Apoptosis in glioma-bearing rats after neural stem cell transplantation

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Research Highlights
(1) Neural stem cells transplanted into tumor-bearing rats can hinder tumor cell growth and proliferation; however, the mechanism remains unclear.
(2) Glioma development and malignant biological characteristics are associated with abnormal signal transduction networks in tumor cells.
(3) This study aimed to explore neural stem cell therapy for glioma from the viewpoint of the Ras/Raf/Mek/Erk pathway. The results showed that transplantation of neural stem cells could inhibit the abnormal activation of Ras/Raf/Mek/Erk signaling, thus promoting apoptosis and potentially treating glioma.

Abstract
Abnormal activation of the Ras/Raf/Mek/Erk signaling cascade plays an important role in glioma. Inhibition of this aberrant activity could effectively hinder glioma cell proliferation and promote cell apoptosis. To investigate the mechanism of glioblastoma treatment by neural stem cell transplantation with respect to the Ras/Raf/Mek/Erk pathway, C6 glioma cells were prepared in suspension and then infused into the rat brain to establish a glioblastoma model. Neural stem cells isolated from fetal rats were then injected into the brain of this glioblastoma model. Results showed that Raf-1, Erk and Bcl-2 protein expression significantly increased, while Caspase-3 protein expression decreased. After transplantation of neural stem cells, Raf-1, Erk and Bcl-2 protein expression significantly decreased, while Caspase-3 protein expression significantly increased. Our findings indicate that transplantation of neural stem cells may promote apoptosis of glioma cells by inhibiting Ras/Raf/Mek/Erk signaling, and thus may represent a novel treatment approach for glioblastoma.

Key Words
neural regeneration; stem cells; Ras/Raf/Mek/Erk signaling pathway; neural stem cells; glioblastoma; C6 glioma cells; Caspase-3; Bcl-2; apoptosis; brain tumor; neuroregeneration

INTRODUCTION
Glioblastoma is the most common intracranial tumor derived from the neuroectoderm, and has high heterogeneity¹. The present recommended treatment plan is radical surgery combined with radiotherapy and chemotherapy; however, prognosis is poor and patients only survive on average for 18 months². Neural stem cells have pluripotent differentiation potentials, and can restore the normal structure and function of the host central nervous system³. After neural stem cells are transplanted into tumor-bearing rats, they can prevent tumor growth and hinder tumor cell growth and proliferation; however, the mechanism remains unclear.
cell growth and proliferation; however, the underlying mechanism remains unclear.

The Ras/Raf/Mek/Erk signaling cascade is a widely activated mitogen-activated protein kinase pathway present in all eukaryotes, and can control cell proliferation, differentiation, survival and apoptosis. Abnormal activation of this cascade is crucial for glioma, and its suppression can effectively inhibit glioma cell proliferation and promote cell apoptosis. However, it is unclear if neural stem cells can inhibit Ras/Raf/Mek/Erk signaling in the treatment of glioma.

Ras/Raf/Mek/Erk signaling is closely associated with apoptosis at the molecular level. Bcl-2 is a downstream substrate of Erk1/2, and allows the survival of poorly differentiated and metaplastic cells to promote tumor growth, ultimately leading to invasive malignant cancer. Increases in Bcl-2 expression are associated with glioma. Bcl-2 acts on Caspase-3 upstream and can inhibit its activation. Caspase-3 expression decreases during the glioma cell apoptosis process.

In this study, we observed Raf-1, Erk, Caspase-3 and Bcl-2 protein expression following neural stem cell transplantation, in a broader attempt to explore the correlation between neural stem cell transplantation, the Ras/Raf/Mek/Erk pathway and apoptosis in glioma cells.

RESULTS

Quantitative analysis of experimental animals
One hundred and twenty Sprague-Dawley rats were randomly divided into three groups, with 40 rats in each group as follows: normal group, no intervention was given; model group, glioblastoma models were established and injected with normal saline; cell transplantation group, glioblastoma models were established and injected with neural stem cells from fetal rats. During the modeling process, one rat in the model group and one rat in the cell transplantation group died, resulting in 118 rats used in the result analysis.

Brain imaging observations of glioblastoma rats
One week after modeling, MRI examination showed that the midline structure, sulci, encephalocele and cisterns were normal in the normal rats, and there were no abnormal brain signals. Tumor lesions were visible in the model rats, with long T1 and T2 signals, and the boundary between lesions was blurry with mild edema present around the lesions. Furthermore, midline structures had shifted, and the ventricular systems were compressed and deformed, which confirmed successful establishment of the model (Figure 1).

![Figure 1](image1.png)

**Figure 1** MRI of brain tissue in normal and glioma model rats.

(A) Normal rat: midline structure is located at the center, with no abnormal brain signals.

(B) Glioma model rat: T1 (B1) and T2 (B2) signals were long, mild edema was observed around the lesion, the midline structure had shifted, and the ventricular system was compressed and deformed. Arrow in B1 indicates tumor.

**Morphology and identification of neural stem cells isolated from fetal rats**

Primary neural stem cells were round and in suspension. After 24 hours of culture, cells began to adhere; at 7–9 days cell spheres formed, showing a rounded shape, uniform size, and suspended proliferation. Adherent cells were detected with anti-nestin immunofluorescence staining, and results showed that cell spheres were positive for nestin and elicited strong yellow-green fluorescence. At 10 days, anti-glial fibrillary acidic protein immunofluorescence staining revealed that there were a large amount of protruding glial fibrillary acidic protein-positive cells, emitting yellow-green fluorescence. This evidence indicates that the cultured and isolated cells had the characteristics of neural stem cells and differentiation potentials (Figure 2).

**Effect of neural stem cell transplantation on Raf-1, Erk, Bcl-2 and Caspase-3 protein expression in tumor tissue from glioma model rats**

One week after stem cell transplantation, western blot analysis showed that Raf-1, Erk and Bcl-2 protein expression in the model group was significantly increased \((P < 0.05)\), while Caspase-3 protein expression was significantly decreased \((P < 0.05)\) compared with the normal group. In the cell transplantation group, Raf-1, Erk and
Bcl-2 protein expression was significantly decreased ($P < 0.05$), while Caspase-3 protein expression was significantly increased ($P < 0.05$) compared with the model group (Figure 3, Table 1).

**Figure 2** Nestin and glial fibrillary acidic protein (GFAP) expression in cultured neural stem cells. (immunofluorescence staining, × 400).

Under fluorescence microscopy, subcultured cells expressed nestin, a marker of neural stem cells (A); while some cells also expressed an astrocyte marker, GFAP (B). Yellow-green fluorescence indicates FITC-labeled nestin and GFAP expression.

**Figure 3** Raf-1, Erk, Bcl-2 and Caspase-3 protein expression in the tumor tissue of glioma model rats after stem cell transplantation.

1: Normal group; 2: model group; 3: cell transplantation group.

Western blot analysis showed that Raf-1, Erk and Bcl-2 expression was low in normal rat brains and upregulated in the tumor tissue of glioma rats, while Caspase-3 expression was reduced in glioma rats; 1 week after stem cell transplantation, Raf-1, Erk and Bcl-2 expression had decreased, and Caspase-3 expression had increased in the tumor tissue of glioma rats.

**Table 1** Raf-1, Erk, Bcl-2 and Caspase-3 protein expression (absorbance ratio to β-actin) in the tumor tissue of glioma model rats at 1 week after stem cell transplantation.

| Group               | Raf-1  | Erk         |
|---------------------|--------|-------------|
| Normal              | 0.527±0.036 | 0.432±0.037 |
| Model               | 0.904±0.028 | 0.726±0.103 |
| Cell transplantation| 0.614±0.035 | 0.516±0.051 |

| Group               | Bcl-2  | Caspase-3  |
|---------------------|--------|-------------|
| Normal              | 0.062±0.015 | 0.324±0.017 |
| Model               | 0.183±0.021 | 0.152±0.018 |
| Cell transplantation| 0.074±0.011 | 0.309±0.012 |

Data are expressed as mean ± SD, with 20 rats per group. *$P < 0.05$, vs. normal group; **$P < 0.05$, vs. model group (one-way analysis of variance followed by least significant difference test).

Quantitative analysis showed that the number of Raf-1-, Erk- and Bcl-2-positive cells and their expression levels in the model group were significantly higher than the normal group ($P < 0.05$), and those in the cell transplantation group were significantly lower than the model group ($P < 0.05$), which was still slightly higher than the normal group ($P < 0.05$). Conversely, Caspase-3 positive cells and its positive expression rate in the model group was significantly lower than the normal group ($P < 0.05$), and those in the cell transplantation group was significantly higher than the model group ($P < 0.05$), which was lower than the normal group ($P < 0.05$; Tables 2–5).

**DISCUSSION**

Glioma is the most common primary intracerebral tumor, accounting for 2% of all malignant tumors in adults. It is characteristics consist of invasive growth, high relapse rate, strong aggression, and abundance in blood vessels\cite{16}. According to WHO classification, patients with grade-3 glioma survive on average for 3–5 years\cite{17}. Glioma occurrence, development and malignant biological characteristics are associated with abnormal signal transduction in tumor cells\cite{18}.
Table 2  Effect of neural stem cell transplantation on Raf-1 expression in tumor tissue of glioma model rats

| Group               | Positive cells (× 400 field) | Positive expression | Positive rate (%) |
|---------------------|------------------------------|---------------------|-------------------|
| Normal              | 23.18±1.49                   | (-) 14 (+) 6 (+++) 0 (++++) 0 | 30.00             |
| Model               | 68.14±2.34ab                 | (-) 2 (+) 2 (++) 9 (++++) 6 | 89.47ab           |
| Cell transplantation | 34.03±1.35ab                 | (-) 10 (+) 3 (++) 5 (++++) 1 | 47.36abc          |

Data are expressed as mean ± SD, and there were 20, 19, 19 rats in the normal, model, cell transplantation groups, respectively. *P < 0.05, vs. normal group; **P < 0.05, vs. model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).

Table 3  Effect of neural stem cell transplantation on Erk expression in tumor tissue of glioma model rats

| Group               | Positive cells (× 400 field) | Positive expression | Positive rate (%) |
|---------------------|------------------------------|---------------------|-------------------|
| Normal              | 20.07±1.28                   | (-) 15 (+) 5 (++) 0 (++++) 0 | 25.00             |
| Model               | 63.52±1.24ab                 | (-) 3 (+) 3 (++) 8 (++++) 5 | 84.21ab           |
| Cell transplantation | 29.84±1.12ab                 | (-) 11 (+) 3 (++) 5 (++++) 1 | 42.10abc          |

Data are expressed as mean ± SD, and there were 20, 19, 19 rats in the normal, model, cell transplantation groups, respectively. *P < 0.05, vs. normal group; **P < 0.05, vs. model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).

Table 4  Effect of neural stem cell transplantation on Bcl-2 expression in tumor tissue of glioma model rats

| Group               | Positive cells (× 400 field) | Positive expression | Positive rate (%) |
|---------------------|------------------------------|---------------------|-------------------|
| Normal              | 21.22±1.15                   | (-) 15 (+) 5 (++) 0 (++++) 0 | 25.00             |
| Model               | 75.11±1.34ab                 | (-) 1 (+) 4 (++) 7 (++++) 7 | 94.74ab           |
| Cell transplantation | 38.06±1.97ab                 | (-) 9 (+) 3 (++) 5 (++++) 2 | 58.82abc          |

Data are expressed as mean ± SD, and there were 20, 19, 19 rats in the normal, model, cell transplantation groups, respectively. *P < 0.05, vs. normal group; **P < 0.05, vs. model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).
Table 5  Effect of neural stem cell transplantation on Caspase-3 expression in tumor tissue of glioma model rats

| Group                  | Positive cells (×400 field) | Positive expression | Positive rate (%) |
|------------------------|-----------------------------|---------------------|-------------------|
|                        | (−) | (+)  | (+++) | (++++) |
| Normal                 | 58.26±1.32                  | 5                  | 3                 | 6                     | 6                   | 75.00               |
| Model                  | 26.18±1.46                  | 13                 | 4                 | 2                     | 0                   | 31.58 ab            |
| Cell transplantation   | 51.27±1.55 ab               | 6                  | 6                 | 5                     | 2                   | 68.42 ab            |

Data are expressed as mean ± SD, and there were 20, 19, and 19 rats in the normal, model, cell transplantation group, respectively. aP < 0.05, vs. normal group; bP < 0.05, vs. model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).

Activation of the Erk1/2 signaling pathway, also called the Raf/Mek/Erk signaling pathway, can cause multiple protein kinase cascade reactions, and transmit extracellular signals into the cells [18]. Under the stimulation of extracellular signals, Ras can be activated by binding with guanosine triphosphate, thereby activating phosphorylation of Raf, Mek and Erk. Then phosphorylated Erk enters the nuclei and triggers activity of transcription factors [26]. Via this signal pathway, extracellular growth and neurotrophic signals are transferred to the cells, causing a series of cellular reactions that can regulate cell proliferation and differentiation [21-23]. Ras/Raf/Mek/Erk signaling pathway disorders are also crucial for tumor occurrence and development [29]. Ras and Raf oncogenic mutations can be detected in many tumors, leading to excess activation of Ras/Raf/Mek/Erk signaling, which contributes to tumor development and malignancy [24]. This protein kinase cascade is the key signaling pathway affecting the abnormal biological characteristics of glioma [26]. Glioma development and malignancy may be the results of abnormal activation of Ras/Raf/Mek/Erk signaling [26]. This signaling pathway exerts the most apparent effect on the promotion of glioma cell survival and proliferation [27]. Moreover, the disorder is also associated with glioma angiogenesis, invasion and metastasis [28]. In glioma, growth factors and their receptors upstream of the Ras/Raf/Mek/Erk signaling pathway, such as epidermal growth factor receptor and platelet-derived growth factor receptor, may be overexpressed as a result of gene amplification or mutation [29-30]. The epidermal growth factor receptor binds with vascular endothelial growth factor-A on the glioma cell surface, thus activating downstream Ras/Raf/Mek/Erk signaling and promoting vascular endothelial cell proliferation, inhibiting vascular endothelial cell apoptosis, and playing a catalytic role in glioma angiogenesis [31]. Excessive activation of this pathway also induces vascular endothelial growth factor expression. Raf-1 and Mek activation can induce the formation of high-level malignant glioma [32-33]. Therefore, suppressing excessive activation of this signaling pathway may kill glioma cells. Raf kinase inhibitors have a positive effect in the treatment of gliomas [34]. Our immunohistochemical assay results showed that Raf-1 and Erk were weakly expressed in normal brain tissue, while a large number of positive granules were observed in the model group. Furthermore, western blot analysis showed that Raf-1 and Erk protein expression in the tumor tissue of model rats was significantly higher than the normal rat brain tissue. This evidence also supports the involvement of abnormal activation of Ras/Raf/Mek/Erk signaling in the pathogenesis of glioma.

Neural stem cells are a type of precursor cells that have high self-renewal capacity and can differentiate into neurons and glial cells, and also generate a variety of nervous tissue cells through unequal division; thus, their differentiation and regulatory mechanisms are crucial in the treatment of nervous system [35-36]. Transplanted neural stem cells may differentiate into new neurons to replace defective or dead neurons, and connect with the surrounding nervous system to establish correct synaptic connection for the recovery of neurological functions [37-38]. Neural stem cells in vivo and in vitro have a strong ability to track glioma [39]. In addition, neural stem cells also exert transdifferentiation [40] and cell fusion [41-42] phenomena. Neural stem cells can specifically target and accumulate around glioma cells, and even track single glioma cells for a long distance [43]. Aboody et al [44] have found that when neural stem cells were transplanted into tumor-bearing rats, they spread throughout the tumor and migrate to other organs, surrounding the tumor to stimulate its growth and development and secreting various factors to reduce tumor size. Neural stem cells also can differentiate into neurons, thus promoting endogenous neurogenesis, generating glial cells, and promoting nerve fibers formation [45]; in addition, neural stem cells also secrete neurotrophic factors, which can promote proliferation, migration and neurogenesis of endogenous neural stem cells, and they play a key role in the reconstruction and functional recovery of neural pathways [46-47]. Scholars [46] showed that transplanted neural stem cells can improve the clinical symptoms of patients with glioblastoma.
could express neurotrophic factors in vivo and promote host axonal growth; our findings suggested that neural stem cells have the potential to replace missing tissue after nervous system injury. In this study, fetal rat hippocampal cells showed the morphology of neural stem cells, and were induced to differentiate into neurons and glial cells, indicating that these cells possessed the characteristics and differentiation potential of neural stem cells. Immunohistochemical staining showed that Raf-1 and Erk expression in the cell transplantation group was significantly lower than in model group; however, Raf-1 and Erk protein expression significantly reduced after transplantation of neural stem cells. We speculate that neural stem cells may inhibit abnormal activation of Ras/Raf/Mek/Erk signaling, thus inhibiting glioma development as consistent with previous findings.

Apoptosis, also known as programmed cell death, is a complex protease cascade reaction involved in multiple protein molecules. The mitochondrial inner membrane potentials depolarize, and mitochondria release apoptosis-inducing factors, which trigger the activation of proteases and induce changes in cell morphology; nuclear condensation is observed, DNA is sheared and degrades, cell membrane ruptures, apoptotic bodies form, and ultimately cell death occurs. The Bcl-2 proteins are important regulatory molecules in the activation of apoptotic proteins, and Bcl-2 plays a crucial role in the inhibition of tumor cell apoptosis. Bcl-2 is expressed during normal cell development, and is scarcely or poorly expressed in the maturation or apoptosis process. Bcl-2, P53 and Bax expression levels may affect tumor cell apoptosis and are regarded as the key evidence for evaluating the severity and pathological grade of malignant tumors. Lyustikman et al. have reported that Ras protein activity increased in the majority of glioma patients, triggering the activation of downstream Raf/Mek/Erk signaling. Sustained activation can upregulate Bcl-2 expression and ultimately immortalize tumor cells. Our findings indicate that Bcl-2 was poorly expressed in normal brain tissue, and greatly expressed in the model group. In addition, Bcl-2 protein expression in the tumor tissue of model rats was significantly higher than that in normal brain tissue. It is reasoned that abnormal activation of Ras/Raf/Mek/Erk signaling pathways in glioma rats may increase Bcl-2 expression. After transplantation of neural stem cells, Bcl-2 protein expression levels were significantly decreased compared with the model group. This indicates that transplanted neural stem cells may inhibit abnormal activation of Ras/Raf/Mek/Erk signaling and decrease Bcl-2 expression, thereby promoting tumor cell apoptosis.

The Caspases are a group of cysteine proteases that contribute to cytokine maturation, cell proliferation and differentiation, as well as cell apoptosis. Caspase activation is necessary in all apoptotic pathways. Caspase-3 is a key protease in downstream pathways triggered by cell apoptosis and is an effector molecule of apoptosis. It is also important in the Caspase cascade and at the final apoptotic execution stage. When Caspase-3 activity is inhibited, apoptotic disorders occur and regulation of cell proliferation and apoptosis are disrupted, which possibly affects tumor occurrence and development as well as biological behavior and prognosis. It is widely acknowledged that suppression of apoptosis is associated with P53 and Caspase-3 expression, and apoptosis mechanisms inevitably lead to tumor and immune system abnormalities. Caspase-3 can inactivate apoptosis inhibitors, degrade intracellular protein substrates, and promote the formation of apoptotic bodies, and its expression is low or absent in tumor cells with low apoptotic rates. Inhibition of Caspase-3 enzyme activity and antagonizing of Caspase-3 functions also suppresses cell apoptosis. Dai and colleagues activated hematoporphyrin monomethyl ether in C6 glioma cells using ultrasound and found Bcl-2 expression decreased, Caspase-3 expression increased, and the apoptotic rate significantly increased. Inhibition of Raf/Mek/Erk signaling can down-regulate Bcl-2 expression and promote the apoptosis of human K562 leukemia cells. After the Mek/Erk pathway was activated, Caspase-3 activity decreased, and the anti-apoptotic ability of chlamydia increased. Immunohistochemical staining results in this study showed that Caspase-3 was highly expressed in normal brain tissue, but poorly expressed in the model group; western blot analysis showed that its protein expression was also significantly lower than that the normal group. This evidence indicates that activation of the Ras/Raf/Mek/Erk signaling pathway may reduce Caspase-3 activity and enhance the anti-apoptotic ability of glioma cells. Furthermore, high Bcl-2 expression and low Caspase-3 expression contributed to inhibition of glioma tumor cell apoptosis and promoted tumor growth. Caspase-3 protein expression levels in the stem cell transplantation group were higher than in the model group, indicating that neural stem cell transplantation enhanced Caspase-3 activity and promoted tumor cell apoptosis. Due to the physiological index vary between human and animals, further studies will focus on the potential effect of neural stem cell transplantation in human. The experimental objects of this study are the rats, which is a different species compared with the human. Further research of the neural stem cells and glioma is required to provide evidence for neural stem cells treatment of the glioma.
In summary, neural stem cell transplantation could inhibit abnormal activation of the Ras/Raf/Mek/Erk pathway, decrease Bcl-2 expression, and promote Caspase-3 activation and glioma cell apoptosis. Therefore, it may represent a novel method of treating glioma.

**MATERIALS AND METHODS**

**Design**

A randomized, controlled animal experiment.

**Time and setting**

Experiments were performed in the Experimental Center, Hubei University of Chinese Medicine, China from January to December 2012.

**Materials**

One hundred and twenty adult male Sprague-Dawley rats, aged 4–6 months and weighing 120 ± 30 g, were used to establish glioma models. One Wistar rat at 14 days of pregnancy was used to prepare fetal rat neural stem cells. All animals were provided by the Experimental Animal Center of Wuhan University, China (license No. SCXK (Hubei) 2008-0005). Experimental protocols were performed according to the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[^67].

**Methods**

**Isolation, culture and identification of neural stem cells**

Embryonic hippocampal tissue isolated from Wistar rats at 14 days of pregnancy was cut into pieces, digested with Dulbecco’s Modified Eagle’s Medium (DMEM)/Nutrient Mixture F12 (Gibco, Carlsbad, CA, USA) and prepared into a suspension. After centrifugation and supernatant removal, cell suspension was dissolved in DMEM/ Nutrient Mixture F12 containing 20 μg/L epidermal growth factor (Sigma, St. Louis, MO, USA), 20 μg/L basic fibroblast growth factor (Sigma) and 2% B27 (Gibco) at 1 × 10^6 cells/mL. Then cells were cultured in medium supplemented with 10% fetal bovine serum (Gibco) in 5% CO₂ at 37°C for 7 days, and incubated with mouse anti-rat nestin monoclonal antibody (1:400; Santa Cruz Technology, Santa Cruz, CA, USA) and rabbit anti-rat glial fibrillary acidic protein polyclonal antibody (1:400; Chemicon) for identification.

**Establishment of glioma models**

C6 glioma cells were cultured in DMEM containing 10% fetal bovine serum (Shanghai Ai Biological Research Co., Ltd., Shanghai, China), and a cell suspension was prepared. Rats in the model group and cell transplantation group were anesthetized intraperitoneally, and the scalp was excised to expose the skull. A hole of 1.2 mm diameter was drilled at 3 mm to the right of sagittal suture and 1 mm advanced to the midpoint of anterior and posterior fontanelle[^88], and injected with 50 μL cell suspension containing 3 × 10⁶ C6 glioma cells, then the scalp was sutured, and 1 × 10⁴ units penicillin was given intramuscularly. One week after modeling, rats in the cell transplantation group and model group were detected with MRI (Philips Gyroscan T5-NT 0.5 T MRI machine; Amsterdam, the Netherlands), to observe glioma growth.

**Transplantation of neural stem cells**

One week after modeling, rats were anesthetized and fixed in a prone position on the stereotaxic instrument. The transplantation site was located at 1 mm to the bregma and 2 mm lateral[^89], and the penetration depth was 5 mm away from the skull surface. A 5 μL suspension containing 1 × 10⁶/μL fetal rat neural stem cells was injected into the brain tissue of tumor-bearing rats, then bone hole was closed with bone wax and the wounds were sutured. Three days before stem cell transplantation, rats in the cell transplantation group (n = 39) were intraperitoneally injected with immunosuppressant cyclosporine (10 mg/kg; North China Pharmaceutical Group Corporation, Shijiazhuang, Hebei Province, China), once per day, until 1 week after cell transplantation. Rats in the model group (n = 39) were injected with cyclosporine and saline. The normal group (n = 40) received no treatment.

**Western blot analysis of Raf-1, Erk, Caspase-3 and Bcl-2 protein expression in tumor tissue**

One week after stem cell transplantation, rats were killed, and brain tissue was harvested, homogenized and trituated into a cell suspension, followed by cell lysis, denaturation and centrifugation at 4°C. After the supernatant was removed, the total protein quantity was determined using the Bradford assay[^89]. Then 10 μg protein was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane, and then blocked in T-TBS containing 50 g/L skimmed milk at room temperature for 2 hours, rinsed with PBS, and incubated with rabbit anti-rat Raf-1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat Erk monoclonal antibody (Life Technologies Inc, Carlsbad, CA, USA), rabbit anti-Caspase-3 polyclonal antibody (Sinovac Biotech Co., Ltd., Shanghai, China), and mouse anti-rat Bcl-2 monoclonal antibody (Seitz Biotechnology...
Co., Ltd., Beijing, China) at 4°C overnight. All antibodies were diluted to 1:1 000. After PBS washing, specimens were incubated with horseradish peroxidase-conjugated goat anti-rabbit/ mouse IgG (1:2 000; Santa Cruz Biotechnology) and reacted with chemiluminescence reagent for 5 minutes, then scanned with an OmniMet 9.5 image analysis system (Buehler Biotechnology, Chicago, IL, USA). The absorbance values were analyzed using Bandscan software (Bio-Rad, Hercules, CA, USA) with β-actin as a reference.

**Immunohistochemical staining of Raf-1, Erk, Caspase-3 and Bcl-2 expression in tumor tissue**

Rats were killed at 1 week after cell transplantation, and brain tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned into slices of 5 μm thickness. After endogenous peroxidase was blocked with 3% hydrogen peroxide, brain tissue was retrieved by microwave heating, blocked with 1% bovine serum for 30 minutes, and incubated at 4°C overnight with the following antibodies diluted at 1:100: rabbit anti-rat Raf-1 monoclonal antibody, rabbit anti-rat Erk monoclonal antibody, rabbit anti-Caspase-3 polyclonal antibody, and mouse anti-Bcl-2 monoclonal antibody. Then specimens were incubated with horseradish peroxidase donkey anti-rabbit/ mouse IgG (1:100; Amyjet Scientific Inc, Wuhan, Hubei Province, China) at 37°C for 30 minutes and at room temperature for 1 hour. Subsequently, specimens were rinsed with PBS three times, stained with 3,3’-diaminobenzidine (Boster, Wuhan, Hubei Province, China), counterstained with hematoxylin, dehydrated, developed, and mounted using gum. Finally brain tissue was observed by optical microscopy (Olympus, Shibuya, Japan).

**Result determination**

Positive cells presented brown-yellow particles in the nucleus or cytoplasm, and the number of positive cells was measured using Image-Pro Plus analysis software (Media Cybernetics Inc., Bethesda, MD, USA) on five high-power fields (400 x magnification) of six pathological slices randomly selected from each rat. The number of blue-stained nuclei cells were counted, and the percentage of positive cells was calculated according to the following formula: number of positive cells/number of positive cells + number of blue-stained cells) x 100%. ≤5%: 0 point, 6–25%: 1 point, 26–50%: 2 points, 51–75%: 3 points [25]. Cells were also evaluated according to the degree of staining (no staining: 1 point, weak staining: 2 points, moderate staining: 3 points, strong staining: 4 points). The final scores = degree of staining × percentage of stained cells. 0 is negative (−), 1 is weak positive (+), 2–3 is positive (++), 4–6 is strongly positive (+++) [17].

**Statistical analysis**

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and measurement data were expressed as mean ± SD. Differences between groups were compared using analysis of variance, and paired comparisons were performed using least significant difference test; count data were expressed as a percentage, and differences between two groups were compared using chi-square test, with P values of < 0.05 considered significant.

**REFERENCES**

[1] Jiang T, Tang GF, Lin Y, et al. Prevalence estimates for primary brain tumors in China: a multi-center cross-sectional study. Chin Med J (Engl). 2011;124(17): 2578-2583.

[2] Chen J, McKay RM, Parada LF. Malignant glioma: lessons from genomics, mouse models, and stem cells. Cell. 2012; 149(1):36-47.

[3] Ahmed AU, Tyler MA, Thaci B, et al. A comparative study of neural and mesenchymal stem cell-based carriers for oncolytic adenovirus in a model of malignant glioma. Mol Pharm. 2011;8(5):1559-1572.

[4] Yang J, Lam DH, Goh SS, et al. Tumor tropism of intravenously injected human-induced pluripotent stem cell-derived neural stem cells and their gene therapy application in a metastatic breast cancer model. Stem Cells. 2012;30(5):1021-1029.

[5] Keshet Y, Seger R. The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. Methods Mol Biol. 2010;661:3-38.

[6] Nakada M, Kita D, Watanabe T, et al. Aberrant signaling pathways in glioma. Cancers. 2011;3(3):3242-3278.

[7] Shepherd RD, Kos SM, Rinker KD. Long term shear stress leads to increased phosphorylation of multiple MAPK species in cultured human aortic endothelial cells. Biochimie. 2009;46(6):529-538.

[8] Kim JY, Kim WJ, Kim H, et al. The stimulation of CD147 induces MMP-9 expression through Erk and NF-kappaB in macrophages: implication for atherosclerosis. Immune Netw. 2009;9(3):90-97.

[9] Zhang R, Banik NL, Ray SK. Combination of all-trans retinoic acid and interferon-gamma suppressed PI3K/Akt survival pathway in glioblastoma T98G cells whereas NF-kappaB survival signaling in glioblastoma U87MG cells for induction of apoptosis. Neurochem Res. 2007; 32(12):2194-2202.

[10] Ohmori T, Asahi S, Sato C, et al. Bcl-2 protein expression and gut neurohormonal polypeptide/amine production in colorectal carcinomas and tumor-neighboring mucosa, which closely correlate to the occurrence of tumor. Histol
Histopathol. 1999;14(1):37-44.

[11] Varela M, Ranunculo SM, Morand A, et al. EGF-R and PDGF-R, but not bcl-2, overexpression predict overall survival in patients with low-grade astrocytomas. J Surg Oncol. 2004;86(1):34-40.

[12] Zhang Z, Su N, Wang Z. Using tissue microarray study of Survivin, Bcl-2 expression in human glioma. Neimenggu Minzu Daxue Xuebao. 2009;15(5):18-20.

[13] Hua Y, Xu R, Han JY, et al. The association of cell cycle and expressions of bcl-2 and bax with pathological grades of glioma. Zhongliu. 2009;6(29):578-582.

[14] Hu MR, Lan Y, Zhang XM. The correlation between Caspase and Bcl-2 in apoptosis and its regulation. Junshi Yixue Kexueyuan Yuankan. 2000;2(24):64-67.

[15] Khan M, Yi F, Rasul A, Li T, et al. Alantolactone induces apoptosis in glioblastoma cells via GSH depletion, ROS generation, and mitochondrial dysfunction. IUBMB Life. 2012;64(9):783-794.

[16] Collins VP. Brain tumours: classification and genes. J Neurol Neurosurg Psychiatry. 2004;75 Suppl 2:i11-2.

[17] Quon H, Abdulkarim B. Adjuvant treatment of anaplastic oligodendrogliomas and oligoastrocytomas. Cochrane Database Syst Rev. 2008;(2):CD007104.

[18] Wang JJ, Wang G, Zhou ZM, et al. Development based on the characteristics of glioma biology and treatment. Zhongguo Yiya Daoakan. 2011;13(3):467-468.

[19] Zhang LZ, Qu QX, Wen K. Regulation of Erk signaling pathway in biological behaviors of tumor cells. Yixue Zongshu. 2011;17(6):836-838.

[20] Sun P, Watanabe H, Takano K, et al. Sustained activation of M-Ras induced by nerve growth factor is essential for neuronal differentiation of PC12 cells. Genes Cells. 2006;11(9):1097-1113.

[21] Qi M, Elion EA. MAP kinase pathways. J Cell Sci. 2005;118(Pt 16):3569-3572.

[22] Chambard JC, Lefloch R, Pouysségur J, et al. Erk implication in cell cycle regulation. Biochim Biophys Acta. 2007;1773(8):1299-1310.

[23] Downward J. Targeting Ras signalling pathways in cancer therapy. Nat Rev Cancer. 2003;3(1):11-22.

[24] Fernández-Medarde A, Santos E. Ras in cancer and developmental diseases. Genes Cancer. 2011;2(3):344-358.

[25] Robinson JP, Vanbrocklin MW, Guilbeault AR, et al. Activated BRaf induces gliomas in mice when combined with Ink4a/Arf loss or Akt activation. Oncogene. 2010;29(3):335-344.

[26] Hambardzumyan D, Squatrito M, Carbajal E, et al. Glioma formation, cancer stem cells, and akt signaling. Stem Cell Rev. 2008;4(3):203-210.

[27] Lemieux E, Boucher MJ, Mongrain S, et al. Constitutive activation of the Mek/Erk pathway inhibits intestinal epithelial cell differentiation. Am J Physiol Gastrointest Liver Physiol. 2011;301(4):G719-730.

[28] Chi AS, Sorensen AG, Jain RK, et al. Angiogenesis as a therapeutic target in malignant gliomas. Oncologist. 2009;14(6):621-636.

[29] Verhaak RG, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRα, IDH1, EGFR, and NF1. Cancer Cell. 2010;17(1):98-110.

[30] Jeukens J, van den Broecke C, Gijsen S, et al. Ras/Raf pathway activation in gliomas: the result of copy number gains rather than activating mutations. Acta Neuropathol. 2007;114(2):121-133.

[31] Takano S. Glioblastoma angiogenesis: VEGF resistance solutions and new strategies based on molecular mechanisms of tumor vessel formation. Brain Tumor Pathol. 2012;29(2):73-86.

[32] Wang L, Wang P, Liu Y, et al. Regulation of cellular growth, apoptosis, and Akt activity in human U251 glioma cells by a combination of cisplatin with CRM197. Anticancer Drugs. 2012;23(1):81-89.

[33] Robinson JP, Vanbrocklin MW, Lastwika KJ, et al. Activated Mek cooperates with Ink4a/Arf loss or Akt activation to induce gliomas in vivo. Oncogene. 2011;30(11):1341-1350.

[34] Neyns B, Sadones J, Chaskis C, et al. Phase II study of sunitinib malate in patients with recurrent high-grade glioma. J Neurol Oncol. 2011;103(3):491-501.

[35] Akerud P, Canals JM, Snyder EY, et al. Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson’s disease. J Neurosci. 2001;21(20):8108-8118.

[36] Conti L, Reitano E, Cattaneo E. Neural stem cell systems: diversities and properties after transplantation in animal models of diseases. Brain Pathol. 2006;16(2):143-154.

[37] Chuang TT. Neurogenesis in mouse models of Alzheimer’s disease. Biochim Biophys Acta. 2010;1802:872-880.

[38] Tayler RM, McCarthny N, Zhao G, et al. Engraftment, migration, and differentiation of genetically modified neural stem cells in the twicher model of progressive demyelination. Exp Neurol. 2002;175(2):434-441.

[39] Kelly S, Bliss TM, Shah AK, et al. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proc Natl Acad Sci U S A. 2004;101(32):11839-11844.

[40] Poleck-Dehlin B, Duell T, Bartl R, et al. Genetic analyses permit the differentiation between reactive malfunctions (‘promyelocyte arrest’) and arising promyelocyte leukemia in a pregnant patient with a history of a medulloblastoma. Leuk Lymphoma. 2004;45(9):1905-1911.

[41] Assanah M, Lochhead R, Ogden A, et al. Glial progenitors in adult white matter are driven to form malignant gliomas by platelet-derived growth factor-expressing retroviruses. J Neurosci. 2006;26(25):6781-6790.

[42] Jessberger S, Clemenson GD Jr, Gage FH. Spontaneous fusion and nonclonal growth of adult neural stem cells. Stem Cells. 2007;25(4):871-874.
3-kinase signaling. Stem Cells. 2008;26(6):1575-1586.

[44] Aboody KS, Najbauer J, Schmidt NO, et al. Targeting of melanoma brain metastases using engineered neural stem/progenitor cells. Neuro Oncol. 2006;8(2):119-126.

[45] Yin ZS, Zhang H, Wang W, et al. Wnt-3a protein promote neuronal differentiation of neural stem cells derived from adult mouse spinal cord. Neuro Res. 2007;29(8):847-854.

[46] Madhavan L, Ourendrik V, Ourendrik J. Neural stem/ progenitor cells initiate the formation of cellular networks that provide neuroprotection by growth factor-modulated antioxidant expression. Stem Cells. 2008;26(1):254-265.

[47] Low CB, Liou YC, Tang BL. Neural differentiation and potential use of stem cells from the human umbilical cord for central nervous system transplantation therapy. J Neurosci Res. 2008;86(8):1670-1679.

[48] Lu P, Jones LL, Snyder EY, et al. Neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol. 2003;181(2):115-129.

[49] Xu R, Hua Y, Zhong P, et al. The role of cell apoptosis in glioma chemotherapy evaluation. Fudan Xuebao: Yixue Ban. 2009;36(6):692-696.

[50] Patel MP, Masood A, Patel PS, et al. Targeting the Bcl-2. Curr Opin Oncol. 2009;21(6):516-523.

[51] Chipuk JE, Moldoveanu T, Llambi F, et al. The Bcl-2 family reunion. Mol Cell. 2010;37(3):299-310.

[52] Fogal V, Sugahara KN, Rusoahl E, et al. Cell surface nucleolin antagonist causes endothelial cell apoptosis and normalization of tumor vasculature. Angiogenesis. 2009;12(1):91-100.

[53] Gu H, Rao S, Zhao J, et al. Gambogic acid reduced bcl-2 expression via p53 in human breast MCF-7 cancer cells. J Cancer Res Clin Oncol. 2009;135(12):1777-1782.

[54] Luystikman Y, Momota H, Pao W, et al. Constitutive activation of Raf-1 induces glioma formation in mice. Neoplasia. 2008;10(5):501-510.

[55] Wu XX, Kakehi Y, Jin XH, et al. Induction of apoptosis in human renal cell carcinoma cells by vitamin E succinate in Caspase-independent manner. Urology. 2009;73(1):193-199.

[56] Porter AG, Jänicke RU. Emerging roles of Caspase-3 in apoptosis. Cell Death Differ. 1999;6(2):99-104.

[57] Meggiato T, Calabrese F, De Cesare CM, et al. C-JUN and CPP32 (CASPASE 3) in human pancreatic cancer: relation to cell proliferation and death. Pancreas. 2003;26(1):65-70.

[58] Körner A, Pazaitou-Panayiotou K, Kelesidis T, et al. Total and high-molecular-weight adiponectin in breast cancer: in vitro and in vivo studies. J Clin Endocrinol Metab. 2007;92(3):1041-1048.

[59] Zarnescu O, Brehar FM, Chivu M, et al. Immunohistochemical localization of Caspase-3, Caspase-9 and Bax in U87 glioblastoma xenografts. J Mol Histol. 2008;39(6):561-569.

[60] Xue LY, Ren LQ, Luo W, et al. Expression of Fas, Fas ligand, Fas-associated death domain protein, Caspase 8 and mutant P53 protein in esophageal squamous cell carcinoma. Zhonghua Yi Xue Za Zhi. 2007;87(3):150-154.

[61] Mignotte B, Vayssiere JL. Mitochondria and apoptosis. Eur J Biochem. 1998;252(1):1-15.

[62] Li YM, Chen H, Li X, et al. A new immunomodulatory therapy for severe sepsis: Ulinastatin Pius Thymosin (alpha) 1. J Intensive Care Med. 2009;24(1):47-53.

[63] Lakhani SA, Masud A, Kuida K, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science. 2006;311(5762):847-851.

[64] Dai S, Hu S, Wu C. Apoptotic effect of sonodynamic therapy mediated by hematoporphyrin monomethyl ether on C6 glioma cells in vitro. Acta Neurochir (Wien). 2009;151(12):1655-1661.

[65] Sancho P, Galeano E, Estañ MC, et al. Raf/Mek/Erk signaling inhibition enhances the ability of dequalinium to induce apoptosis in the human leukemic cell line K562. Exp Biol Med (Maywood). 2012;237(8):933-942.

[66] Du K, Zheng Q, Zhou M, et al. Chlamydial antiapoptotic activity involves activation of the Raf/Mek/Erk survival pathway. Curr Microbiol. 2011;63(4):341-346.

[67] The Ministry of Science and Technology of the People’s Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

[68] Paxions G, Watson C. The Rat Brain in Stereotaxic Coor- dinates. 6th ed. San Diego: Academic Press. 2005.

[69] Bao CF, Liu X, Wei J, et al. Quantitative analysis of c-Jun N-terminal kinases’ expression in mouse brain during fetal development. Shuli Yiya Zue Xazhi. 2009;22(2):152-153.

[70] Li GS, Zhang DR, Liu D, et al. The expression and si- gnificance of p34cdc2 in non-small cell lung cancer. Zhong- guo Yike Daxue Xuebao. 2002;31(2):104-108.

[71] Xuan Y, Lin Z. Expression of Indian Hedgehog signaling molecules in breast cancer. J Cancer Res Clin Oncol. 2009;135(2):235-240.

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