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Low Temperature Induced De-Differentiation of Astrocytes

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Abstract Radial glial cells are astrocyte precursors, which are transiently present in the developing central nervous system and transform eventually into astrocytes in the cerebral cortex and into Bergmann glia in the cerebellum. Previous reports indicate that the transformation from radial glia to astrocytes can be reversed by diffusible chemical signals derived from embryonic forebrain in vitro and by freezing injury in vivo. But there is no direct evidence proving that mature astrocytes can de-differentiate into radial glial cells. Here we show that purified astrocytes could de-differentiate into radial glial-like cells (RGLCs) in vitro with freeze-thaw stimulation. RGLCs had the expression of markers for radial glia including Nestin and Pax6, and astrocyte markers, the glial fibrillary acidic protein and Vimentin. Cortical neurons, when co-cultured with RGLCs, migrated along the processes of RGLCs at an average speed of 26.26 ± 3.36 μm/h. Moreover, the proliferation of RGLCs was significantly promoted by epidermal growth factor (EGF) at the concentration of 10–30 ng/ml. These results reveal that low temperature induces astrocytes to de-differentiate into immature RGLCs, which provides an in vitro model to investigate mechanisms of astroglial cells de-differentiation. J. Cell. Biochem. 99: 1096–1107, 2006.

Key words: radial glial-like cells (RGLCs); de-differentiation; neuronal migration; radial glia

Radial glia are morphologically characterized as a typical bipolar cell with long, thin, unbranched processes that terminate in conical end feet, which guide the migration of neurons from the proliferative zone to the cortical plate in the cerebral cortex [Rakic, 1972; Voigt, 1989]. Radial glia expresses a series of specific markers, including Nestin, Vimentin, RC2, brain-lipid-binding protein (BLBP), glutamate astrocyte-specific transporter (GLAST), Pax6, and antigens recognized by D4 polyclonal antisera [Hockfield and McKay, 1985; Misson et al., 1988; Edwards et al., 1990; Cameron and Rakic, 1994; Feng et al., 1994; Shibata et al., 1997; Götz et al., 1998; Hartfuss et al., 2001]. Traditionally, after neurogenesis and the completion of neuronal migration, radial glia undergo morphological and biochemical changes, and differentiate into astrocytes in the cerebrum and Bergmann glia in the cerebellum [Schmechel and Rakic, 1979; Culican et al., 1990; Hartfuss et al., 2001; Barry and McDermott, 2005]. There are a variety of transitional forms of cells during the transformation from radial glia to astroglia in vivo, as demonstrated by Golgi staining, gold sublimate, and glial fibrillary acidic protein (GFAP) immunocytochemistry staining [Pixley and de Vellis, 1984; Hartfuss et al., 2001; Alves et al., 2002]. Recent reports also show that radial glia are neuronal precursors after neurogenesis [Parnavelas and Nadarajah, 2001; Götz et al., 2002; Heins et al., 2002; Malatesta et al., 2003].

Traditionally astrocytes are considered as mature, fate-determined cells, which provide important structural, metabolic and trophic

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support to neurons [Fields and Stevens-Graham, 2002; Doetsch, 2003]. However, several previous reports show that freezing injury, traumatic CNS injury induce the appearance of some radial glia-like fibers, which expresses Nestin in the adult rodents [Hatten, 1985; Rosen et al., 1992; Rosen et al., 1994; Hunter and Hatten, 1995; Shibuya et al., 2002; Huttmann et al., 2003]. Embryonic neurons transplanted into adult brain induce a transient rejuvenation of the host Bergmann glia or astrocytes into radial glia like cells that might mediate the active migration of transplanted neurons and neural precursors [Soriano et al., 1997; Leavitt et al., 1999]. The transforming growth factor-α (TGF-α), neuregulin, diffusible factors derived from embryonic cortical neurons or immortalized multipotent neural precursor cells could also induce astrocytes to re-express Nestin and radial glia like morphology both in vitro and in vivo [Hunter and Hatten, 1995; Zhou et al., 2001; Patten et al., 2003; Schmid et al., 2003].

These previous findings indicate that astrocytes in rat neocortex can be induced to de-differentiate into Nestin expressing and radial glial like cells by freezing injury stimuli to the cortial plate. In the present study mature astrocytes were purified from postnatal cerebral cortex cultures and underwent the freeze-thaw stimulation. After this treatment, we found that purified mature astrocytes were induced to de-differentiate into radial glial-like cells (RGLCs), which were morphologically, immunocytochemically and functionally similar to radial glia in vivo. Our results provide a simple means to obtain large quantities of immature RGLCs from the de-differentiation of mature astrocytes.

MATERIALS AND METHODS

Antibodies

The following primary antibodies were used: mouse anti-rat Nestin (PharMingen); mouse anti-Vimentin (Sigma); mouse anti-GFAP (Sigma); rabbit anti-GFAP (Sigma); mouse anti-MAP2 (Chemicon) and mouse anti-BrdU (Sigma); rabbit anti-Pax-6 (Covance Inc.); mouse anti-O4 (Boehringer Mannheim); mouse anti-neurofilament 160 (Sigma) and Mouse anti-RC2 (Developmental Studies Hybridoma Bank at the University of Iowa).

Purification of Astrocytes

Primary astrocyte cultures were prepared with neonatal Sprague–Dawley rats. Purified astrocyte cultures from the cerebral cortex were prepared as described previously with modifications [Kornblum et al., 1990; Louis et al., 1992]. Briefly, the neocortex of newborn rats was dissected out free of meninges, and incubated in 0.125% trypsin for 25 min at 37°C. The tissue was then gently triturated. Dissociated cells were plated at the density of 2.0×10^5 viable cells/cm^2 on culture flask coated with poly-D-lysine (Sigma). Cells were fed every 3–4 days with DMEM-F12 (GibcoBRL) (1:1, v/v) supplemented with 10% (v/v) heat-inactivated horse serum (HS, GibcoBRL) for 9–10 days. The flasks were then shaked overnight in a rotary shaker at 200 rpm. The remaining adherent cells were split 1:3 and then treated with 2×48 h pulses of 10^{-5} M arabinosylcytosine (Ara-C, Sigma). The purity of astrocytes was more than 99% based on the GFAP staining.

Freezing and Thawing of Astrocytes

The freeze-thaw treatment was described previously with modifications [Das et al., 1983; Kawamoto and Barrett, 1986]. Briefly, the astrocytes were collected from the culture flasks using trypsin, and resuspended in 1 ml of freezing medium (3×10^5 cells/ml) consisting of DMEM-F12 supplemented with 20% fetal calf serum (FCS, GibcoBRL) and 10% dimethylsulfoxide (DMSO, Sigma). The cell suspension was incubated at 4°C for 15 min, −20°C for 1 h, and then stored at −80°C for later thawing experiments. In thawing experiments, the cell suspensions were thawed in 37°C water bath without agitation, immediately washed twice with D-Hanks, and centrifuged for 3 min at 800*g*. The cells were resuspended and plated at a density of 1,000 cell/mm^2 on poly-D-lysine-coated glass coverslips and grown in DMEM-F12 medium containing 10% FCS. Twenty-four hours after plating, 10% FCS-containing medium was changed with Neurobasal™ Medium supplemented with B27 (GibcoBRL) (NB + B27 medium). Purified astrocytes treated with different concentration as (0.1, 0.3, 1, 3, and 10%, respectively) of DMSO in serum-containing medium or serum-free medium were used as control.

Immunocytochemical Staining

The cultures were rinsed briefly, fixed with 4% paraformaldehyde for 30 min at room temperature. After blocking non-specific binding with 2% normal goat serum for 1 h at room temperature, the culture was incubated with primary antibodies overnight at 4°C, and then secondary antibodies, fluorescein-conjugated goat-anti-mouse IgG or rhodamine-conjugated goat-anti-mouse IgG (Jackson ImmunoRes Lab, Inc.) for 60 min at 37°C. The cultures were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For double labeling of bromodeoxyuridine (BrdU, Sigma) and other antigens, the cultures were pulsed with 10 μM BrdU for the last 3 days. After fixation the cultures were treated with 2N hydrochloric acid (HCl), and then 0.1 M sodium tetraborate (pH 8.5). Since both antibodies for BrdU and Nestin are monoclonal IgG antibody, cultures were first stained with BrdU antibody and its secondary antibody, FITC-conjugated goat-anti-mouse IgG (BD). After thoroughly washing with PBS, the same culture was stained with Nestin antibody and its rhodamine-conjugated goat-anti-mouse IgG (BD).

Western Blot Analysis

The freeze-thawed cells, purified astrocytes, cerebellar tissue, or rat brain tissue were lysed in an ice-cold RIPA buffers, as described elsewhere [Du et al., 2000]. The lysates were mixed with sample buffer for SDS–PAGE. Samples were boiled for 5 min. Equal amounts of total proteins were loaded to 8.5% SDS–PAGE, and then immunoblotted with the primary antibodies: mouse anti-Nestin (1:800), mouse anti-Vimentin (1:1,000), rabbit anti-GFAP (1:1,500) or rabbit anti-Pax6 (1:500). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:10,000, Pierce) were used as secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Pierce).

Migration of Neurons on RGLC Processes

The freeze-thawed cells were plated in low density on poly-D-lysine-coated glass coverslips for at least 3 days. Freshly dissociated embryonic 18 (E18) cerebral cortical neurons were added to the freeze-thawed cells culture at a ratio of 5–10 neurons per one freeze-thawed cell [Hatten et al., 1984; Edmondson and Hatten, 1987; Gasser and Hatten, 1990]. The cultures were incubated overnight, and then photographed at 37°C on an Olympus IMT-2 inverted microscope with an Olympus OM-2 camera. Observations were made on the co-cultured cells that met the following three criteria: (1) the process of a bipolar RGLC was longer than 100 μM; (2) neurons, identified by their characteristic cell body, nucleus size and shape, were riding on this RGLC process; (3) these neurons were positioned close to the cell body of RGLC. Because neurons migrated along the straight RGLCs fibers, the images were acquired every 15 min during a period of 30–60 min. The averaged speed of neuronal migration during the experiment was then analyzed.

RESULTS

Astrocytes De-Differentiated Into RGLCs in Response to Freezing Treatment

After the freeze-thaw treatment, approximately $31.46 \pm 2.89\%$ of the total cells cultured in NB + B27 medium remained viable (surviving cells/total cells per dish, 24 h after plating). We called these surviving cells freeze-thawed cells. Morphological analysis was performed after freeze-thawed cells were cultured in serum-free NB + B27 medium for 3 days. In the freeze-thawed cells there were bipolar cells ($59.09 \pm 0.81\%$), multipolar astrocytes ($33.98 \pm 0.55\%$) and epithelial-like astrocytes ($6.43 \pm 0.36\%$) (Fig. 1C). The morphology of freeze-thawed cells was significantly different from that of purified astrocytes (Fig. 1A). The bipolar processes could be as long as 1000 μm and their terminals usually formed end-foot-like structures (Fig. 1D,E). These morphological features were similar to those of the regular radial glial cells during development and these bipolar-shaped freeze-thawed cells with long, thin and unbranched processes were thus termed as RGLCs.

To exclude the possibility that the morphological characteristics of RGLCs were caused by culture conditions, purified astrocytes were cultured in the same NB + B27 medium for 4 days. No notable difference was found between astrocytes cultured in normal serum-containing medium and in the serum-free medium, and no bipolar cell was found in either culture (Fig. 1A,B). The morphology of the RGLCs and that of the astrocytes cultured in serum-free medium were significantly different

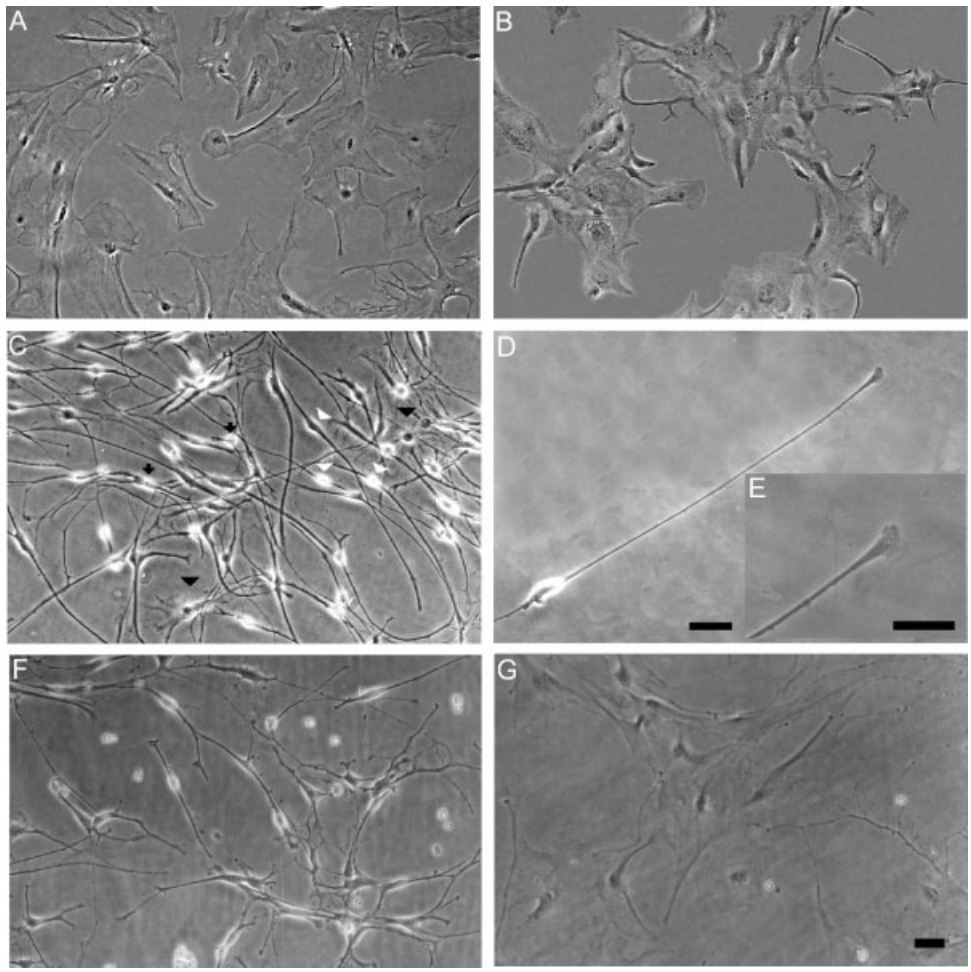

Fig. 1. The morphological difference between radial glial-like cells (RGLCs) and purified astrocytes. Phase-contrast image of purified astrocytes cultured in horse serum-contained medium is shown in **A**, purified astrocytes cultured 4 days in serum-free medium in **B** and the freeze-thawed cells in serum-free medium in **C**. In the freeze-thawed cells there were bipolar cells (indicated with white arrowhead), multipolar astrocytes (indicated with black arrow) and epithelial-like astrocytes (indicated with black arrowhead). The RGLCs with long, thin, unbranched process (**D**), and end-foot-like structure (**E**). The transformation of RGLCs to astrocytes. The medium of RGLCs at 9 *div* was changed into serum-free medium or 10% HS containing medium, respectively. The RGLCs in serum-free medium (**F**) and in 10% HS-containing medium (**G**) after 24 h medium change. Scale bar = 10 μm.

(Fig. 1B,C). When cultured in serum-free medium, RGLCs maintained their de-differentiated phenotype for up to 32 days. However in the presence of horse serum, the typical morphology of RGLCs disappeared within 24 h, and RGLCs rapidly transformed into astrocytes (Fig. 1F,G). We also tested the effect of DMSO, which was supplemented in the freezing medium, on astrocytes cultured in serum-free medium or in serum-containing medium, both of which containing different concentrations of DMSO as 0.1, 0.3, 1, 3, and 10%, respectively. We found that there were no significant difference on the morphology of astrocytes cultured in the medium with/without different concentration of DMSO (0.1, 0.3, 1, and 3%), and all astrocytes died in the presence of high concentration of DMSO (10%) (data not shown). These results demonstrate that the freeze-thaw treatment of astrocytes cause more than half of the surviving cells becoming RGLCs that morphologically resembled the radial glial cells in vivo.

RGLCs Were Immunoreactive to Nestin, Vimentin and GFAP

To investigate the immunocytochemical characteristics of RGLCs, we stained the freeze-thawed cells with specific antibodies. We found

that all cells in 9-day culture of freeze-thawed cells were immunoreactive to antibody against Nestin (Fig. 2C), a specific marker for immature precursor cells for neurons and glia [Voigt, 1989; Lendahl et al., 1990]. Purified astrocytes were Nestin-negative when cultured 9 days in serum-containing medium (Fig. 2A). However, when cultured 9 days in NB+B27 medium, purified astrocytes were Nestin-positive, although still maintaining their typical morphological features (Fig. 2B). In addition, RGLCs expressed Vimentin and GFAP, which are markers for radial glia and mature astrocytes (Fig. 2F,I). Astrocytes cultured both in NB+B27 and in culture medium containing horse serum were Vimentin and GFAP positive (Fig. 2D,E, G,H), which is consistent with previous reports [Schnitzer et al., 1981; Pixley and de Vellis, 1984].

The expression of molecular markers in both freeze-thawed cells (including RGLCs, as described above) and astrocytes were confirmed by Western blot analysis. As shown in Figure 3A, after 9 days in vitro (*div*), the freeze-thawed cells expressed Nestin, GFAP and Vimentin. Astrocytes grown 9 days in serum-free medium also expressed Nestin in addition to GFAP and Vimentin, while cultured 9 days in the medium containing 10% horse serum astrocytes only expressed GFAP and Vimentin.

It has been reported that type 1 astrocytes, but not type 2, expressed nestin mRNA and protein [Gallo and Armstrong, 1995]. In our data, purified astrocytes were Nestin-negative

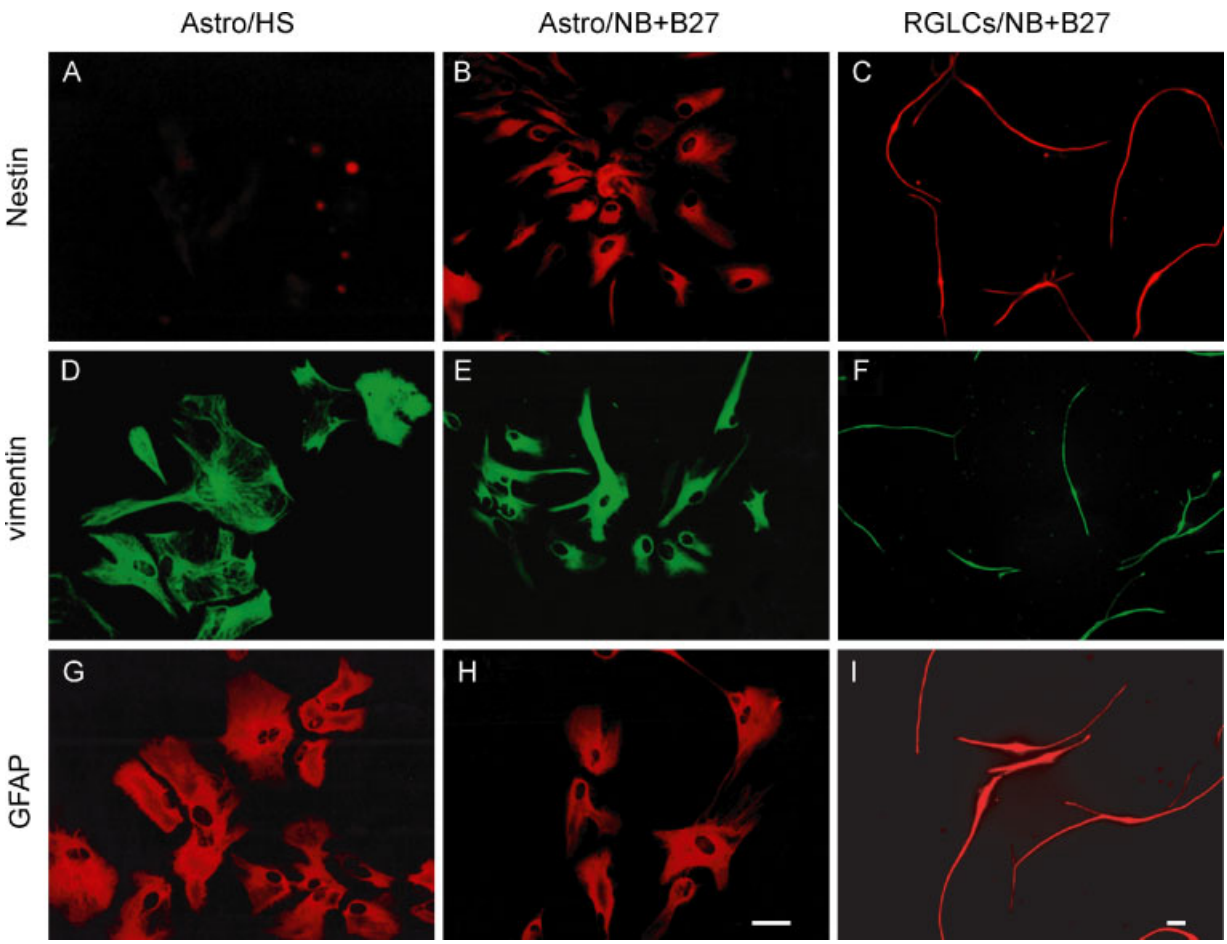

Fig. 2. The immunocytochemical difference between RGLCs and purified astrocytes after 9 days in vitro. Immunoreactivities of astrocytes and RGLCs to Nestin are shown in **A**–**C**, to Vimentin in **D**–**F** and to GFAP in **G**–**I**, respectively. The RGLCs were Nestin, Vimentin and GFAP immunopositive, the purified astrocytes cultured in horse serum-contained medium (Astro/HS) were Nestin negative but Vimentin and GFAP positive. The purified astrocytes cultured in serum-free medium (Astro/NB + B27) were Nestin, Vimentin and GFAP positive, which was the same as RGLCs. The scale bar shown in H is also applied to A, B, D, E and G, and that in I is also used in C and F. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

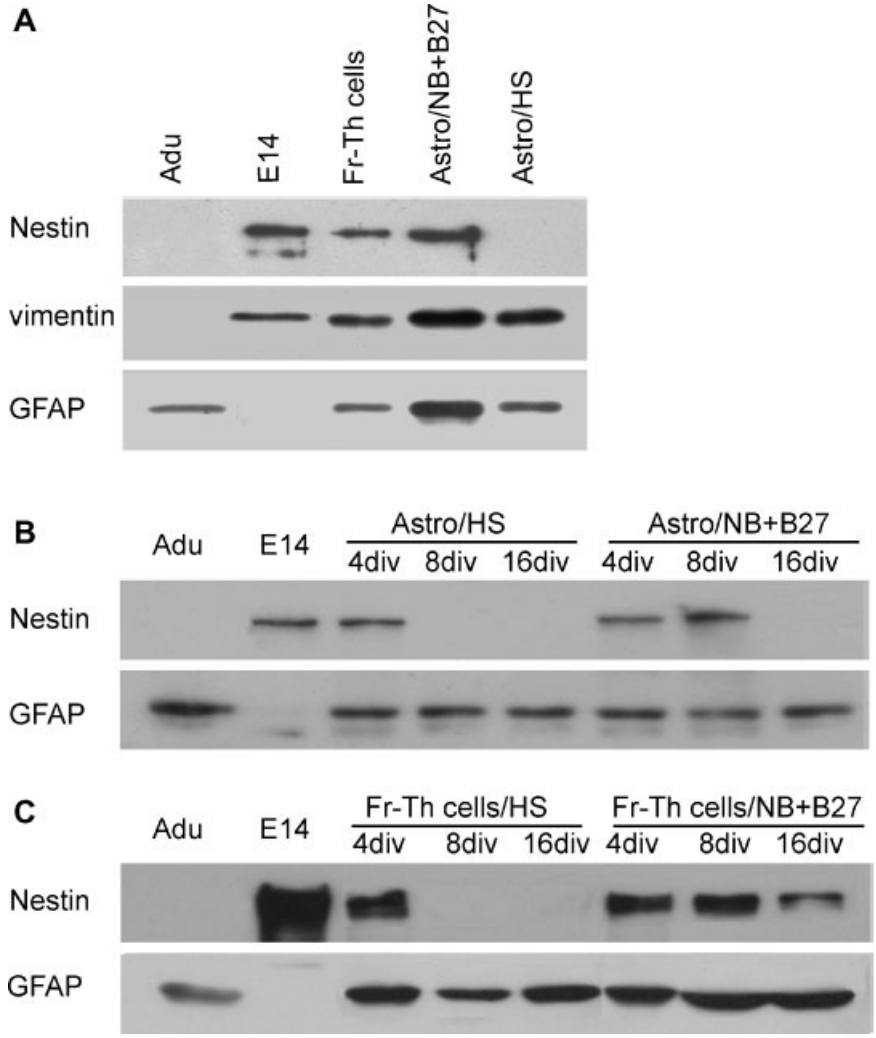

Fig. 3. Western immunoblotting showing expressions of Nestin and GFAP in freeze-thawed cells and purified astrocytes. **A**: Expression of Nestin in freeze-thawed cells and purified astrocytes. Purified astrocytes cultured in serum-contained medium (Astro/HS) were Nestin negative, but purified astrocytes cultured in serum-free medium (Astro/NB + B27) and the freeze-thawed cells (Fr-Th cells) were Nestin positive. **B**: Time course of Nestin expression in purified astrocytes. Nestin expression was detectable in 4 *div* astrocytes cultured in HS, and 4 *div*, 8 *div* astrocytes cultured in NB + B27 medium. **C**: Time course of Nestin expression in freeze-thawed cells. Nestin expression was detectable in 4 *div*, 8 *div*, and 16 *div* freeze-thawed cells cultured in serum-free medium (Fr-Th cells/NB + B27), but only in 4 *div* freeze-thawed cells cultured in serum-containing medium (Fr-Th cells/HS). Cerebellum extracts of adult SD rat (Adu) and cortex extracts of E14 rat (E14) were used as antibodies controls. GFAP expression was detectable in all purified astrocytes and the freeze-thawed cell cultures and used as loading control.

when cultured in serum-containing medium and were Nestin-positive when cultured in serum-free medium. Our hypothesis was that the Nestin expression in astrocytes was time dependent in different medium. To understand the time course expression of Nestin in astrocytes, the Nestin expression was examined at different time points in astrocytes and in freeze-thawed cells. We found that when grown in serum-containing medium, astrocytes expressed Nestin at 4 *div* but not at other time points such as 8 *div* and 16 *div*, while those grown in serum-free medium expressed Nestin until 8 *div* but not at 16 *div* (Fig. 3B). The freeze-thawed cells cultured in horse serum-containing medium expressed Nestin at 4 *div*, but not after 8 *div*, (when RGLCs had lost their typical morphology and transformed into astrocytes) whereas those grown in serum-free medium still expressed Nestin until 16 *div* (Fig. 3C). Taken together, these results suggest that RGLCs have Nestin expression, which is different from purified mature astrocytes.

RGLCs Were Immunoreactive to Pax6, a Marker for Radial Glia

To further elucidate the immunocytochemical characteristics of RGLCs, we stained freeze-thawed cells with a radial glia-specific marker Pax6 [Götz et al., 1998; Englund et al., 2005]. We found that all freeze-thawed cells were immunoreactive to antibody against Pax6 (Fig. 4C). Purified astrocytes growing in horse serum-

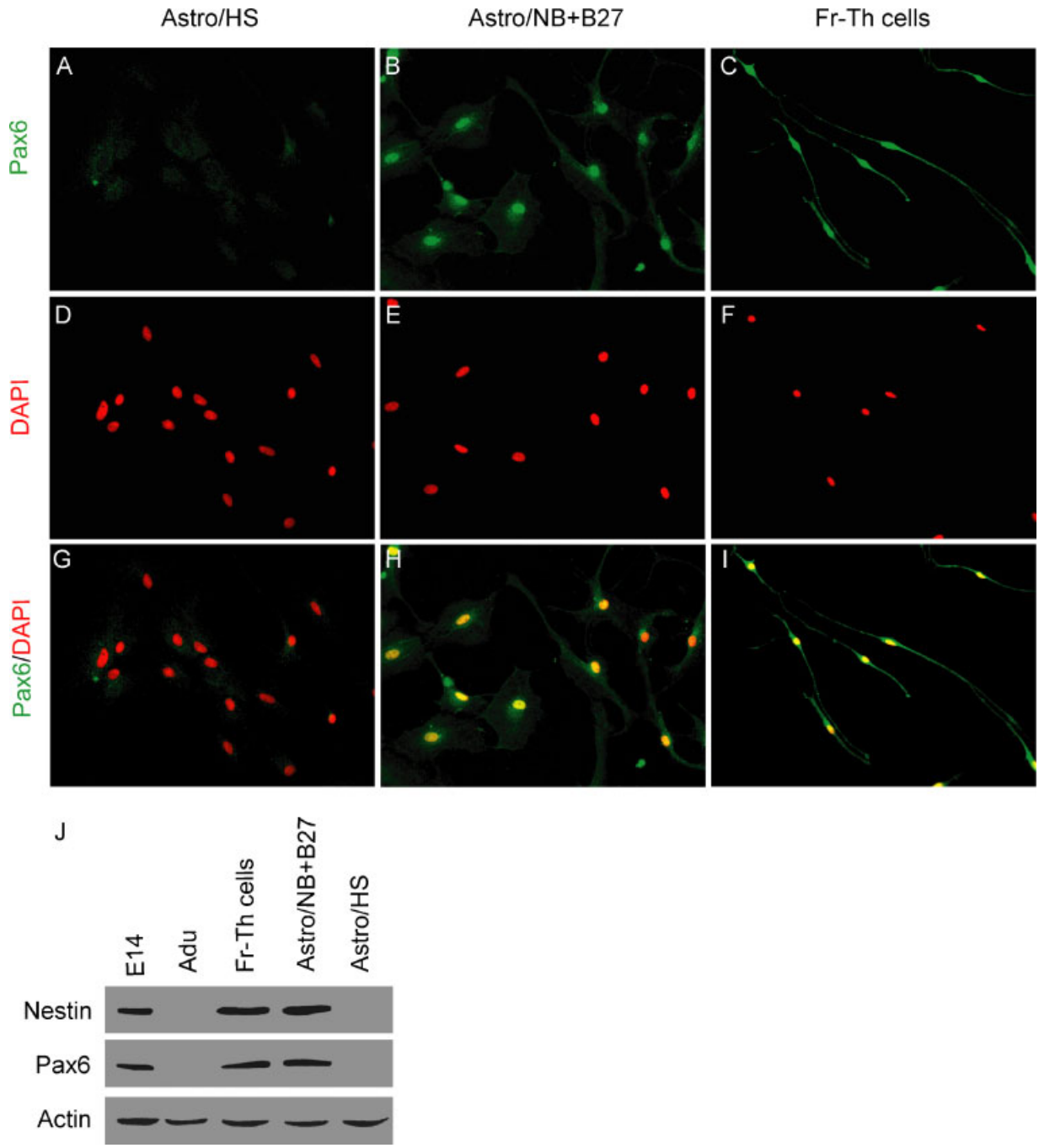

Fig. 4. Expression of Pax6 in RGLCs and purified astrocytes. Astrocytes cultured in 10% horse serum medium (Astro/HS), in serum-free medium (Astro/NB + B27) and freeze-thawed cells (Fr-Th cells) were stained with anti-Pax-6 antibody at 4 *div* (**A**–**C**), and with the nuclear dye 4′-6-Diamidino-2-phenylindole (DAPI), which was converted from blue to red for better visualization (**D**–**F**). The merged pictures are shown in **G**–**I**. **J**: Western immunoblotting of Pax6 in freeze-thawed cells, purified astrocytes cultured in NB+B27, and purified astrocytes cultured in HS-containing medium at 4 *div*. Cortex extracts from adult SD rats (Adu) and E14 rats (E14) were used as controls. Nestin and Actin immunoblot was used as control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

containing medium were Pax6-negative (Fig. 4A), but those cultured in NB+B27 medium were Pax6-positive (Fig. 4B). The expression of Pax6 in both freeze-thawed cells and astrocytes were confirmed by Western blot analysis. As shown in Figure 4J, after 9 days in vitro, the freeze-thawed cells and astrocytes grown in serum-free medium expressed Pax6 as well as Nestin, while astrocytes in medium containing 10% horse serum expressed neither Pax6 nor Nestin. Actin antibody was used as control. We didn't get any specific staining with the antibody against another radial glia-specific marker RC2 both in freeze-thawed cells and control astrocytes, due to the species specificity of the RC2 antibody we used.

To distinguish our RGLCs from oligodendrocytes and neurons, freeze-thawed cells were immunostained with oligodendrocyte marker O4, and neuronal markers neurofilament 160 and TuJ1. Astrocytes were used as control. Our data indicate that RGLCs and astrocytes were immunoreactively negative to neurofilament antibody NF160 and TuJ1 as well as to oligodendrocyte antibody O4 (data not shown). These results indicate that RGLCs have the immunocytochemical characteristics for radial glia (Nestin and Pax6) and astrocytes (Vimentin, GFAP), but not that for oligodendrocyte and neuron.

Embryonic Neurons Migrated on RGLC Processes

During the early stage of neural development, neonatal neurons migrate along the bipolar processes of radial glia to their final destinations [Rakic, 1972; Hatten and Liem, 1981; Hatten, 1984; Edmondson and Hatten, 1987; Gasser and Hatten, 1990]. Since the RGLCs derived from astrocytes exhibited similar morphology as the radial glia and expressed radial glia-specific antigens, it is reasonable to hypothesize that RGLCs would be able to mediate neuronal migration.

To test this possibility, dissociated neurons prepared from the cerebral cortex of E18 rat were co-cultured with 4-day old freeze-thawed cell cultures, and neurons were allowed to settle down overnight. We found that embryonic cortical neurons attached to and migrated along the processes of RGLCs. An example is shown in Figure 5A in which one neuron was migrating along a RGLC process toward its distal end. The migrating cells were identified as neurons by staining them with anti-MAP2 antibody (Fig. 5C). Quantitative analysis showed that the speed of neuronal migration varied from 1 to 60 μm/h, and the average speed was 26.26 ± 3.36 μm/h ($n = 20$). Most neurons (~65%) migrated at the speed of 10–30 μm/h. However, about 15% neurons migrated at the higher speed of 50–60 μm/h (Fig. 5B). In some cases there were two or three neurons migrating along one single RGLC process, they moved in the similar or sometimes in the opposite directions at different speeds (data not shown). As a control, dissociated embryonic cortical neurons were added to purified astrocyte cultures, and all neurons were found staying on top of astrocytes without any migration (Fig. 5D,E), which was consistent with previous reports [Hatten and Liem, 1981; Hatten, 1984]. These findings suggest that freeze-thawed cells derived from astrocytes recapture the biological function of radial glia in vivo in guiding the migration of neonatal neurons.

EGF Promoted RGLCs Proliferation

Epidermal growth factor (EGF) is a polypeptide mitogen for stem cells, neuronal precursor cells, radial glial cells and astrocytes in vitro [Anchan et al., 1991; Labourdette and Sensenbrenner, 1995; Gregg and Weiss, 2003]. To determine whether RGLCs could proliferate as radial glia does in vivo, we examined the proliferative ability of RGLCs in response to EGF. For this, freeze-thawed cells were grown in serum-free medium containing different concentrations of EGF. BrdU (10 μM) was used to label cells undergoing mitosis. After 3 days of EGF treatment, the cultured cells were fixed and stained with antibodies against BrdU and Nestin, and the number of double-stained bipolar RGLCs in 20 fields randomly chosen across the 22 mm square coverslip was counted under a fluorescence microscope (Fig. 6A). The results of three independent experiments were analyzed, and for each experiment, three or four dishes of cultured cells were used for each concentration of EGF tested. The statistical results indicate that the number of dividing RGLCs increase markedly after 10 or 30 ng/ml EGF treatment (Fig. 6B), suggesting that EGF treatment markedly promotes the proliferation of RGLCs, as it does of radial glial cells in the developing brain.

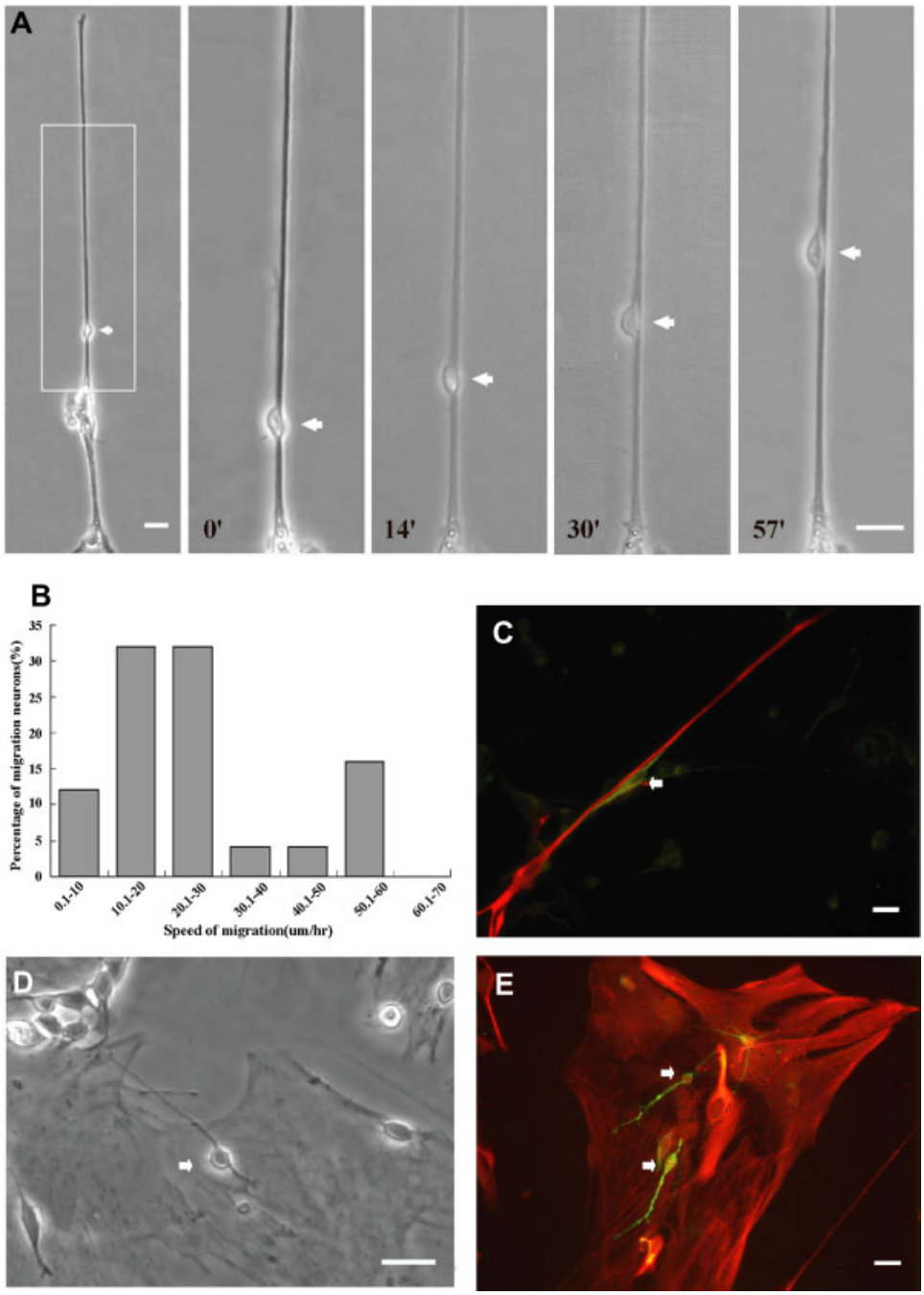

Fig. 5. Role of RGLCs in neuronal migration. **A**: Time-lapse images show the migration of an E18 cortical neuron (indicated by white arrow) along a RGLC process. **B**: Distribution of the migrating speed of neurons (μm/h) along the RGLC processes in vitro. E18 cerebral cortical neurons migrated along RGLC processes at an average speed of 26.26 ± 3.36 μm/h (Mean ± SEM, n = 25). **C**: Co-cultured RGLCs and migrating neurons stained with MAP2 and Nestin antibody. **D**: Co-culture of astrocytes and neurons. Neurons co-cultured with purified astrocytes did not migrate. **E**: Astrocytes and co-cultured neurons double-stained with MAP2 and Nestin antibody. White arrows indicate neurons in C–E. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

In mammals the mechanisms of transformation from radial glial cells to astrocytes are still far from clear. It has been shown that mature astrocytes could be induced to de-differentiate into Nestin-expressing phenotypes by some soluble signals or freezing injury stimuli [Hatten, 1985; Rosen et al., 1992; Rosen et al., 1994; Hunter and Hatten, 1995; Soriano et al., 1997;

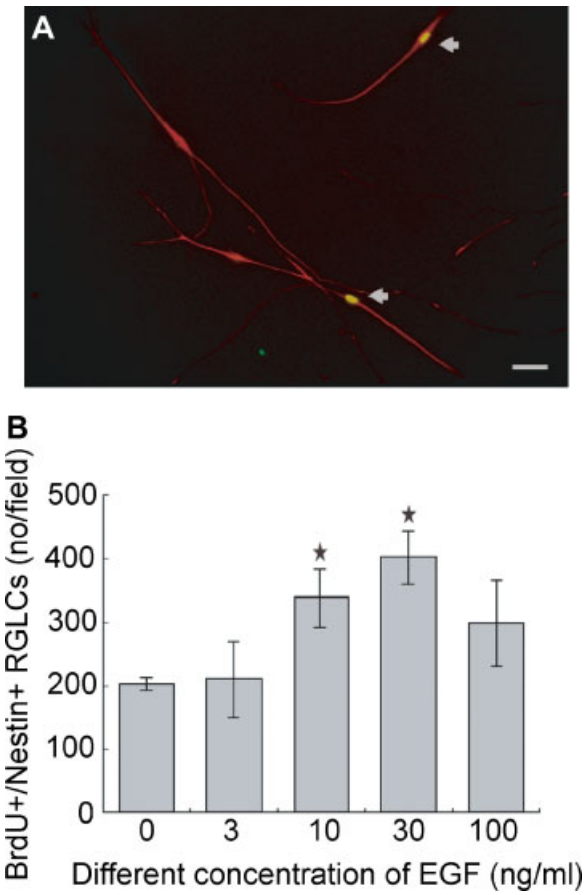

Fig. 6. The proliferative promotion of RGLCs by epidermal growth factor (EGF). **A**: Bipolar RGLCs double-stained with mouse anti-BrdU (FITC-conjugated) and mouse anti-Nestin (Rhodamine-conjugated) antibody. White arrows indicate the double-stained RGLCs. **B**: Quantification of the BrdU and Nestin double-stained bipolar RGLCs cultured in media containing different concentrations of EGF. EGF of 10 and 30 ng/ml concentration significantly increased the proliferation of RGLCs. Data represent mean ± SEM values of three independent experiments. (N = 3, n = 4, $*P < 0.01$, Students *t*-test). Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Leavitt et al., 1999; Zhou et al., 2001; Shibuya et al., 2002; Huttmann et al., 2003]. It is also reported that astrocytes may act as neuronal stem cells in adult mammalian brain [Doetsch et al., 1999; Lim and Alvarez-Buylla, 1999; Seri et al., 2001; Mori et al., 2005]. Resent reports have shown that the progenitors of the granule cells express astrocytic and radial glial markers, proliferate and differentiate into neurons mainly in the hilus during the early postnatal period [Imura et al., 2003, 2006; Namba et al., 2005]. In this study, we introduce a physical freeze-thaw method that directly induces mature astrocytes to de-differentiate into RGLCs. RGLCs have the expression of markers for radial glia including Nestin and Pax6, and astrocyte markers, the GFAP and Vimentin, but not those of neurons (NF160 and TuJ1) and oligodendrocytes (O4). Cortical neurons, when co-cultured with RGLCs, migrate along the processes of RGLCs at an average speed of 26.26 ± 3.36 μm/h. Moreover, the proliferation of RGLCs is significantly promoted by EGF at the concentration of 10–30 ng/ml. And these astrocyte-derived RGLCs could be transformed again into astrocytes. This study indicates that mature astrocytes have its own multipotent to de-differentiate into radial glial cells, and that RGLCs represent a rejuvenated member of the astroglia lineage. Thus, freeze-thaw treatment of astrocytes may provide a good in vitro model to explore the mechanisms of re-differentiation from the de-differentiated of RGLCs, which would provide insight into the molecular mechanisms of radial glia differentiation in vivo.

In the present study astrocytes express Nestin transiently in the beginning several days of culture, as shown in the results of our Western blot analysis, which is consistent with previous reports that type 1 astrocytes, but not type 2, expressed nestin mRNA and protein [Gallo and Armstrong, 1995]. It is important to note that astrocytes express Nestin at 4 *div*, but not at 8 *div* and 16 *div* when grown in serum-containing medium, while those grown in serum-free medium expressed Nestin until 8 *div* but not at 16 *div*. A possible address of the results is that the stimulation from passage would induce the purified astrocytes active, and then they will have a transient expression of Nestin.

It has been reported that astroglial receptor systems, in combination with glial geometry, promote CNS neural migration [Fishman and Hatten, 1993]. These authors also show that CNS neurons could migrate on glass fibers coated with astroglial membranes at a rate of 5.5 μm/h and with the ECM component laminin at 7.2 μm/h. These rates are slower than neuronal migration on living astroglia (11.79 ± 0.718 μm/h) [Fishell and Hatten, 1991], and much slower than neuronal migration on RGLC fibers in our system. Our quantitative analysis shows that the average speed of neuronal migration is 26.26 ± 3.36 μm/h (n = 20), suggesting that RGLCs seem to have much better ability to lead neuronal migration than that of astroglia.

In conclusion, on the basis of morphological and immunocytochemical features, physiological function in guiding neuronal migration and proliferative property, we believe the astrocyte-derived RGLCs induced by freeze-thaw treatment are de-differentiated, rejuvenated immature RGLCs. They still maintain some immunocytochemical features of astrocytes but do not express antigens specific to neurons and oligodendrocytes. The freeze-thaw treatment of astrocytes provides a simple way of producing RGLCs and might also be used for investigating mechanisms of differentiation and de-differentiation.

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