Induction of mesenchymal stem cell-like transformation in rat primary glial cells using hypoxia, mild hypothermia and growth factors

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Abstract. The transformation of rat primary glial cells into mesenchymal stem cells (MSCs) is intriguing as more seed cells can be harvested. The present study aimed to evaluate the effects of growth factors, hypoxia and mild hypothermia on the transformation of primary glial cells into MSCs. Rat primary glial cells were induced to differentiate by treatment with hypoxia, mild hypothermia and basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Immunohistochemistry and western blotting were then used to determine the expression levels of glial fibrillary acidic protein (GFAP), nestin, musashi-1, neuron specific enolase (NSE) and neuronal nuclei (NeuN), in each treatment group. bFGF and EGF increased the proportion of CD44$^+$ and CD105$^+$ cells, while anaerobic mild hypothermia increased the proportion of CD90$^+$ cells. The combination of bFGF and EGF, and anaerobic mild hypothermia increased the proportion of CD29$^+$ cells and significantly decreased the proportions of GFAP$^+$ cells and NSE$^+$ cells. Treatment of primary glial cells with bFGF and EGF increased the expression levels of nestin, Musashi-1, NSE and NeuN. Anaerobic mild hypothermia increased the expression levels of Musashi-1 and decreased the expression levels of NSE and NeuN in glial cells. The results of the present study demonstrated that bFGF, EGF and anaerobic mild hypothermia treatments may promote the transformation of glial cells into MSC-like cells, and that the combination of these two treatments may have the optimal effect.

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Introduction

Cerebral ischemia and the hypoxia that arises from this condition are the direct causes of stroke and other cerebral diseases (1). Over recent years, mild hypothermia therapy and stem cell therapy have been introduced as a treatment option for patients suffering from these conditions (2). For example, randomized, double-blind, Phase III clinical trials have been carried out by two European groups to investigate the potential effects of hypothermia therapy over 6 months, demonstrating that treatment involving mild hypothermia resulted in good neurological recovery (3,4). Additionally, experiments of cerebral infarction in a rat model demonstrated that mild hypothermia treatment can decrease the area of infarction and achieve good levels of recovery in terms of nerve function (5). In addition, there have been several successful cases of stem cell therapy and mild hypothermia combined with stem cell therapy for stroke in infants (6-8).

A previous study has suggested that basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) can promote the transformation of bone marrow-derived mesenchymal stem cells (MSCs) or adipose MSCs into neural cells (9). Glial cells are primary cells that can be isolated and cultured from the cerebral cortex; these represent a heterogeneous mixture of cell types that have the ability to proliferate and differentiate (10). The development of new induction methods to improve the efficiency of glial cell transformation into neural stem cells, MSCs would allow us to produce greater numbers of specific cells for neural repair treatments.

Previous studies injected the brains of rats with MSCs and achieved good levels of repair in the central nervous system (11,12). In addition, a range of efficient methods have now been described to facilitate the acquisition of mesenchymal stem cells; these cells are important as they have the ability to differentiate into neural cells (13,14). However, a previous study investigated the transformation of rat primary glial cells into MSCs (15). The present study hypothesized that treatment with mild hypothermia or growth factors may promote the differentiation of glial cells into MSCs, thus supplementing nerve cells that died due to hypoxia. The aim of the present study was to use a rat model to investigate the

effects of cytokine induction and anaerobic mild hypothermia on the differentiation of primary glial cells.

Materials and methods

Primary isolation of rat glial cells. All animal procedures were approved by the Ethics Committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University (approval no. 2017BBG70066; Nanchang, China). All experiments involving animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (16). A total of ten specific pathogen-free neonatal Sprague-Dawley rats (1-3 days post-natal; 6-8 g; male:female, 1:1) were provided by the Animal Center of Jiangxi Provincial People's Hospital Affiliated to Nanchang University. The animals were housed in a specific pathogen-free environment at a temperature of $23\pm2°C$, with 45-65% humidity, 12-h light/dark cycles, and free access to food and water. The animals were sacrificed by cervical dislocation and immersed in 75% alcohol for 2-3 min on an ultra-clean table. D-Hank's solution (Gibco; Thermo Fisher Scientific, Inc.), containing a mixture of penicillin (10,000 U/ml) and streptomycin (10 mg/ml), was added to four petri dishes (labeled 1-4). First, the sterilized rats were transferred to dish number 1. Their heads were removed and placed in dish number 2. The skulls were then separated at the brainstem, and the brain was quickly transferred to dish number 3. The cortex was then freed and the meninges on the cortex were fully removed with tweezers and transferred to dish number 4. The cortical tissue was then rinsed carefully in D-Hank's solution and then transferred to a vial containing penicillin (10,000 U/ml) and D-Hank's solution. The tissue suspension was then cut into tissue fragments that were ~1 mm^3 in size and then transferred to a centrifuge tube. An equivalent volume of 0.25% trypsin was added to the centrifuge tube, the contents were mixed carefully and then allowed to digest at 37°C for 15 min. The digestion was terminated by adding three equivalent volumes of complete culture medium including DF12 (cat. no. 1859228; Gibco; Thermo Fisher Scientific, Inc.) and 20% fetal bovine serum (FBS; cat. no. 04-007-1A; Biological Industries). The cell suspension was then mixed by gentle blowing with a suction tube and filtered with a 200-mesh, and the isolated cells were centrifuged at 850 x g for 3 min at room temperature. The supernatant was discarded and the cell precipitation was suspended in DF12 medium with 20% FBS. Finally, the cells were cultured with 5% CO_2 at 37°C until the first passage prior to subsequent experimentation.

Hypoxia, mild hypothermia and growth factor treatments. Cells at 70% confluency were divided into eight groups for culture in normal culture medium (DF12 with 20% FBS) as follows: Control-normal (astrocytes cultured at 37°C with 5% CO_2); control-hypothermia (33°C, 5% CO_2, 12 h); control-hypoxia (37°C, 95% N_2, 5% CO_2, 12 h); control-hypothermia + hypoxia; bFGF + EGF-normal; bFGF + EGF-hypothermia (33°C, 5% CO_2, 12 h); bFGF + EGF-hypoxia (37°C, 95% N_2, 5% CO_2, 12 h); and bFGF + EGF-hypothermia + hypoxia. Cells in the bFGF + EGF groups received 10 ng/ml bFGF (cat. no. P1020-500; Novoprotein) and 20 ng/ml EGF (cat. no. REGFP-05011; Cyagen Biosciences, Inc.) for 7 days.

Hypothermia involved a reduction in the culture temperature from 37°C to 33°C for 12 h. To mimic a hypoxic environment, cells were cultured in 95% N_2 + 5% CO_2 for 12 h. The cells were treated for a total of 7 days and collected for subsequent experiments.

Immunocytochemical staining. Cells were fixed in 4% para-formaldehyde at room temperature for 1 h. Following antigen retrieval by heating in a microwave in Tris/EDTA (pH 9.0), 3% hydrogen peroxide was used to block endogenous peroxidase activity for 15 min at room temperature. After non-specific blocking in 5% BSA (HyClone; GE Healthcare Life Science) at room temperature for 2 h, the cells were incubated overnight at 4°C with primary antibodies against nestin (cat. no. OM264981; OmniAb; 1:250), glial fibrillary acidic protein (GFAP; cat. no. ab33922; Abcam; 1:500), neuronal nuclei (NeuN; cat. no. ab177487; Abcam; 1:500), Musashi-1 (cat. no. bs-20241r; BIOSS; 1:250) and neuron specific enolase (NSE; cat. no. bs-10445r; BIOSS; 1:500). The following morning, the cells were washed with PBS and then incubated with an HRP-conjugated goat anti-rabbit IgG H&L secondary antibody (cat. no. ab6721; Abcam; 1:500) at 37°C for 30 min. Subsequently, the cells were washed with PBS, and stained with 3,3'-diaminobenzidine for 5-10 min at room temperature. Cells were then re-washed in PBS for 1 min and stained with hematoxylin for 3 min at room temperature; this staining was then converted to a blue color using ethanol hydrochloride. Finally, cells were observed by light microscopy. ImagePro Plus (version 6.0; Media Cybernetics, Inc.) was used to measure the mean optical density of positive cells within three randomly selected fields of view at high magnification (x200).

Flow cytometry. Instrument parameters were adjusted by fluorescence homology control. Cells were washed twice with PBS and digested with 0.25% trypsin containing 0.02% EDTA. The cell suspension was then transferred into a 10-ml centrifuge tube. Cells were collected from each treatment group by centrifugation at 850 x g for 3 min at room temperature. Next, 1 ml of PBS solution was added to each tube and centrifuged at 1,700 x g for 1 min at room temperature. The supernatant was discarded, and $5x10^5$ cells were collected in 300 ml PBS. Subsequently, 300 μl 2X binding buffer and 5 μl of the following antibodies were added to each tube: Mouse anti-CD90-FITC (cat. no. 561973; BD Biosciences; 1:100), mouse anti-CD29-PE (cat. no. 102207; BioLegend, Inc.; 1:100), mouse anti-CD44-PE-cy7 (cat. no. ab4679; Abcam; 1:100) and mouse anti-CD105-APC (cat. no. 17-1051-80; eBioscience; Thermo Fisher Scientific, Inc.; 1:100). The tubes were mixed carefully and incubated at room temperature in the dark for 10 min. Multichannel fluorescence counting was then performed on a FACSCalibur flow cytometer (BD Biosciences). The combinations of CD105/CD90 and CD29/CD44 were used in the flow cytometry experiments. Data were analyzed using FlowJo software (version 7.6; FlowJo, LLC).

Western blotting. Cells from each treatment group were mixed with RIPA solution (Beyotime Institute of Biotechnology) and incubated at 4°C for 30 min to create a lysed suspension containing protein extract. The extracts were then centrifuged

at 8,500 x g for 10 min at 4°C; the supernatant, containing the total protein extract, was retained for analysis. Next, a bicinchoninic acid kit (Beyotime Institute of Biotechnology) was used to determine the concentration of each protein extract. Proteins (20 μg) were then separated by 10% SDS-PAGE, and then transferred to PVDF membranes. After blocking in 5% skimmed milk at room temperature for 2 h, membranes were then incubated overnight at 4°C with a range of primary antibodies, including: Nestin (cat. no. OM264981; OMNIAB; 1:250), GFAP (cat. no. ab33922; Abcam; 1:500), NeuN (cat. no. ab177487; Abcam; 1:500), Musashi-1 (cat. no. bs-20241r: BIOSS; 1:250), NSE (cat. no. bs-10445r; BIOSS; 1:500) and GAPDH (cat. no. ab8245; Abcam; 1:1,000). The following morning, the membranes were washed with PBS and incubated with an HRP goat anti-rabbit IgG H&L secondary antibody (cat. no. ab6721; Abcam; 1:500) or HRP goat anti-mouse IgG H&L secondary antibody (cat. no. ab6789; Abcam; 1:500) at room temperature for 1-2 h. The membranes were then developed using ECL exposure solution (cat. no. SW2010-1; Beijing Solarbio Science & Technology Co., Ltd.). Quantity one software (version 4.6; Bio-Rad Laboratories, Inc.) was used to analyze the gray value of specific bands of interest.

Statistical analysis. Data are presented as the mean ± SD of six repeats. Comparisons among multiple groups were analyzed using two-way ANOVA followed by a Bonferroni post-hoc test. All data were analyzed using SPSS (version 19.0; IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference

Results

Glial cell identification. Data relating to the immunohistochemical detection of GFAP are shown in Fig. 1. Primary cultured glial cells expressed GFAP, indicating that these cells exhibited low levels of differentiation. Some of these cells expressed strong immunofluorescence for GFAP; these cells were polygonal in shape and were bifurcated, thus indicating that they were astrocytes. However, ~40% cells in the staining were GFAP- cells, which were small in size. These cells may not be in healthy conditions.

Identification of differentiation clusters by flow cytometry. Flow cytometry was used to detect clusters of differentiated cells (CD44, CD29, CD90 and CD105) associated with MSCs. Under normal conditions, bFGF and EGF treatment increased the proportion of CD105$^+$ cells from <1 to ~30% ($P < 0.05$; Figs. 2 and 3A), and also significantly increased the proportion of CD44$^+$ and CD90$^+$ cells compared with the control group ($P < 0.05$; Figs. 2, 3B and C). However, the proportion of CD29$^+$ cells significantly decreased from ~60 to ~40% following bFGF and EGF treatment compared with the control group under normal conditions ($P < 0.05$; Figs. 2 and 3D). The combined treatment of anaerobic mild hypothermia, bFGF and EGF led to an increase in the proportion of CD29$^+$ cells to ~60% compared with the control group ($P < 0.05$; Figs. 2 and 3D). Additionally, anaerobic mild hypothermia treatment increased the proportion of CD44$^+$, CD90$^+$ and CD105$^+$ cells compared with the normal group ($P < 0.05$; Figs. 2 and 3A-C), although anaerobic mild
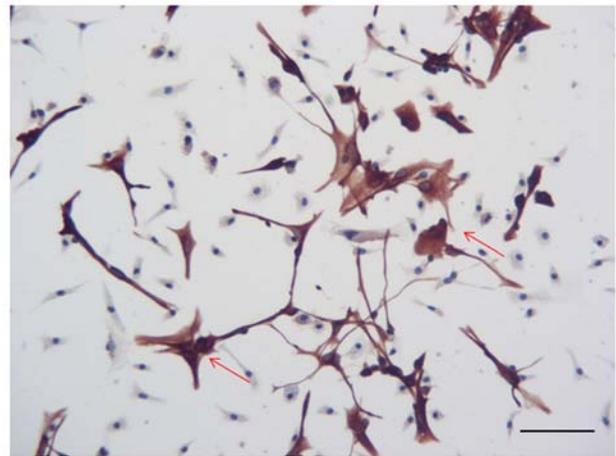
Figure 1. Primary cultured glial cells express glial fibrillary acidic protein. Immunocytochemical methods were used to successfully identify astrocytes (marked by red arrows). Blue color indicates nuclei stained by hematoxylin. Scale bar, 20 μm.

hypothermia treatment with bFGF and EGF did not show a synergistic effect with regards to increasing the proportion of CD44$^+$ and CD90$^+$ clusters compared with the normal group ($P > 0.05$; Figs. 2 and 3A-C).

Growth factor and anaerobic mild hypothermia treatment decreases the expression levels of NSE, NeuN and GFAP, but promotes the expression levels of nestin and Musashi-1. Cells from the control-normal group and the bFGF + EGF-hypoxia + hypothermia group were tested by immunocytochemistry to determine the expression levels of a range of marker genes that are specific for neural cells, including GFAP, nestin, musashi-1, NSE and NeuN (Fig. 4). Compared with normal cultured glial cells, cells that were treated with anaerobic mild hypothermia, bFGF and EGF demonstrated a decrease in the proportions of GFAP$^+$, NSE$^+$ and NeuN$^+$ cells, but an increase in the proportions of Musashi-1$^+$ and Nestin$^+$ cells; additionally, these cells tended to grow flat on the wall of culture dishes with fewer clusters (Fig. 4).

Western blotting was performed to detect specific cellular markers. Growth factor treatment (bFGF and EGF) led to an increase in the expression levels of nestin and Musashi-1, and a decrease in the expression levels of NSE and NeuN under normal conditions compared with control cells (Figs. 5 and 6). Anaerobic mild hypothermia treatment increased the expression levels of Musashi-1, but decreased the expression levels of NSE and NeuN in glial cells compared with the normal group. Additionally, anaerobic mild hypothermia treatment significantly decreased GFAP expression compared with the control-normal group ($P < 0.05$; Figs. 5 and 6). Additionally, the combination of growth factors and anaerobic mild hypothermia treatments had synergistic effects on the inhibition of GFAP when compared with the bFGF + EGF + normal group ($P < 0.05$; Figs. 5 and 6).

Discussion

According to the classical biological view, cell differentiation is unidirectional; in normal tissues, stem cells can differentiate
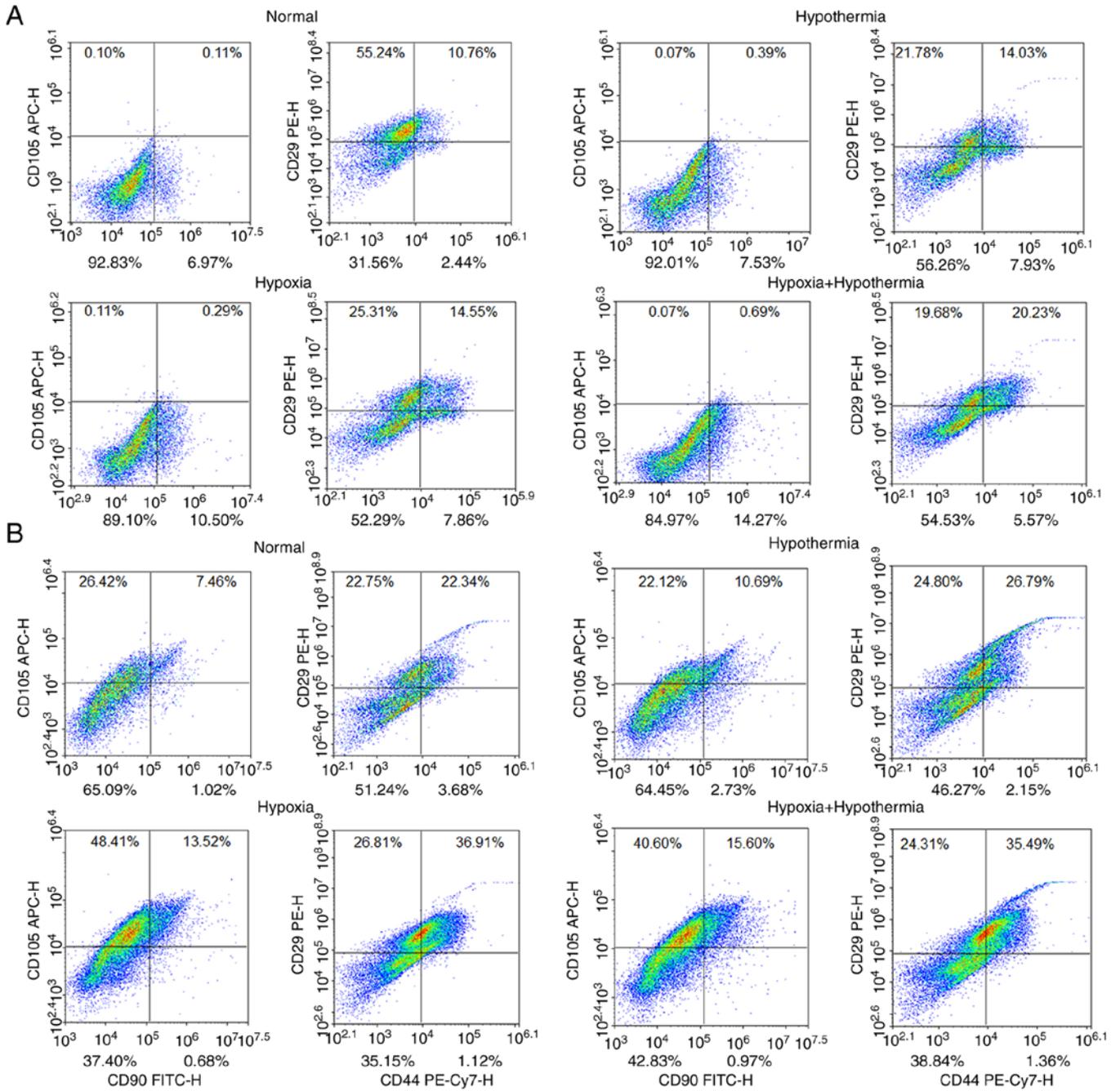
Figure 2. Identification of CD molecules by flow cytometry. Representative flow cytometry images of CD molecules (CD44, CD29, CD90 and CD105). (A) Control group. (B) bFGF + EGF group. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

into a range of different cell types, such as neurons and glial cells (4). Although cells can be induced to differentiate into stem cells by a range of artificial techniques (17), it appears that under normal physiological conditions, differentiated cells cannot revert back to stem cells. However, this view has been challenged. A previous study found that glial cells in dental pulp can undergo a form of 'reverse differentiation' and revert back into stem cells (15). This discovery not only challenged the traditional concept, but also created a new option for the treatment of disease. The current study identified differences between growth factor treatment and mild hypothermia treatment with regards to the induction of glial cell differentiation, and that the combination of growth factor

treatment and mild hypothermia treatment could improve the proportion of stem cell-like cells derived from a glial cell population.

Neuronal lineage markers and CD molecules were selected as indicators to evaluate the differentiation of glial cells under a range of different treatments involving growth factors, anaerobic conditions and mild hypothermic conditions. Nestin is known to be predominantly expressed in neural stem cells, but not in mature neurons; therefore, nestin is an effective marker for neural stem cells (18). Neuroblasts (neuroprogenitors) are monopotent stem cells with a certain potential for differentiation; these cells differentiate mainly into neurons, astrocytes and oligodendrocytes (19). GFAP,
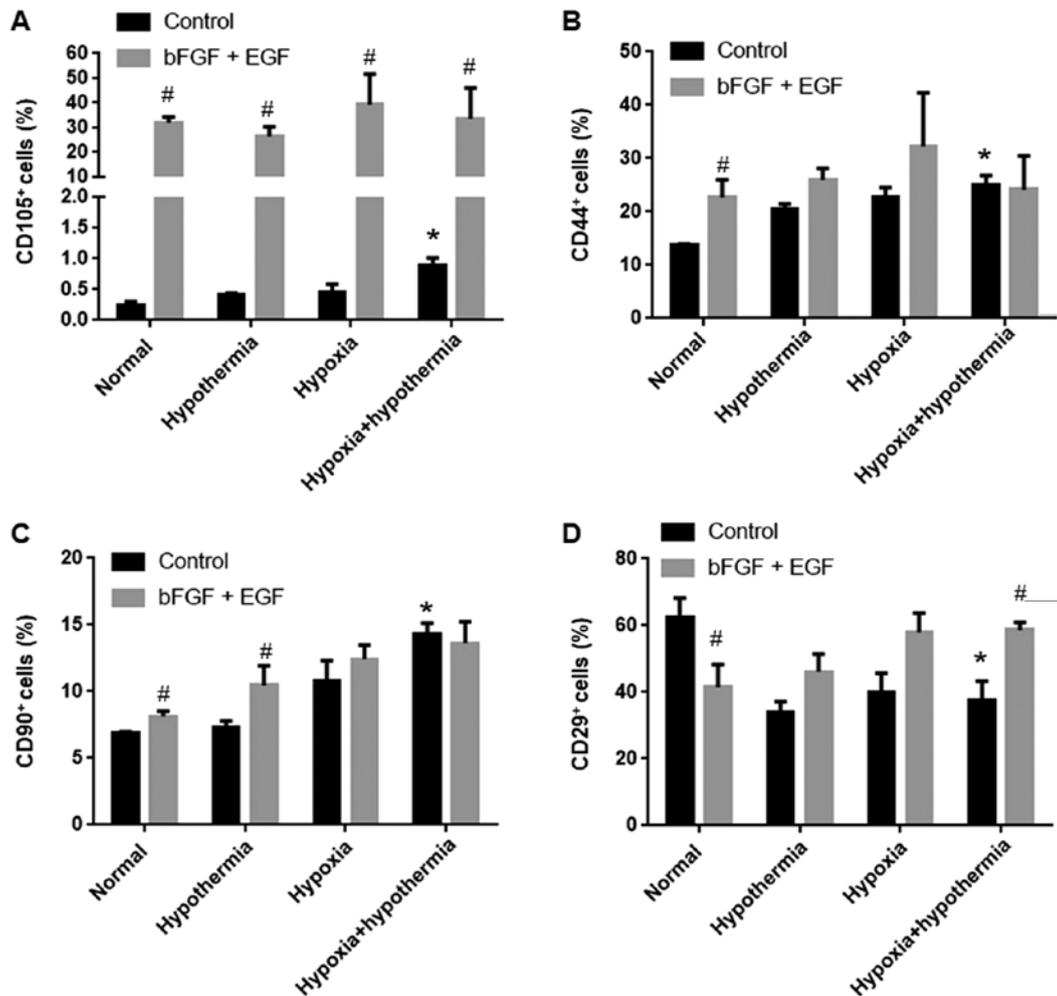
Figure 3. Quantification of the relative proportions of cells positive for CD44, CD29, CD90 and CD105. (A) CD105, (B) CD44, (C) CD90 and (D) CD29. [*]P<0.05 vs. control-normal; [#]P<0.05 vs. corresponding control. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

an established marker for neuroblasts, is also expressed in astrocytes (20) and is therefore commonly used to identify neuroblasts containing nestin. Musashi-1, an RNA-binding protein, is mainly expressed in mitotic neural stem cells and is therefore used to identify neural stem cells and neuroblasts (21). NeuN antigen is a nucleoprotein that serves a vital role in neurons (22); the positive expression of NeuN antigen indicates that neurons are no longer undergoing mitotic events (23). NSE is a cytoplasmic protein that is mainly expressed in mature neurons and also represents an effective marker for differentiated neurons (24). The detection of CD molecules is an effective method with which to identify MSCs (25). According to the International Society for Cellular Therapy, the simplest identification criterion for human MSCs is the presence of adherent human cells that exhibit positivity for CD90/Thy1 and CD105/Endoglin, as determined by flow cytometry (26,27). However, this simple criterion cannot be readily applied to the CDs of non-human MSCs. For example, MSCs from mouse adipose tissue can be CD105^{-}, and MSCs from rat bone marrow can be CD90^{-} (28). Therefore, the identification of mouse-derived MSCs should also consider positivity for CD29 and CD44 (29,30).

The indicators selected for the present study provide information from two different perspectives. One perspective is to reflect the growth of MSCs in the nervous system by detecting the positivity of MSC transforming clusters on the surface of glial cells in response to different treatments. The other perspective is to measure the expression levels of genetic and protein markers in glial cells in response to different treatment conditions. The central nervous system also contains MSCs; when a large number of neural cells die, MSCs proliferate and then differentiate to help repair the nervous system injury (31). This is the theoretical basis of stem cell therapy, in which MSCs are injected into the brain. Growth factor induction is a common and effective way of promoting this proliferation and to increase the proportion of MSCs produced (29). In order to identify MSCs in an accurate manner, it is important to maintain a high ratio of positivity in specific CD clusters (32). In the present study, CD105^{+} cells accounted for <0.5% of all glial cells in the normal state; this increased to ~1% after hypoxia and mild hypothermia treatments, thus indicating that mild hypothermia treatments can slightly increase the proportion of MSCs after hypoxia. Additionally, the present study demonstrated that this treatment increased the proportions of cells that were positive for CD105 and CD90 (to 30 and 20%, respectively), although the proportion of cells that were positive for CD44 and CD90 did not
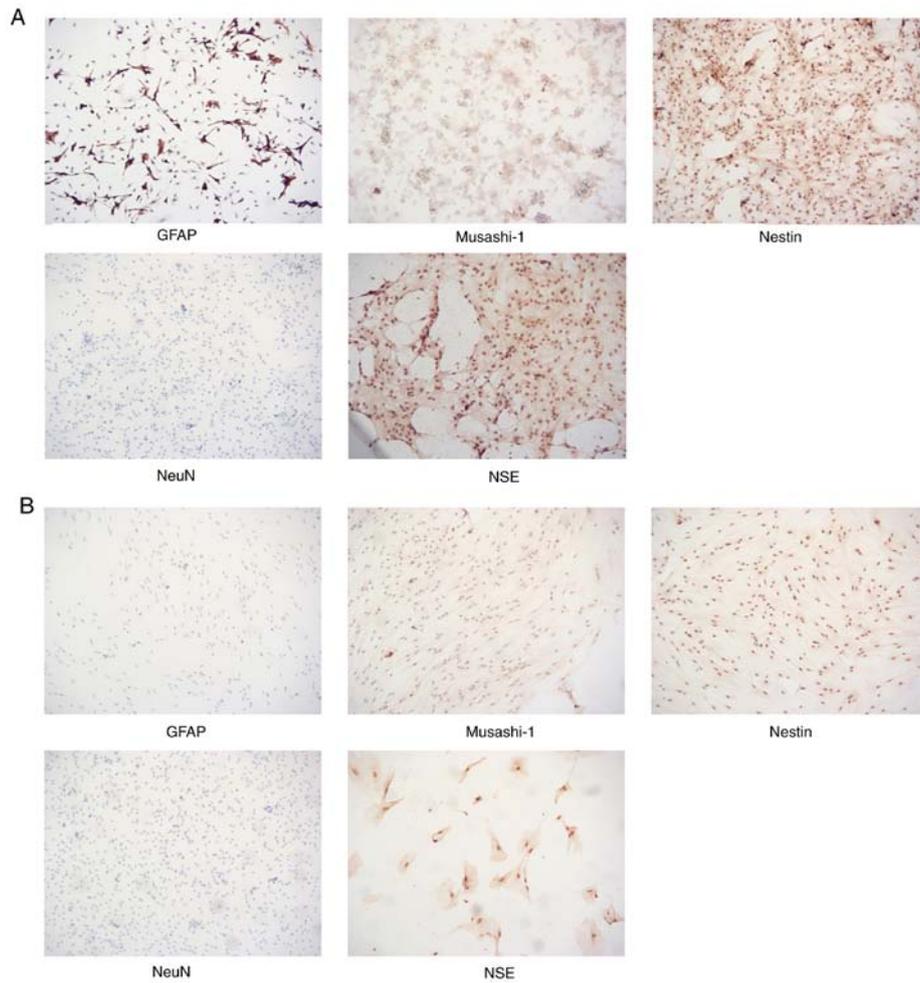
Figure 4. Immunocytochemical detection of cellular markers. (A) Control-normal group, (B) bFGF + EGF-hypoxia + hypothermia group. The target protein is indicated by brown staining. Magnification, x100. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; NSE, neuron specific enolase; NeuN, neuronal nuclei.
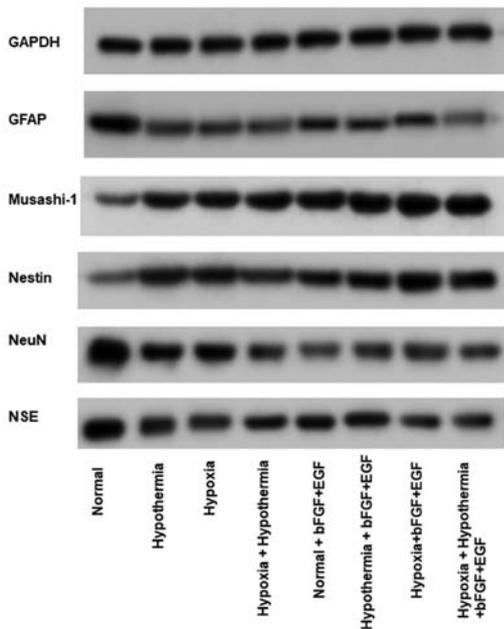
Figure 5. Detection of cellular markers by western blotting. Representative western blots for GFAP, nestin, musashi-1, NSE and NeuN, after treatment with bFGF + EGF and hypoxia + hypothermia. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; NSE, neuron specific enolase; NeuN, neuronal nuclei.

change significantly. This indicated that growth factors may induce the proliferation of cells that are positive for both CD105 and CD29. However, these cells may not be MSCs; instead, these cells may be somatic cells exhibiting greater levels of differentiation. The proportion of MSCs that were positive for CD90 was ~15%. This is in accordance with the present immunocytochemistry results, which demonstrated that glial cells in the group receiving growth factors adopted a morphology that was more similar to fibroblasts.

During normal body function, different generations of cells must undergo processes that turn mature cells into stem cells (33). Although research relating to the induction of stem cells by acid has been questioned recently (34), there is no essential difference between the basic concept of this research and stem cell induction. The present study demonstrated that changes in the cellular environment may induce mature cells to become stem cells, although the identity of these specific environmental factors remains unknown. The results of the present study demonstrated that hypoxic mild hypothermia treatment or growth factor treatment upregulated the expression levels of nestin and Musashi-1 in differentiated neurons and downregulated the expression levels of NSE and NeuN in mature neurons. Hypoxia treatment, combined with mild hypothermia and growth factor treatment, further enhanced
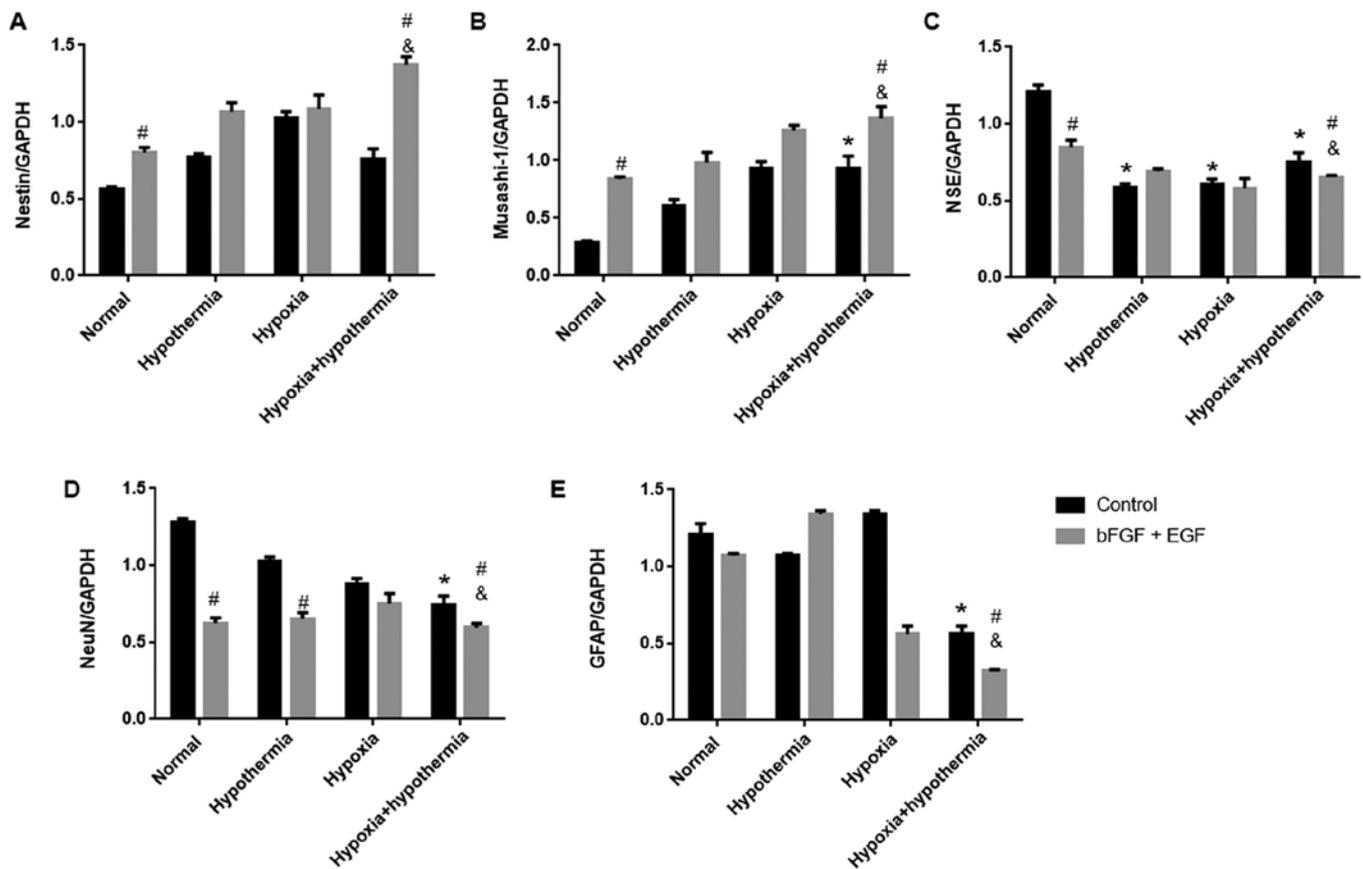
Figure 6. Quantitative data of the expression levels of different cellular markers. (A) nestin, (B) musashi-1, (C) NSE, (D) NeuN, (E) GFAP. [*]P<0.05 vs. control-normal; [#]P<0.05 vs. corresponding control; [&]P<0.05 vs. control-bFGF + EGF. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; NSE, neuron specific enolase; NeuN, neuronal nuclei.

this effect. Additionally, the results of the present study demonstrated that the relative expression levels of GFAP were inhibited by mild hypothermia or growth factor treatment after hypoxia treatment. It is possible that neural stem cells are positive for GFAP, nestin and Musashi-1, whereas neuroblasts are positive for nestin and Musashi-1 (35). The observed decrease in GFAP expression may indicate that neural stem cells that are derived from MSCs have the ability to rapidly differentiate into nerve cells.

Previous studies have demonstrated that it is difficult to identify MSCs from various rat tissues using surface markers alone (36,37). In the present study, nestin and Musashi-1 were selected as biomarkers for MSCs (38) and the induction of MSC-like transformation in rat primary glial cells by hypoxia, mild hypothermia and growth factors from the morphological aspect was verified. Moreover, CD29 and CD44 positivity supported the MSC-like characteristics, at least to some extent. However, these biomarkers and surface markers are not sufficient to prove the specific features of MSCs. Future work should aim to stimulate transformed cells into different types of neurons (39), which could provide further support for the hypothesis of the current study.

In conclusion, the results of the present study suggested that treatments involving bFGF and EGF, and anaerobic mild hypothermia may promote the transformation of glial cells into MSC-like cells, and that these effects were optimized when the two treatments were combined.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HW, WZ and GH performed the experiments and analyzed the data. HW and CS designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University (Nanchang, China; approval no. 2017BBG70066).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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