; NT-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 A2B5 (Fig. 1F), implying these cells are early GP.

Purification and characterization of GRP cells
As PDGFRα and A2B5 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 IgG1 | 1:100 | Chemicon |
| Vimentin | Mouse IgG1 | 1:200 | DAKO |
| PSA-NCAM | Mouse IgM | 1:200 | Chemicon |
| Tuj1 | Mouse IgG1 | 1:150 | Chemicon |
| MAP2 | Rabbit IgG | 1:600 | Chemicon |
| A2B5 | Mouse IgM | 1:200 | Chemicon |
| PDGFRα | Rabbit IgG | 1:200 | Santa Cruz |
| O4 | Mouse IgM | 1:200 | Chemicon |
| MBP | Rabbit IgG | 1:200 | DAKO |
| CD44 | Mouse IgM | 1:40 | DAKO |
| BrdU | Mouse IgG1 | 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’ | 51 | 178 | XM_001108559 |
| FGFR3 | f5’GGGGCGAGTGGCAGAAGTGT 3’ | 62 | 431 | XM_001100919 |
| Nestin | f5’CGCTGGAGGAGCAGAATGAG 3’ r5’CCAGCGACGTCTCCATGTGT 3’ | 55 | 531 | NM_006617 XR_020684 |
| OCT-4 | f5’GACAACAAATGAGAACCCTCA3’ r5’CACATCCTTCTCTAGCCCAA3’ | 54 | 185 | NM_002701 NM_013633 |
| Pax6 | f5’CATGCAGAACAGTCACAGCGG 3’ r5’CCCATCTGTTGCTTTTCGCTA3’ | 60 | 414 | NM_000280 NM_013627 |
| Pax7 | f5’TGCGCTTACCAAGGAGACCGAGG3’ r5’CCAGATTCGGCCTGAT3’ | 54 | 310 | AF254422 |
| PDGFRα | f5’CCAGGCGCTTCTGCTAC3’ r5’TCATCCGGACACCTCC3’ | 54 | 280 | JR_017084 |
| PDGFRβ | f5’CTCACTTCATCTCCTCCAC3’ r5’GTCTGGTTGAGGCGAGT3’ | 55 | 435 | XM_001107595 |
| Sox2 | f5’AGCATGATGCGAGGAGAG3’ r5’GGAGTGGGAGGAAGAGG3’ | 54 | 270 | NM_003106 NM_011443 |
| Sox9 | f5’GGTGTCTACAAGGCTACGACT3’ r5’TGGTGCCCTTCTACCCAGCACT3’ | 59 | 321 | NM_00132868 |
| Sox10 | f5’GCTGGTCAAGGAACTGAC3’ r5’TGAGTGGAGGGGAGGT3’ | 57 | 194 | XM_525950 |
| Vimentin | f5’GCCGACAAAGCGGAGGAGG3’ r5’GTGGGTGGCTACAGGAGG3’ | 52 | 383 | XM_00936558 X65697 |
| GAPDH | f5’TGAATTGCGAGGATGCAAGAGAG3’ r5’TGATGTCAGGAGGAGG3’ | 57 | 449 | NM_002046 |

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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 Aβ5, 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.

**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: Aβ5+/GFAP+ type II astrocytes (Fig. 3A) bearing projections in the presence ofCNTF/bFGF or LIF/bFGF; flat Aβ5/ GFAP+ type I astrocytes (Fig. 3B) with BMP4/ bFGF, expressing FGFR3 by RT-PCR (data not shown); and MBP+ oligodendrocytes (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 Aβ5, 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 Aβ5, a glial progenitor marker in mice and humans [3, 4, 6,11, 12], indicates that they
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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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.