Research Article • DOI: 10.2478/s13380-013-0135-0 • Translational Neuroscience • 4(3) • 2013 • 378-384

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Translational Neuroscience

NEURAL LINEAGE DEVELOPMENT IN THE RHESUS MONKEY WITH EMBRYONIC STEM CELLS

Hongwei Chen[1,2,3,5,*], Qiang Wei[1,2,5], Jing Zhang[1,2,5], Tao Tan[1,2,5], Rongrong Li[1,2,5], Jilin Chen[4]

[1]*Laboratory of Reproductive and Developmental Biology, Kunming Primate Research Center, and Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, P. R. China,*
[2]*Yunnan Key Laboratory of Animal Reproductive Biology, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, P. R. China,*
[3]*School of Life Sciences and Chemistry, Zhaotong University, Zhaotong, Yunnan 657000, P.R. China*
[4]*Zhaotong University, Zhaotong, Yunnan 657000, P.R. China*
[5]*Graduate University of Chinese Academy of Sciences, Beijing 100049, P. R. China*

Received 25 August 2013
accepted 31 August 2013

Abstract

There are three controversial and undetermined models of neurogenesis and gliogenesis from neuroepithelial cells in the early neural tube; the first in which neurons and glia were proposed to originate from a single homogenous population, the second from two separate pools of committed glial and neuronal progenitors, or, lastly, from transit radial glial (RG). Issues concerning embryonic neural lineage development in primates are not well understood due to restrictions imposed by ethics and material sources. In this study, early neural lineage development was investigated *in vitro* with rhesus monkey embryonic stem cells (rESC) by means of immunofluorescence with lineage specific markers. It was revealed that neural differentiation likely progresses in a sequential lineage restriction pathway from neuroepithelial stem/progenitor cells to neurons and glia via RG and intermediate precursors: neuronal precursors and glial progenitors. In conclusion, our results suggest that the early neural lineage development of rESC *in vitro* supported the model in which neuroepithelial cells develop into RG capable of generating both neurons and glia. This work should facilitate understanding of the mechanism of development of the nervous system in primates.

Keywords

• Differentiation • Neurogenesis • Gliogenesis • Neuroepithelial stem/progenitor cell (NEP) • Radial glia (RG)

Introduction

During neurulation, part of the ectodermal cells is specified to form the neuroepithelium of the neural tube from which neurons and glia are derived, making up the nervous system [1]. Although it is generally believed that neurogenesis arises earlier than gliogenesis [2], most issues concerning neural stem cell (NSC) lineage development are still argumentative and undetermined. Historically, neuroepithelial cells (NE) in the early neural tube were believed to produce two separate pools of committed glial and neuronal progenitors to generate glial cells and neurons, respectively [3,4]. Later, the other alternative hypothesis of a single homogeneous proliferative cell population that generates both neurons and glia was proposed [5,6] and supported [7-12]. Recent data further imply that NE cells either produce or transform into radial glia (RG) cells, which then develop into neurons and glia [13-15]. However, this knowledge is fragmentary and less understood in primates, including humans [16], due to restrictions imposed by ethics and material sources, unlike in rodents.

Embryonic stem cells (ESC) are believed to be a good model to study cell differentiation and hold great prospects for regenerative medicine because of their pluripotency, i.e. the ability to differentiate *in vitro* into all derivatives of the three embryonic germ layers constituting the body [17-19]. Many neural cell types have been derived from ESC, including neural stem/progenitor cells [20], RG [21-24], neuronal restricted precursors and glial restricted precursors (GRP) [25], neuronal subtypes and oligodendrocytes [26-29]. The neural progenitors from ESC even display properties of distinct regionalization responsible for neural patterning [30-32]. The *in vitro* induction of neural differentiation in ESC also closely mimics molecular mechanisms of embryonic brain development [33]. All these findings make it possible to use rESC as a model for investigating cell lineage relations to better understand primate, including human, embryonic neurogenesis and gliogenesis because the rhesus monkey has the advantage over rodents of being closely related to humans in terms of genetics and physiology [34].

In this work, neural lineage development in the rhesus monkey was systematically elucidated *in vitro* with rESC for the first time. The results revealed that NEP predominantly switched into RG and differentiated into neurons and glia via intermediate neuronal precursors (NP) and glial progenitors (GP), respectively.

Materials and methods

ESC culture and neuroectoderm differentiation

The procedures for the preparation of a culture of rESC were described previously [18]. Briefly, the rhesus monkey ESC line R366.4 (a generous gift from Professor J.A. Thomson, Wisconsin University, Madison, WI, USA) was grown on γ-ray irradiated mouse embryonic fibroblast feeder layers in 0.1% gelatin-coated tissue culture plates (Becton Dickinson & Company,

* *E-mail: chenhw02@mail.kiz.ac.cn*

Franklin Lakes, NJ, USA). The rESC culture medium consisted of DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 15% defined fetal bovine serum (dFBS) (Hyclone, Logan, UT, USA), 1% nonessential amino acids (Invitrogen), 0.1mM 2-mercaptoethanol (Sigma, St. Louis, MO), and 2mM L-glutamine (Sigma). The medium was changed daily, and the ESC colonies were split every 5-7 days by incubation with 10mg/ml dispase (Invitrogen) and then replated after centrifugation onto dishes with fresh mouse embryonic fibroblast feeder cells.

To directly differentiate rESC into neuroectoderm, a multi-step embryoid body (EB)-based differentiation method [35] was adopted and slightly modified. In brief, rESC colonies collected after incubation with dispase were first cultured in suspended drops of rESC differentiation medium (using 10% dFBS instead of 15% dFBS; 35µl/drop, n=1400±303) to form EB on the inner surface of 100-mm culture dish lids (BD) for 2 days. Then the EB were transferred to another tissue culture dish in a suspension culture in rESC differentiation medium. The EBs were maintained for a total of 5 days (2 days in suspended drops and 3 days in suspension) were plated onto 0.1% gelatin-coated culture dishes in rESC differentiation medium. After 24h of culture to allow cell attachment and surface spreading, the medium was replaced with a ITSFn medium for 1-4 days containing DMEM/F12 (1:1, Invitrogen) supplemented with 1x ITS supplement (Invitrogen) and fibronectin (5µg/ml, Sigma), with medium replenishment every 2 days. Then N2 medium consisting of DMEM/F12, laminin (1 µg /ml, Sigma) and basic fibroblast growth factor (bFGF, 10ng/ml, Sigma) supplemented with 1x N2 supplement (Invitrogen) was added for 6-9 more days.

NEP/RG culture

After being in culture in N2 medium for 6-9 days, neural rosettes (NR, P0) were mechanically dissociated or digested by collagenase IV (1mg/ml, Invitrogen) for obtaining NEP. To expand NEP/RG, cell clumps were mechanically divided every 6 days and then plated onto poly-ornithine (PLO, 0.01%)/laminin (20µg/ml) coated dishes with a NEP/RG proliferation medium which consists of DMEM/F12 plus bFGF (20ng/ml), epidermal growth factor (EGF, 20ng/ml, Chemicon, Temecula, CA), 1x N2 supplement, 1x B27 supplement (Invitrogen), lmM L-glutmaine for P1 only, and 50 Units/ml penicillin/streptomycin (Sigma).

NEP/RG could be stored in 90% newborn calf serum plus 10% DMSO in liquid nitrogen and successfully thawed and recovered afterwards.

Differentiation of NEPs/RG in Vitro

Because NEP/RG clumps did not survive over 3 days in NEP/RG proliferation medium with bFGF and EGF withdrawn, cells were induced to differentiate in an alternative terminal differentiation medium (TDM) or GRP-conditioned medium obtained from regular expansion culture of GRP (unpublished). TDM is a mixture of the modified NEP/RG proliferation medium, in which bFGF and EGF were substituted by neurotrophin-3 (20ng/ml, R&D systems, Minneapolis, MN) and ciliary neurotrophic factor (20ng/ml, PeproTech, Rocky Hill, NJ), and the rhesus fetal cortex (approximate 3 month old) cell-derived conditioned medium, which consisted of DMEM/F12 plus 10% FBS (Invitrogen), in a 1:1 (v/v) ratio.

Immunocytochemistry

For immunocytochemistry, cells were rinsed in phosphate-buffered saline (PH=7.4) three times and fixed in 4% PFA for 15-20 minutes. After treatment with 0.4% triton-X 100 for 10 min (for cytoplasmic proteins only), the cells were blocked with 5-10% goat serum for half an hour at room temperature (20°C). Primary antibodies (Table 1) were used at room temperature for 1h or at 4°C overnight. Secondary antibodies conjugated with 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 the room temperature for an hour. Cell nuclei were stained with Hoechst 33258 or propidium iodide (PI, Sigma). The cells were examined using a confocal laser scanning system (LSM 510 META; Carl Zeiss, Jena, Germany).

Statistical analysis

The results are presented as means ± the standard error of the mean (SEM). Statistical analysis was performed using the One-Way ANOVA with statistical significance defined as $P<0.05$.

Table 1. Primary antibodies used for immunofluorescence.

Antigen	Species/Type	Dilution	Source
Oct-4	Rabbit IgG	1:100	Santa Cruz
Pax6	Rabbit IgG	1:800	Chemicon
Nestin	Mouse IgG1	1:100	Chemicon
Musashi-1	Rabbit IgG	1:60	Chemicon
Vimentin	Mouse IgG1	1:200	DAKO
BLBP	Rabbit IgG	1:50	PeproTech
GLAST	Guinea pig IgG	1:100	Chemicon
PSA-NCAM	Mouse IgM	1:200	Chemicon
ß-tubulin III	Mouse IgG1	1:150	Chemicon
MAP2	Rabbit IgG	1:600	Chemicon
A_2B_5	Mouse IgM	1:200	Chemicon
O4	Mouse IgM	1:200	Chemicon
MBP	Rabbit IgG	1:100	DAKO
CD44	Mouse IgG1	1:40	DAKO
GFAP	Rabbit IgG	1:600	Chemicon

Abbreviations: BLBP, brain-lipid-binding protein; GLAST, astrocyte-specific glutamate transporter; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated-protein 2; MBP, myelin basic protein.

Results

Direct differentiation of rESC into neuroectoderm cells

The undifferentiated rESC (Figure 1A) were efficiently directed towards differentiating into neuroectoderm cells by means of forming EB (Figure 1B) as described previously [35]. Cellular characteristics of morphology and specific marker expression implied that they represented three distinct neural lineage populations: pseudostratified Pax6$^+$ NEP (Figure 1E) in a structure of NR (Figure 1C,D-J) at high cell density in the central part; outwards migrating fibroblast-like cells at low cell density at the periphery (data not shown); and transient PSA-NCAM$^+$ (Figure 1H) and/or ß-tubulin III$^+$ (Figure 1I, J) NPs with epidermal morphology at intermediate cell density between the former two parts. Neither the pluripotency marker (Oct-4, Figure 1D) nor terminal differentiation markers (microtubule-associated protein 2, MAP2, glial fibrillary acidic protein, GFAP, and O4) were detected in these cells. Notably, NEP/RG markers Nestin (Figure 1F, 2A), Vimentin (Figure 1G, 2B) and brain-lipid-binding protein (BLBP, Figure 2B) also were hardly detected in the NRs at this stage by immunostaining.

Sequential lineage transition from NEP to RG, NP and GP

It was further demonstrated that NEPs that derived from NRs sequentially restricted their differentiation potential. Morphologically, early cell clumps consisted of neuroepithelial cells with a distinct colony boundary (Figure 2E), while late cell clumps comprised neural stem/progenitors with radial processes outgrowing (Figure 2F). The majority of NEPs (P1) expressed NEP markers Pax6 (87.23±6.62%), Nestin (79.12±7.46%) (Figure 2A,G), Musashi-1 and Sox2 (data not shown), but only 16.13±4.17% cells were immunopositive for Vimentin (Figure 2B,K), and other RG markers such as BLBP (Figure 2I), astrocyte-specific glutamate transporter (GLAST, Figure 2B) were not detected. In the subsequent expansion culture, the proportion of cells stained with NEP markers decreased (Figure 2A) while RG specific markers increased (Figure 2B). Nestin$^+$ cells decreased to 2.13±0.14% at P8 and about 70% cells lost Pax6 expression from P5 to P8 (Figure 2A). In contrast, the percentages of cells expressing Vimentin, BLBP, or GLAST (P8) rose to 87.17±7.49% (Figure 2B,L), 91.53±4.37% (Figure 2B,J,L), and 78.02±8.41% (Figure 2B), respectively. These results suggested that most of the NEPs dynamically produced or transformed into RG cells.

Along with RG differentiation, neurogenesis also occurred in NEP/RG (Figure 2C). Between 10-20% of the NEPs spontaneously differentiated into PSA-NCAM-positive NP gradually from P1 to P8, with a peak of 20.40±3.03% at P5 (Figure 2C). Co-localization of Nestin and PSA-NCAM directly demonstrated that NP could originate from NEP (detected at P2). Young neurons positive for ß-tubulin III (Figure 2M,N), the progeny of PSA-NCAM-positive NP, significantly came into being at P3 (Figure 2N)-P8.

Gliogenesis took place later than neurogenesis. Neither astroglial nor oligodendroglial cells were detected until P8 (Figure 2D,P) although A_2B_5 was expressed in some cells at P1 (Figure 2O).

Expansion and differentiation of NEP/RG

NEP/RG at high cell density (Figure 2E,F) could propagate for over 2 months with bFGF and EGF stimulation until they lost high cell-density contact. Long term-cultured NEP/RG (P10) kept their high neurogenic potential when they were induced to differentiate in the terminal differentiation medium. In a week, 72.51±8.95% of cells differentiated into mature MAP2$^+$ neurons (Figure 3A), 10.32±3.01% of cells into GFAP$^+$ astrocytes (Figure 3B), or a minority of cells into O4$^+$ oligodendrocytes (Figure 3C). In

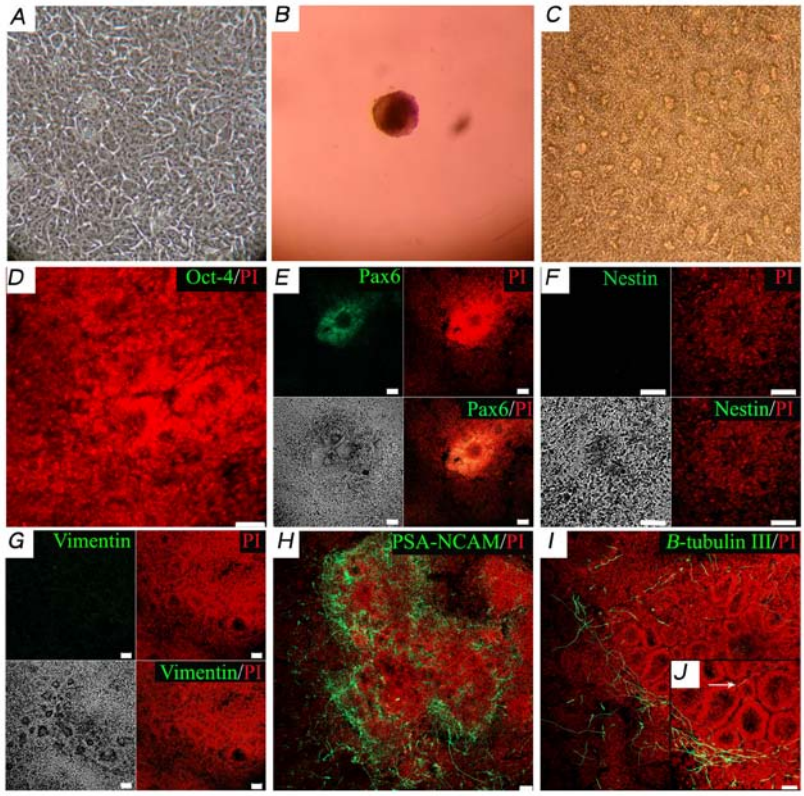

Figure 1. Direct differentiation of rhesus monkey embryonic stem cells (rESC) into neuroectodermal cells. With a lineage selection method (A-C), rESC were directed towards differentiating into distinct neural populations expressing specific cell markers (D-J). A, undifferentiated rESC; B, EB on day 5; C, neural rosettes (NR); D, no cells were immunoreactive for Oct-4. NR comprised of neuroepithelial stem/progenitor cells (NEP) expressing Pax6 (E) but not Nestin (F) and Vimentin (G). Transient cells expressed neuronal precursor maker PSA-NCAM (H), ß-tubulin III (I); J, higher magnification of I with arrow indicating ß-tubulin III-positive neuron in NEP. (E). Nuclei were stained by PI (D-J). Magnification: 200x (A), 100x (C), and 40x (B). Scale bar: 50 µm (D-J).

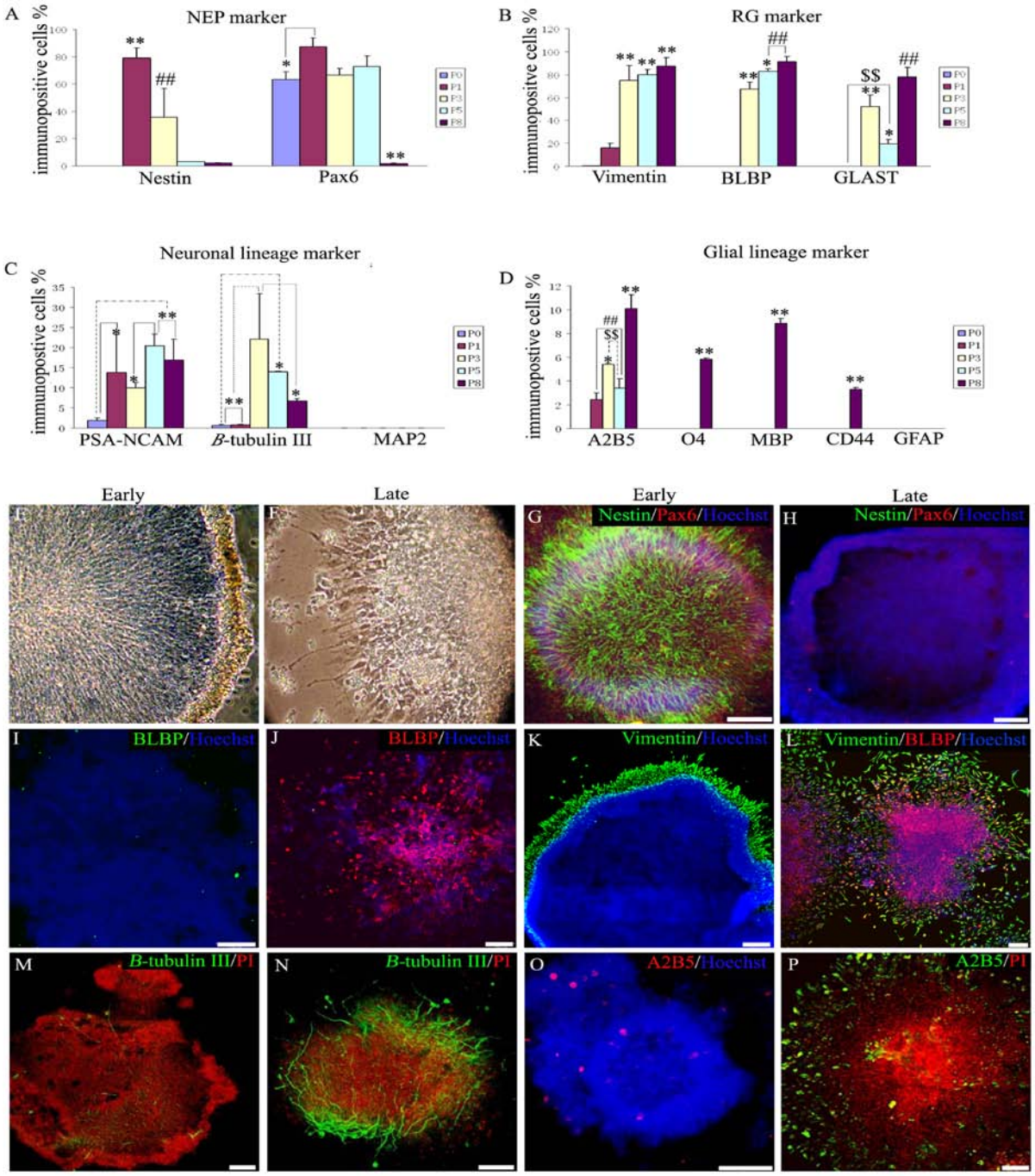

Figure 2. Lineage transition from neuroepithelial stem/progenitor cells (NEP) to radial glia (RG), neuronal precursors (NP) and glial progenitors (GP). The expression analysis of lineage specific markers (A-D) showed that early NEPs with a distinct colony boundary (E) that were derived from neural rosettes (NRs) (P0) expressed Nestin and Pax6 (A, G). NEP then dynamically transitioned into RG (B, I-L) with outgrowing radial processes (F), expressing Vimentin (K, L) and brain-lipid-binding protein (BLBP, J, L). Meanwhile, the neuronal lineage represented by ß-tubulin III$^+$ young neurons (M, N) progressively came into being (C). Glial lineage (O, P) apparently took place later (D). Nuclei were stained by PI (M, N, P) or Hoechst (G-L, O). Magnification: 200x (E, F). Scale bar: 100μm (G-P). The statistical data (A-D) come from 6-17 NR or NEP/RG clumps with over 1,000 cells. **, ## and $$ represent P<0.01; * represents P<0.05.

contrast, if the NEPs/RG differentiated in the GRP-conditioned medium for 4 weeks, the ratio of differentiated ß-tubulin III$^+$ neurons vs. GFAP$^+$ astrocytes reversed to 13.46±9.65% vs. 71.84±8.00% (Figure 3D), which means the majority of NEP/RG switched into gliogenic

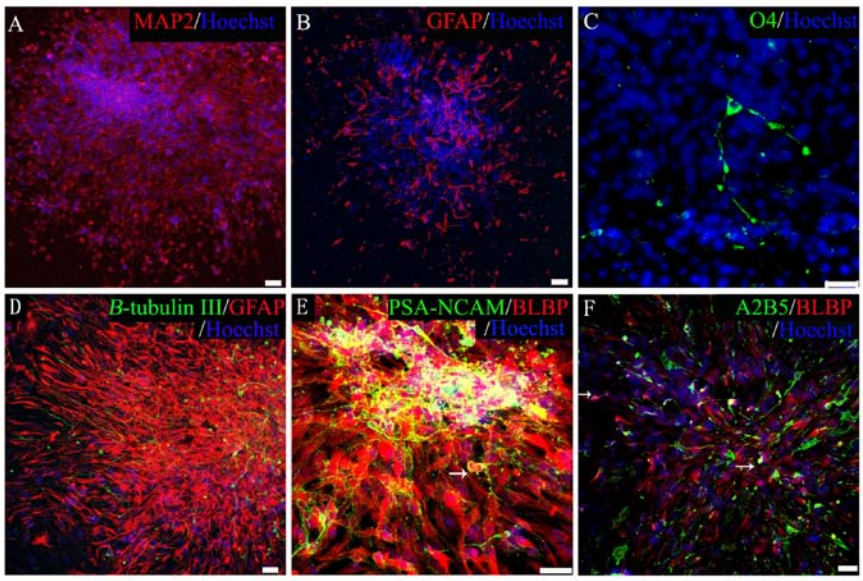

Figure 3. Differentiation of long-term culture NEP/RG *in vitro*. Under the terminal differentiation medium (A-C), NEP/RG differentiated into neurons ($MAP2^+$, A), astrocytes ($GFAP^+$, B) and oligodendrocytes ($O4^+$, C) in a week. When differentiated in the glial restricted precursor-conditioned medium for 4 weeks (D-F), the majority of cells were $GFAP^+$ astrocytes with a few ß-tubulin III-positive cells (D). Some $BLBP^+$ RG cells were also stained with PSA-NCAM (arrow in E) and A_2B_5 (arrow in F). Nuclei were stained by Hoechst. Scale bar: 50 μm.

precursors. Additionally, PSA-NCAM (Figure 3E) and A_2B_5 (Figure 3F) were found to be co-localized with BLBP in some differentiating cells.

Discussion

In the present study, neural lineage development with rESC *in vitro* was reported comprehensively and the results indicated that the lineage transition proceeded sequentially from NEP to RG, NP and GP in a temporally organized manner *in vitro*.

Lineage restriction development was the most outstanding change in rhesus NEP expansion (Figure 2). Temporally, the majority of NEP dynamically produced or transformed into RG, then into NP and GP (Figure 2), which was different from the model that neurons and glial cells directly originated from committed neuronal and glial precursors derived from early NE, respectively [3,4]; however, our data are consistent with the newly proposed NSC lineage differentiation theory [14], although the coexistence of neuronal and glial precursor cells in the cerebral ventricular zone at the time of peak neurogenesis during midgestation in the monkey occipital lobe [36,37] was once accepted to favor the primitive model. The apparent appearance of approximate 10-20% $PSA\text{-}NCAM^+$ NP (Figure 2C) before RG specific markers (Vimentin, BLBP, GLAST) obviously started to be expressed (P0, P1) (Figure 2B) implied these neuronal precursors might be produced by early NEP. This hypothesis was confirmed by the co-localization of Nestin and PSA-NCAM in the cells at P2, which was also observed in the neuronal restricted precursors isolated in embryonic mouse neuroepithelium [38]. However, the hypothesis could not exclude that some neurons originated directly from the NEP with reactivity to Pax6 (Figure 1E) but not Nestin (Figure 1F) because the earliest immature unipolar neurons (Figure 1J) were found in fresh NR before most of the NSC and RG markers were expressed in the NEP (Figures 1F,G, 2B). Pax6 was expressed earlier than Nestin in rhesus NEP (Figure 1E,F), which is similar to human ESC [39]. In contrast to rodents, human "pioneer neurons" already appear before the neural tube completely closes [40]. However, whether these earliest neurons directly originating from the early rhesus NEP are unique in primates or are just a special case in cell culture *in vitro* needs to be further clarified. Besides, terminal differentiation of NEP/RG (Figure 3A) indicated that RG kept strong neurogenic ability and differentiated into neurons, which probably was mediated via dividing intermediate neuronal precursors (Figure 3E). This novel evidence proved that rhesus RG are neuronal precursors, as in mice [13,15,41] and human [42,43] RG. The results also suggested that the neurogenic potential of RG is conserved in vertebrates; but such competence of RG could switch into gliogenic capability at later stages when the cells express GFAP [45], which might explain undetected GFAP expression of the RG at early differentiation stages in the present study (Figure 2D). All the above results suggested that neurons could originate from NEP, RG and NP.

Similar to development in other vertebrates [2,11], rhesus gliogenesis (Figure 2D) arose apparently later than neurogenesis. Astroglial or oligodendroglial cells were not detected until P8 (Figure 2D,P). Additionally, RG cells were astrocyte ancestors (Figure 3) as previously described [24]. The transition from RG to astrocytes was likely mediated by $BLBP^+/A_2B_5^+$ GP (Figure 3F), which was also discovered in mouse glial differentiation [45,46]. Recent work by Howard *et al.* [47] indeed demonstrated that RG cells in the developing human brain could differentiate into oligodendrocytes via oligodendrocyte progenitors. The results suggested that glial cells could originate from either RG or GP. Taken together, the neural lineage development with rESC was proposed that NEP predominantly switch into RG and then directly or indirectly differentiate into neurons or glia.

In summary, the neural lineage development of rESC was systematically elucidated for the first time. It was demonstrated that early neurogenesis and gliogenesis in rhesus monkey are consistent with the model in which NE develop into RG that have the capability of generating both neurons and glia. This fundamental knowledge will help to uncover the mechanism of nervous system development in primates.

Acknowledgements

This work was supported by research grants from the National Basic Research Program 2006CB701505 and 2007CB947701, the Chinese Academy of Sciences KSCX2-YW-R-47, and the Chinese National Science Foundation 30570906 and 30700159. We are thankful to Professor Weizhi Ji for support and direction. We appreciate Dr. B. Bavister for deliberated revision of the manuscript. The authors declare no conflict of interest.

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