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Astragaloside VI Promotes Neural Stem Cell Proliferation and Enhances Neurological Function Recovery in Transient Cerebral Ischemic Injury via Activating EGFR/MAPK Signaling Cascades

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Abstract

Radix Astragali (AR) is a commonly used medicinal herb for post-stroke disability in Traditional Chinese Medicine but its active compounds for promoting neurogenic effects are largely unknown. In the present study, we tested the hypothesis that *Astragaloside VI* could be a promising active compound from AR for adult neurogenesis and brain repair via targeting epidermal growth factor (EGF)-mediated MAPK signaling pathway in post-stroke treatment. By using cultured neural stem cells (NSCs) and experimental stroke rat model, we investigated the effects of *Astragaloside VI* on inducing NSCs proliferation and self-renewal in vitro, and enhancing neurogenesis for the recovery of the neurological functions in post-ischemic brains in vivo. For animal experiments, rats were undergone 1.5 h middle cerebral artery occlusion (MCAO) plus 7 days reperfusion. *Astragaloside VI* (2 μg/kg) was daily administrated by intravenous injection (i.v.) for 7 days. *Astragaloside VI* treatment promoted neurogenesis and astrogenic formation in dentate gyrus zone, subventricular zone, and cortex of the transient ischemic rat brains in vivo. *Astragaloside VI* treatment enhanced NSCs self-renewal and proliferation in the cultured NSCs in vitro without affecting NSCs differentiation. Western blot analysis showed that *Astragaloside VI* up-regulated the expression of nestin, p-EGFR and p-MAPK, and increased neurosphere sizes, whose effects were abolished by the co-treatment of EGF receptor inhibitor gefitinib and ERK inhibitor PD98059. Behavior tests revealed that *Astragaloside VI* promoted the spatial learning and memory and improved the impaired motor function in transient cerebral ischemic rats. Taken together, *Astragaloside VI* could effectively activate EGFR/MAPK signaling cascades, promote NSCs proliferation and neurogenesis in transient cerebral ischemic brains, and improve the repair of neurological functions in post-ischemic stroke rats. *Astragaloside VI* could be a new therapeutic drug candidate for post-stroke treatment.

Keywords *Astragaloside VI* · Transient cerebral ischemia · Neural stem cell · Proliferation · Epidermal growth factor

Chemical compound studied in this article
Astragaloside VI (PubChem CID: 71448940)

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Abbreviations

EGF	Epidermal growth factor
NSCs	Neural stem cells
MCAO	Middle cerebral artery occlusion
SVZ	Subventricular zone
LV	Lateral ventricles
DG	Dentate gyrus
AsVI	*Astragaloside VI*
TCM	Traditional Chinese medicine

Introduction

Ischemic stroke is a common disease leading to the neurological deficit and cognitive impairments [1]. Hippocampus is the most vulnerable brain region associated with cognitive functions for learning and memory during cerebral ischemia injury

[2–6]. Until now, no effective treatment is available to improve brain repair and neurological recovery [7].

Adult neurogenesis is a rigorous process of immature precursor cells to produce new neurons/glial cells in adult brains with evidence from both animal stroke models [8, 9] and stroke patients [10, 11]. Neurogenesis comprises a complex process starting from the proliferation of neural progenitor cells, followed the differentiating commitments into different phenotypes and functional neurons, eventually integrating into neurological network systems. The spontaneous neurogenesis occurs in the adult mammalian central nervous system throughout life, which is restrictive at subventricular zone (SVZ) and subgranular zone (SGZ) in the hippocampus and cortex [12, 13]. However, the spontaneous neurogenesis is insufficient for functional recovery in the post-stroke disability [14]. Targeting endogenous neural stem/progenitor cells (NSCs/NPCs) for promoting adult neurogenesis becomes a promising strategy for neurogenesis and brain repair for stroke treatment [14–16].

Many intrinsic and extrinsic factors from NSCs/NPCs and neurogenic niches participate in the network regulations of neurogenesis at different neurogenetic stages for regeneration therapy [17]. Among them, epidermal growth factor (EGF)-mediated signaling pathway plays critical roles in promoting neurogenesis [18–20]. EGF is the principal mitogen in the process of NSCs proliferation [21], cell-fate decision, and even NSCs dedifferentiation [22–24]. EGF binds the specific ligands of epidermal growth factor receptor (EGFR) [25, 26] and the activated EGFR forms a dimer to stimulate its intrinsic intracellular protein tyrosine kinase activity. EGFRs are abundantly expressed in the SVZ area [27, 28], and regulate the migration of neuronal progenitors [29, 30]. EGFR is responsible for PI3K activation and NSCs survival. Autophosphorylation of EGFR initiates ERK/MAPK pathway, leading to DNA synthesis and cell proliferation [31]. EGFR and fibroblast growth factor receptor (FGFR) synergistically regulate the ERK/MAPK pathway [32]. Ischemia/hypoxia stimulations could activate EGFR in putative stem cells/progenitors and mediate neurogenesis in experimental ischemic stroke models [33, 34]. MAPK can be activated by phosphorylation of threonine and tyrosine residues in response to brain ischemia [35]. MAPK contributes to the maintenance of neural stem cells [36]. MAPK phosphorylation supports neuronal survival in the dentate gyrus zone [37]. Therefore, EGFR/MAPK signaling cascades are important therapeutic targets to induce neurogenesis and brain repair in the treatment of post-stroke disability.

Radix Astragali (AR) is a commonly used medicinal herb in traditional Chinese medicine, and the major component of the classic herbal prescription Buyang Huanwu decoction for stroke treatment [38]. We systematically reviewed the relationships between AR's chemical structures and their bioactivities in various biological systems [39]. AR extract had neuroprotective effects against cerebral ischemia injury [40]. Many active compounds isolated from AR showed neuroprotective effects in different experimental stroke animal models. For example, our previous study revealed that *Calycosin-7-O-β-D-glucoside*, a representative isoflavone in AR, has neuroprotective effects on the blood-brain barrier (BBB) integrity and neuroprotective effects against cerebral ischemia-reperfusion injury [40]. Astragalosides, a group of triterpene glycosides, have the potentials for neuroprotection and improving spatial memory [39]. AR extract reversed Abeta (25–35)-induced memory loss and prevented the loss of axons and synapses in the cerebral cortex and hippocampus in mice. *Astragalosides I*, *II*, and *IV* promoted axonal regeneration, reconstruction of neuronal synapses, and prevention of Abeta (25–35)-induced neuronal death in cultured neurons [41]. *Astragaloside IV* also protected the BBB integrity and promoted angiogenesis in cerebral ischemia-reperfusion injury [42–45]. However, whether the active compounds from AR promote adult neurogenesis in post-stroke brain repair is unknown yet. In our pilot study, we screened the bioactivities of *Astragalosides* on promoting proliferation of NSCs and cell growth in cultured NSCs. *Astragaloside VI* revealed the most promising effects on inducing NSCs proliferation in primary cultured NSCs (Supplementary 1, 2). Herein, we tested the hypothesis that *Astragaloside VI* could promote adult neurogenesis for inducing brain repair and recovery of neurological function via activating EGF-mediated EGFR/MAPK signaling cascades by using in vitro cultured NSCs and in vivo experimental stroke animal model.

Materials and Methods

Animals and Cerebral Ischemia-Reperfusion Model

Adult male Sprague-Dawley (SD) rats weighing 260–280 g were provided by the Laboratory Animal Units at the University of Hong Kong. All of the animal protocols were approved and regulated by Animal Care and Ethical Committee, University of Hong Kong (No.1844-09). Efforts were taken to minimize the number of animals used and their suffering. Rats were maintained under the controlled temperature (20 ± 2 °C) and allowed free access to food and water. Focal cerebral ischemia was induced by the intraluminal suture middle cerebral artery occlusion (MCAO) method [46, 47]. In brief, rats were anesthetized with 4% isoflurane in the gas of 70% N_2O with 30% O_2 in whole experiments. The left-sided carotid arteries were exposed through a midline cervical incision. The left external carotid artery was dissected and isolated distally by coagulating its branches and placing a distal ligation prior to the transaction. A piece of 3–0 monofilament nylon suture, with its tip rounded by gentle heating, was introduced via the lumen of the left external carotid artery stump and the left internal carotid artery to embed into the left anterior cerebral

artery so that the left middle cerebral artery was occluded at its origin. An abrupt reduction in regional cerebral perfusion to less than 30% of the baseline value was considered to achieve focal ischemia. During the cerebral ischemia-reperfusion process, blood flow was monitored by using a Laser Doppler (Supplementary 3). At 1.5 h of cerebral ischemia, the intraluminal suture was withdrawn from the left anterior cerebral artery and the left internal carotid artery to permit reperfusion. For the sham control group, the surgical process was the same as the MCAO group without suture occlusion.

Primary Cultured Neural Stem Cells

C17.2 cells, the multi-potent neural progenitor cell line isolated from postnatal mouse cerebellum, were obtained from American Tissue Culture Collection. Cells were maintained in Iscove's modified Dulbecco's medium (Invitrogen) containing 2% L-glutamine and EDTA with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS) on the condition of 37 °C with a humidified atmospheric air (78% N_2 and 21% O_2) supplied with 5% CO_2. Primary cultured neural stem cells were harvested from the cortex in the embryonic 14–16 day Sprague-Dawley rats. Briefly, fore cortex in rat embryos was dissected and ground into single cells by filtering through a nylon mesh (70 μm) and re-suspended in DMEM/F12 medium (Invitrogen). Cells were seeded in the density of 1×10^5 cells/ml in DMEM/F12 with 2% B27 (Invitrogen), bFGF (10 ng/ml, Sigma), EGF (10 ng/ml, Sigma), and 1% PS. Cells were incubated at 37 °C in a humidified atmosphere containing 78% N_2 and 21% O_2 supplied with 5% CO_2.

Experimental Protocols and Drug Treatments

Astragaloside VI was purified and identified by Professor Guowei Qin, Shanghai Institute of Meteria Medica with the purity of 99% as determined by high-performance liquid chromatography analysis (Supplementary 4). In the in vivo animal experiments, *Astragaloside VI* (2 μg/kg wt., dissolved in saline) was intravenously injected into the rats at 2 h after MCAO ischemia once a day for 7 days. The same volume of saline was used as vehicle control. Rats in each group were given an intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU) (20 mg/kg) twice per day for 2 days. All of the rats were euthanized at the day after the last injection of BrdU. For in vitro study, *Astragaloside VI* was dissolved in PBS and added to the culture medium. C17.2 cells or primary cultured neural stem cells were seeded into the 24-well plate. In EGF-deprived experiments, primary cultured neural stem cells were incubated with *Astragaloside VI* (10, 100 nM) in the DMEM/F12 media deprived of EGF or normal DMEM/F12 media. In the mechanistic study, EGFR inhibitor Gefitinib or ERK inhibitor PD-98059 (10 μM) were added to the culture medium. After incubated for 2 h, cells were rinsed with medium for three times. The experiments included the following groups: normal medium group, Gefitinib-treated group, PD98059-treated group, *Astragaloside VI* (10 nM)-treated group, *Astragaloside VI* (10 nM) plus Gefitinib-treated group, *Astragaloside VI* (10 nM) plus PD98059-treated group, *Astragaloside VI* (100 nM)-treated group, *Astragaloside VI* (100 nM) plus Gefitinib-treated group, and *Astragaloside VI* (100 nM) plus PD98059-treated group. BrdU assay, neurosphere assay, and Western blot analysis were conducted afterward.

Tissue Preparation and Cryosections

Rats were sacrificed at 7 days after MCAO ischemia-reperfusion injury. Brain tissues were harvested, fixed in 4% PFA at 4 °C overnight, and sank in 30% sucrose for another 24 h and stored at 4 °C. The brain tissues were cut into serial sections at 20 μm thickness by using microtome (Leica) and spread over microscopy slides. The brain sections were prepared in "1 in 6." In detail, one section was picked up from every six consecutive sections by 100-μm intervals to avoid repeated counting of cells.

TTC Staining

Brain tissues were rapidly collected and were sliced into five equally coronal sections (2–3 mm thickness) with the aid of a brain matrix. The sections were incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) at 37 °C for 15 min with gentle shaking and then were fixed with 4% paraformaldehyde overnight. The stained slices were photographed and the size of the infarct was quantified using ImageJ software (NIH, USA).

Immunocytochemistry

Sections were rinsed with 0.1 M PBS. DNA denature procedure would proceed for BrdU detection. Sections were dipped into 2 N HCl in 37 °C for 1 h and put into 0.1 M borate buffer for 10 min. After rinsed in PBS, sections were incubated with blocking buffer (PBS with 10% goat serum and 0.5% Triton-100) for 2 h. The sections were incubated with the primary antibodies including rat anti-BrdU(1:300, AbDSerotee, MCA2060), mouse anti-NeuN(1:400, Millipore, MAB377), rabbit anti-DCX (1:300, Cell Signaling, 4604), and rabbit anti-SOX2 (1300, Millipore, AB5630) overnight in a cold room followed by rinsing with PBS and incubated with the appropriate secondary antibodies (Alexa Fluor 488 or 568-conjugated IgG 1:300, Invitrogen) in room temperature for 2 h. Sections were mounted with mounting medium (Dako) and the fluorescent imaging was observed and recorded with Carl Zeiss Axio Observer Z1 fluorescent imaging system and analyzed by the Image J software (NIH, USA).

MTT Assay

Cell viability was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). Cells (1×10^4/ml) were seeded into 96-well plates and cultured for 1 day and then incubated with *Astragaloside VI* (1, 10, 100,1000 nM) or *Astragaloside I*, *II*, *III*, *V* (50 nM, 500 nM, 5 μM, 50 μM) for 48 h. Cells were then incubated with MTT (0.5 mg/ml) at 37 °C for 4 h. After removing the medium gently and adding 100 μl DMSO into each well, we quantified the formazan dye by using a microplate reader (Model 680, Bio-Rad) at the absorbance of 490 nm and the optical density (OD) value was used for cell viability.

BrdU Assay

Neural progenitor cell line C17.2 cells (1×10^4 cells/ml) were incubated with BrdU (10 μM) at 37 °C for 2 h and then washed with culture medium three times. Cells were incubated with *Astragaloside VI* (5, 10, 20, 100 nM) or vehicle for 48 h. After fixed in 4% PFA for 30 min and washed with PBS, the cells were treated with 2 N HCl at 37 °C for 1 h, and following incubation with 0.1 M borate buffer for 10 min. After incubated with blocking buffer (PBS plus 10% goat serum, 0.1% Triton-100) for 2 h, the cells were incubated with primary antibody BrdU (1400, AbDSerotee) at 4 °C overnight, rinsed with PBS, and then incubated with secondary antibody (Alexa 488 Goat anti-rat 1:400, Invitrogen) at room temperature for 2 h. After rinsed with PBS, cells growing in the coverslips were mounted on the glass slides with the fluorescent mounting media (Dako). Fluorescent imaging was recorded with Carl Zeiss Axio Observer Z1 fluorescent imaging system and analyzed by ImageJ software (NIH, USA). BrdU-positive cells were calculated by an investigator who was blind to experimental design.

Neurosphere Assay

Primary cultured NSCs (1×10^4cell/ml) were seeded in 24-well-plates with DMEM-F12 with 2% B27, 1% PS, 1% L-glutamine, 10 ng/ml EGF, and 10 ng/ml BFGF. Cells were incubated with different dosages of *Astragaloside VI* (10, 100, 1000 nM) or vehicle medium for 48 h. Coverslips were fixed by 4% PFA for 20 min, rinsed with PBS, and incubated with blocking buffer (PBS plus 10% goat serum and 0.1% Triton-100) 2 h. Then, the coverslips were incubated with primary antibody nestin (mouse 1:300, Millipore) at 4 °C overnight, washed with PBS, and incubated with secondary antibody (Alexa 568 Goat anti-mouse 1:300, Invitrogen) at room temperature for 2 h in the dark. The coverslips were rinsed with PBS and incubated with DAPI for 10 min to identify the neurosphere formation. Photos were taken under the microscope by Carl Zeiss Axio Observer Z1 fluorescent imaging system. Fifteen fields were randomly chosen in every well. The quantities of the neurosphere and the largest diameter of the sphere in every field were counted.

Western Blot Analysis

Cells and brain tissues were harvested with lysis buffer solution (RIPA Buffer, Sigma-Aldrich), phosphatases inhibitor cocktail (P5726, Sigma-Aldrich), and protease inhibitor cocktail (P8340, Sigma-Aldrich). The lysate was centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was separated. Equal amounts of protein were subjected to SDS-PAGE analysis, transferred onto a PVDF membrane, and probed with primary antibodies against nestin (mouse 1:300, Millipore, MAB377), EGFR (rat 1:500, Millipore, 06-847), p-EGFR (rat 1:500, Millipore, 09-310), GAPDH (1:1000, Millipore, ABS16), p44/42MAPK (1:1000, Cell Signaling, 9102), and p-p44/42MAPK (1:1000, Cell Signaling, 9101). After washing, the membranes were incubated with corresponding secondary antibody HRP-conjugated goat anti-rabbit IgG (1:1000, Santa Cruz, sc-2030) and goat anti-mouse IgG (1:1000, Santa Cruz, sc-2005). Band intensity was quantified with ImageJ software (NIH, Bethesda, MD, USA).

Water Maze Test

Morris water maze test was used for assessing learning and memory function, which was performed by an investigator who was blinded to the experimental design. The non-toxic ink was added to make the water opaque. Animals were brought into the conducting room 30 min prior to the test. Rats were subjected to four trials per day for five consecutive days. In each trial, rats were given 60 s to find the platform. Escape latency was recorded when rats reached the platform. If the rat could not reach the platform within 60 s, it was recorded as 60 s. Rats were permitted to stay on the platform for another 15 s once they found it. Rats were dried and returned to its cage. Four-minute inter-trial rest was given to the rats. A probe trial was conducted on the sixth day. The round tank was divided into four equal quadrants. The target circle was defined as an area within 20 cm diameter as measured from the center of the platform. The number of times that one rat crossed area within 90 s was recorded as the platform crossing frequencies.

Motor Test

Motor behavioral tests were performed before MCAO and at 7 days after MCAO by an investigator who was blinded to the experimental groups. An accelerating rotarod was used to measure rat motor function. The rats were placed on the rotarod cylinder, and the time for which the animals remained on the rotarod was measured. The speed was slowly increased from 4 to 40 rpm within 5 min. A trial was ended if the animal fell off the rungs or gripped the device and spun around for two consecutive revolutions without attempting to walk on the rungs. The animals

were trained 3 days before MCAO operation. The duration (in seconds) on the device was recorded with rotarod measurement at 1 day prior to surgery. Motor test data are presented as a percentage of mean duration (three trials) on the rotarod compared with the internal baseline control (before surgery).

Statistical Analysis

Data were expressed as mean ± S.D. SPSS 16.0 software was used for statistical analysis. One-way analysis of variance (ANOVA) was used for multiple group designed experiments for statistical analysis of the variances followed by Student-Newman-Keuls (SNK) Q test. For two independent group designed experiments, unpaired Student's t test was used to determine the difference. Significance was set at a probability value of $p < 0.05$.

Results

Astragaloside VI Decreased Infarct Volume in MCAO Ischemic Rat Brain

We firstly investigated the neuroprotective effects of *Astragaloside VI* on reducing infarction size in the ischemia-reperfused rats by using TTC staining. Both MCAO vehicle and *Astragaloside VI* treatment rats were subjected to 1.5 h of transient MCAO ischemia plus 24 h of reperfusion, whereas sham control rats conducted similar operation but without MCAO ligation. Each rat brain was cut into five slides and strained by TTC for measuring infarction volume. Sham control rats had no infarction area in the brains (data not shown). As showed in Fig. 1, the MCAO vehicle rats had a mean infarct size around 35%. The rats with *Astragaloside VI* treatment had mean infarct size around 8% which was significantly smaller than the MCAO vehicle rats. The results suggest that *Astragaloside VI* has neuroprotective effects against cerebral ischemia-reperfusion injury.

Astragaloside VI Promoted the Proliferation of NSCs in the Post-Ischemic Brain In Vivo and Cultured NSPCs In Vitro

We investigated the effects of *Astragaloside VI* on promoting NSCs proliferation in the post MCAO ischemic rat brains in vivo. As showed in Fig. 2a–d, the transient MCAO ischemic vehicle group had significantly higher rates of BrdU/SOX2-positive staining cells in the subventricular zone (SVZ) and lateral ventricles (LV) than the sham control group, indicating the spontaneous proliferation of NSCs in the ischemic brain area. Notably, the *Astragaloside VI* treatment group had significantly higher rates of BrdU/SOX2-positive staining cells than the MCAO ischemic vehicle group. We also investigated NSCs proliferation by detecting BrdU incorporation, neurosphere formation, and MTT assay in the in vitro-cultured NSCs (Fig. 3). *Astragaloside VI* treatment dose-dependently increased in the rates of BrdU-positive staining cells, the diameters of neurosphere, and cell viability (Fig. 3). Those results indicate that *Astragaloside VI* could promote NSCs proliferation in both MCAO ischemic brains in vivo and cultured NSCs in vitro.

Astragaloside VI Promoted Neurogenesis and Astrogenesis in Post-Ischemia Brains

We then addressed the question whether *Astragaloside VI* could promote neuronal differentiation in post-ischemia brains in vivo. DCX is a commonly used biomarker for neuronal differentiation [48]. NeuN is a neuronal nuclear antigen used as the mature neuron marker. As shown in Fig. 4a–d, the MCAO vehicle group had significantly higher rates of BrdU/DCX and BrdU/NeuN-positive staining cells in the SVZ and dentate gyrus zone respectively than the sham control group. *Astragaloside VI* treatment group had significantly higher rates of BrdU/DCX-positive staining and BrdU/NeuN-positive staining cells in the SVZ and dentate gyrus zone than the MCAO vehicle group. We also investigated the astrogenesis-promoting effects by using GFAP as a biomarker for astrogenesis (Fig. 4e, f). Similarly,

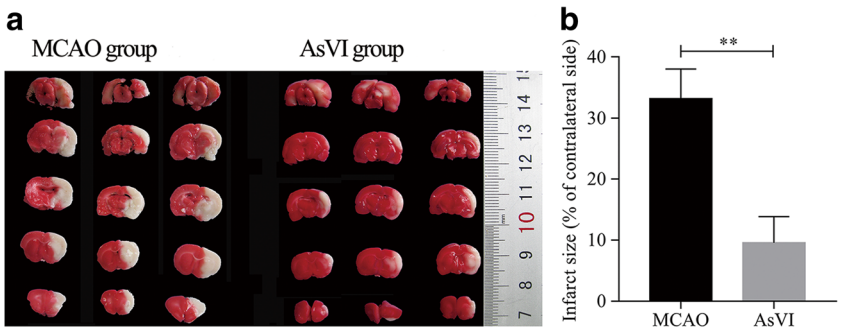

Fig. 1 *Astragaloside VI* decreases infarct volume in post-ischemic rat brains in vivo. Rats were subjected to 1.5 h of middle cerebral artery occlusion (MCAO) ischemia plus 24 h of reperfusion. *Astragaloside VI* (AsVI, 2 μg/kg wt.) was intravenously injected into the rats once daily for 1 day. **a** Representative TTC staining in surgery and treatment group. **b** Quantitative analysis on the infract size between MCAO surgery and treatment group (Mean ± SEM, $n = 6$ rats). $^{**}p < 0.01$

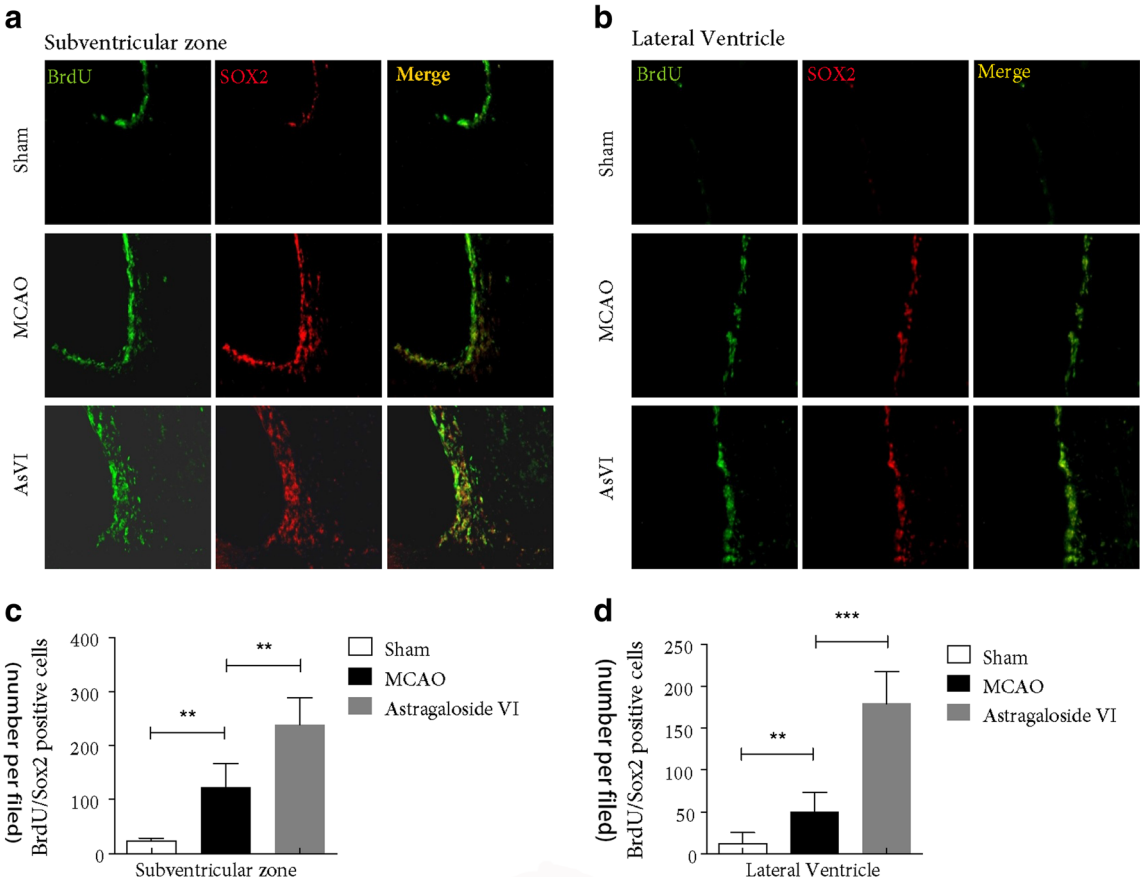

Fig. 2 *Astragaloside VI* promotes NSCs proliferation in SVZ and LV in post-ischemic brains in vivo. Rats were subjected to 1.5 h of middle cerebral artery occlusion (MCAO) ischemia plus 7 days of reperfusion. *Astragaloside VI* (AsVI, 2 μg/kg wt.) was intravenously injected into the rats once daily for 7 days. **a** Representative immunofluorescent staining images of BrdU (green) and SOX2 (red) in subventricular zone (SVZ) of post-ischemic rat brains. **b** Representative immunofluorescent staining images of BrdU (green) and SOX2 (red) in lateral ventricles (LV) of post-ischemic rat brains. **c** Quantitative analysis on the results of BrdU/SOX2 double-positive cells in SVZ (Mean ± S.D., $n = 6$ rats in each group). $^{**}p < 0.01$. **d** Quantitative analysis on the results of BrdU/SOX2 double-positive cells in LV (Mean ± S.D., $n = 6$ rats in each group). $^{**}p < 0.01$;$^{***}p < 0.001$

the MCAO vehicle group had significantly increased rates of BrdU/GFAP dual-positive staining cells in the DG, cortex, SVZ, and LV than the sham control group. *Astragaloside VI* treatment group had remarkably higher rates of BrdU/ GFAP-positive staining cells than MCAO vehicle group. These results suggest that *Astragaloside VI* could promote both neurogenesis and astrogenesis in the post-ischemic brains in vivo.

Astragaloside VI Exerted No Effect on Neuronal and Astrocytic Differentiation in Cultured NSCs In Vitro

We then investigated the effects of *Astragaloside VI* on promoting the differentiation of NSCs into mature neurons and astrocytes in primary cultured NSCs by detecting Tuj1 and GFAP-positive staining populations. After treated with PBS or *Astragaloside VI* (10, 100, 1000 nM) for 7 days, we conducted triple immunostaining experiments to identify the expressions of Tuj1 (green), GFAP (red), and DAPI (blue) (Fig. 5). No significant difference in the percentages of Tuj1-positive cells and GFAP-positive cells were found in the control group and *Astragaloside VI* treatment group. These results suggest that *Astragaloside VI* has no effect on the neuronal and astrocytic differentiation of NSCs in vitro.

Astragaloside VI Exhibited EGF-Like Effects on Promoting NSCs Growth and Inducing EGFR-Mediated MAPK/ERK Signaling Pathway

To explore the underlying mechanisms of *Astragaloside VI* in promoting NSCs proliferation, we investigated the neurosphere formation in cultured NSCs with or without trophic factor EGF in the culture medium. EGF containing medium was used as positive control. As showed in Fig. 6a, b, the EGF-depriving negative control group (Ctrl, left) had a significantly smaller diameter of the neurospheres (60 μm) than EGF-positive control group (150 μm, EGF, right). *Astragaloside VI* treatment dose-dependently increased the diameters of the neurospheres

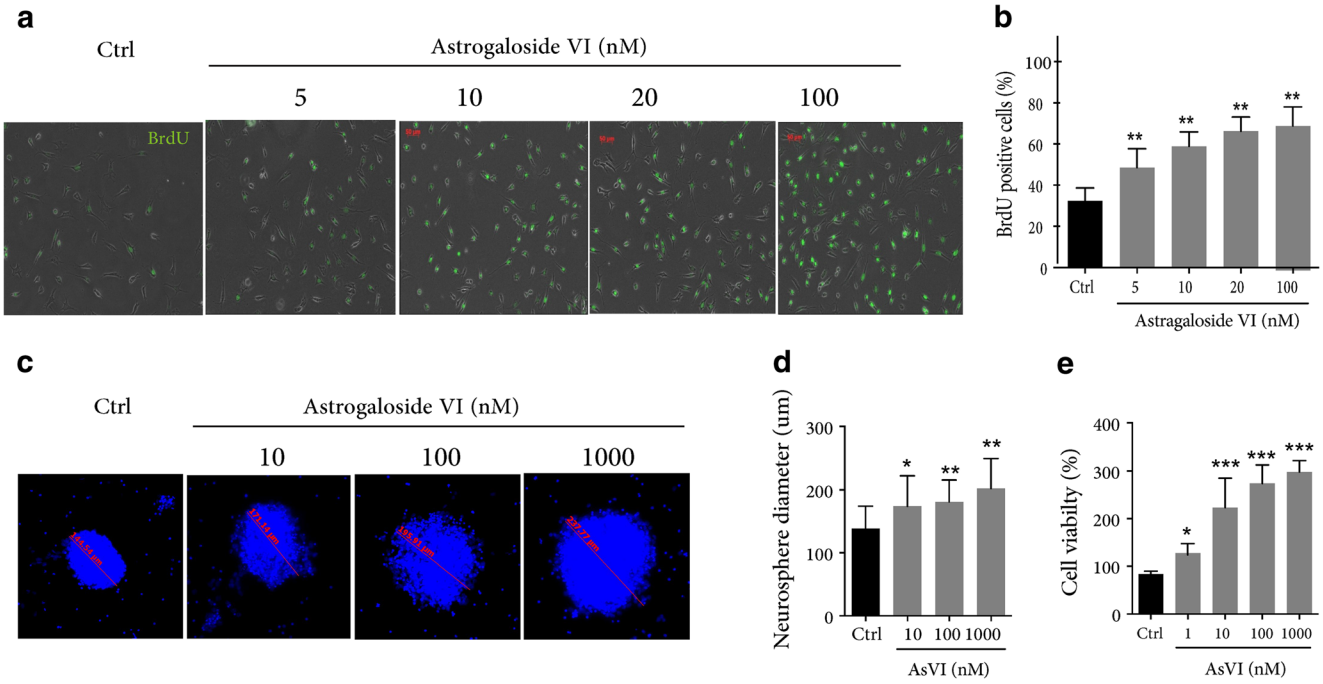

Fig. 3 *Astragaloside VI* promotes the self-renewal of NSCs and the proliferation of neural progenitor cells (NPCs) in vitro. C17.2 cells were treated with PBS or *Astragaloside VI* (5, 10, 20, 100 nM) for 24 h. **a** Representative images of BrdU immunoreactivity, *scalar bar*, 50 μm. **b** Quantitative analysis of BrdU-positive staining cells with or without *Astragaloside VI* treatment. The rates of BrdU-positive cells were counted in a blinded manner with ImageJ software (Mean ± S.D., $n = 9$ independent test). $^{**}p < 0.01$. **c** Representative results of neurosphere assay. Double immunostaining with DAPI and nestin in primary cultured NSCs. **d** Quantitative analysis of neurosphere diameters in the cultured NSCs with or without *Astragaloside VI* treatment. Diameters of neurospheres were measured in a blinder manner with ImageJ software. Values are the lengths of diameter ($n = 9$ independent test). $^{*}p < 0.05$; $^{**}p < 0.01$. **e** MTT assay for cell viability. NPCs were treated with PBS or *Astragaloside VI* (0, 1, 10, 100, 1000 nM) for 48 h. Values of each group were normalized by the absorbance value of control group (Mean ± S.D., $n = 9$ independent test), $^{*}p < 0.05$; $^{***}p < 0.001$

in the EGF-depriving negative control group. The group of *Astragaloside* VI treatment at 100 nM had a similar diameter of the neurospheres to the EGF-positive control group.

We also investigated the effects of *Astragaloside VI* on phosphorylated EGF receptor, phosphorylated MAPK, and nestin expression in the ischemic brains with 1.5 h MCAO plus 7 days reperfusion (Fig. 6c). Western blot analysis revealed that *Astragaloside VI* treatment remarkably up-regulated the expression of nestin, the phosphorylated EGF receptor, and phosphorylated MAPK in the hippocampus of the ischemic brains. These results suggest that *Astragaloside VI* has an EGF-like effect on promoting NSCs growth and neurosphere formation in cultured NSCs in vitro and activating EGF receptor and MAPK signaling cascades in MCAO transient ischemic brains in vivo.

EGFR Inhibitor Gefitinib and ERK Inhibitor PD98059 Abolished the Effects of *Astragaloside VI* on the Proliferation of NSCs

We further tested whether EGFR would be necessary for the bioactivity of *Astragaloside VI* for promoting NSCs proliferation. Gefitinib, the EGFR inhibitor, was used in the experiments of both post-ischemic brains in vivo (Fig. 7a–d) and primary cultured NSCs in vitro (Fig. 7e–i). Gefitinib co-treatment almost abolished the effects of *Astragaloside VI* on the NSCs proliferation in the ischemic brain tissues in vivo. The BrdU/SOX2-positive staining cells were unidentical in the SVZ and LV regions in the transient MCAO ischemic brains with co-treatment of Gefitinib and *Astragaloside VI*. Consistently, in vitro NSCs experiments yielded similar results to the in vivo experiments. As showed in Fig. 7e, f, the control group had the mean diameter of the neurosphere around 62 μm, whereas *Astragaloside VI* treatment groups at the doses of 10 and 100 nM had the mean diameters of the neurospheres at 85 and 100 μm respectively. Co-treatment of Gefitinib and *Astragaloside VI* had the decreased mean diameter of the neurospheres at 37 μm. As showed in Fig. 7g, h, *Astragaloside VI* treatment significantly increased the BrdU-positive percentages and the effects were abolished by co-treatment of Gefitinib (10 nM) in BrdU incorporation assay. Furthermore, in Fig. 7i, Western blot experiments revealed that Gefitinib blocked the effects of *Astragaloside VI* on the inductions of the expressions of nestin, *p*-EGFR, *p-p44/42* MAPK, and *p*-AKT in the primary cultured NSCs in vitro. We then used ERK inhibitor PD98059 and conducted neurosphere assay to further confirm that the roles of MAPK/ERK signaling in the neurogenic effects of *Astragaloside VI* on promoting NSCs proliferation (Fig. 8). Pre-treatment of PD98059 (10 nM) abolished the effects of

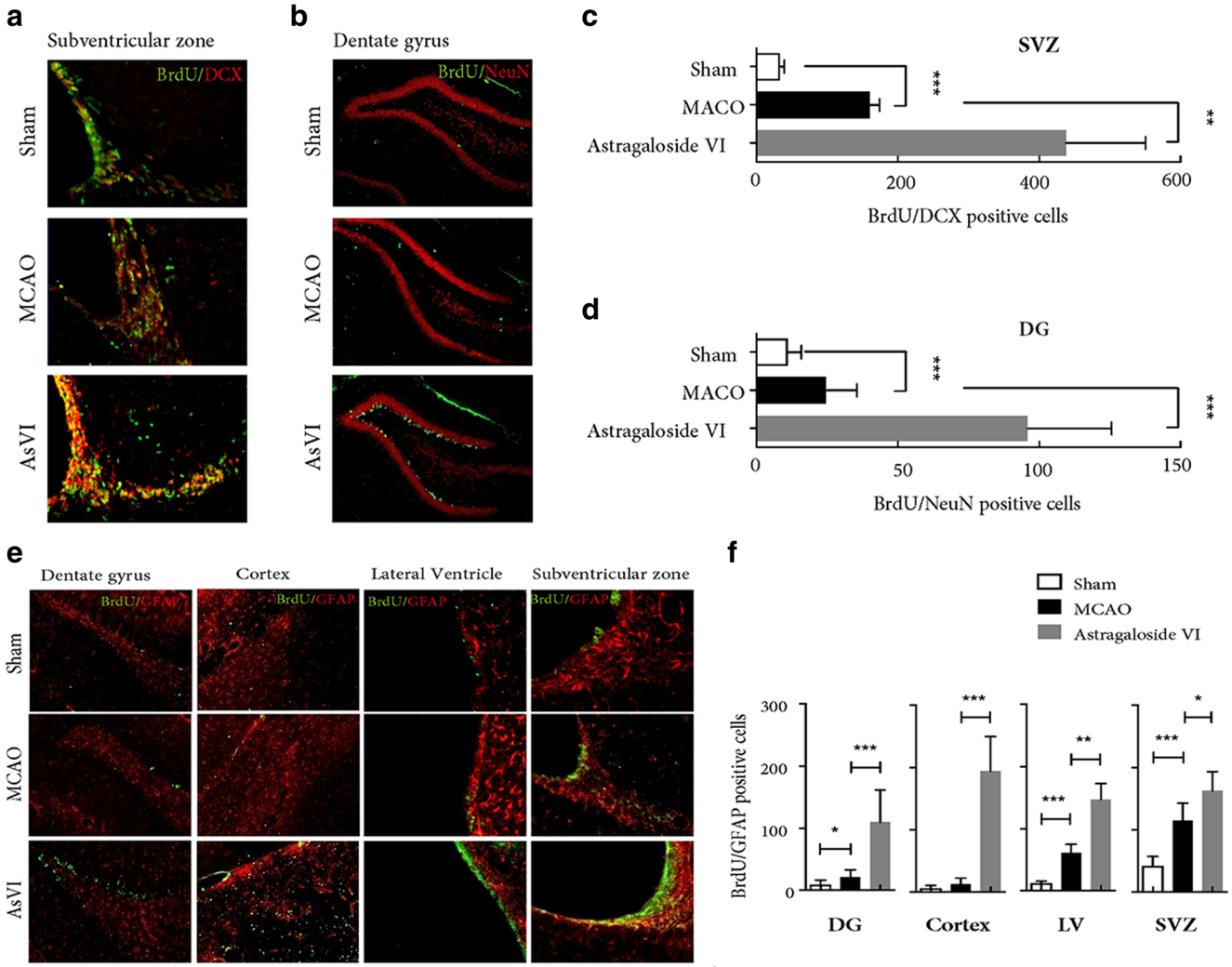

Fig. 4 *Astragaloside VI* promotes neurogenesis and astrogenesis in post-ischemic brains in vivo. Rats were subjected to 1.5 h of MCAO ischemia plus 7 days of reperfusion. *Astragaloside VI* (2 μg/kg wt.) was intravenously injected into the rats once daily for 7 days. Neurogenesis was identified by dual immunofluorescent staining of BrdU/DCX and BrdU/NeuN in the brain sessions. Astrogenesis was identified by dual immunofluorescent staining of BrdU/GFAP in the brain sessions. **a** Representative immunofluorescent staining images of BrdU (green) and DCX (red) in SVZ. **b** Representative immunofluorescent staining images of BrdU (green) and NeuN (red) in ischemic DG of transient MCAO ischemic rat brains. **c** Quantitative analysis on the rates of BrdU/DCX-positive immunostaining cells in SVZ (Mean ± S.D., $n = 6$ rats per group). $^{**}p < 0.01$; $^{***}p < 0.001$. **d** Quantitative analysis on the rates of BrdU/NeuN-positive staining cells in DG (Mean ± S.D., $n = 6$ rats per group). $^{***}p < 0.001$. **e** Representative immunofluorescent staining images of BrdU (green) and GFAP (red) dual-positive staining in the ischemic DG, cortex, LV, and SVZ. **f** Quantitative analysis on the BrdU/DCX-positive cells at DG, cortex, LV, and SVZ respectively (Mean ± S.D., $n = 6$ rats), $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Astragaloside VI on the neurosphere formation in the primary cultured NSCs. Therefore, these results suggest that *Astragaloside VI* could activate EGFR-mediated MAPK/ERK signaling cascades in the promotion of NSCs proliferation.

Astragaloside VI Improved Learning Ability, Memory, and Motor Function Recovery in Post Cerebral Ischemic Rats

The hippocampus plays a central role in learning and memory and cerebral ischemia could impair spatial memories [49–52]. We subsequently conducted a Morris water-maze test to elucidate the effects of *Astragaloside VI* on promoting learning ability and memory function. The experiments were performed on day 7 after 1.5 h MCAO ischemia. Each rat was trained four times per day for five consecutive days. The escape latencies on the hidden platform were recorded. Figure 9a–c showed different performance parameters in the sham control, MCAO vehicle group, and *Astragaloside VI* groups. The reduced escape latencies with the increased training days represents the learning ability. The sham control rats memorized well the position of the hidden platform and had shorter and shorter swimming escape latency to reach the hidden platform during the training courses. The MCAO vehicle group had unchanged escape latency during the training period for 5 days, indicating the deficiency of learning capacity. *Astragaloside VI* group had

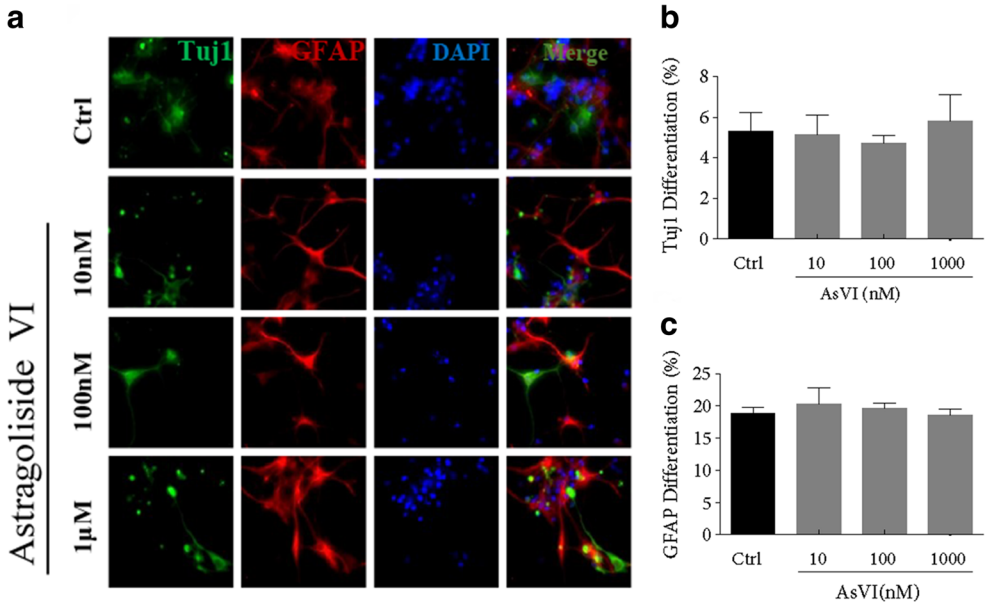

Fig. 5 *Astragaloside VI* has no effect on the differentiation of NSCs in primary cultured *NSCs* in vitro. Cells were treated with PBS or *Astragaloside VI* (10, 100, 1000 nM) for 7 days and the expressions of Tuj1 (green), GFAP (red), and DAPI (blue) were identified by triple immunostaining experiments. **a** Representative immunofluorescent images with DAPI (blue), tuj1 (green), and GFAP (red) staining in primary cultured NSCs. **b** Quantitative analysis on the Tuj1-positive cells in control and *Astragaloside VI* treatment groups. **c** Quantitative analysis on the GFAP-positive cellsin control and *Astragaloside VI* treatment groups (Means ± S.D., $n = 9$ independent tests)

restored significantly learning ability similar to the sham control group. Meanwhile, spatial memory was measured by a probe test at day 6 after the final training trial. The platform was removed during the test and the platform crossing latency and the frequency of crossing target quadrant were recorded. The MCAO vehicle group had significantly longer platform crossing latency time and shorter duration in the target quadrant than the sham control group. *Astragaloside VI* treatment group had significantly lower crossing latency and higher frequency of swimming across the platform in the certain quadrant than the MCAO vehicle group. These results indicate that *Astragaloside VI* could improve the learning ability and restore the memory ability in the post MCAO brain ischemic rats.

We finally investigated the motor function by using the rotarod test. As showed in Fig. 9d–g, the MCAO ischemic vehicle group had significantly lower levels of the average speed, rotating speed, rotating duration, and running distance than the sham control group. However, the *Astragaloside VI* group had significantly higher levels of the average speed, rotating speed, and had a longer duration and running distance than MCAO ischemic vehicle group. Therefore, we conclude that *Astragaloside VI* could promote the recovery of motor function in the post-ischemic rats.

Discussion

In the present study, we report that *Astragaloside VI*, an active compound from a medicinal herb *Radix Astragali*, has neuroprotective effects by reducing infarction size in acute cerebral ischemia-reperfusion injury. Importantly, we prove that *Astragaloside VI* could be an EGFR promoter to inducing neurogenesis in post-ischemic brain injury. The conclusion was supported by the results from the rat model of 1.5-h transient cerebral ischemia in vivo as well as the cultured NSCs in vitro. By detecting BrdU/SOX2-positive staining cells, we found that *Astragaloside VI* treatment increased the newborn neurons at DG zone, SVZ, and cortex in the post-ischemic rat brains in vivo. By detecting BrdU incorporation, neurosphere formation, and MTT assay, we further confirmed that *Astragaloside VI* promoted NSCs self-renewal and proliferation in the cultured NSCs in vitro. The underlying mechanism could be through targeting EGFR and activating EGFR-mediated MAPK/ERK signaling cascades. By dual staining of BrdU/DCX and BrdU/GFAP, we found that *Astragaloside VI* induced neurogenesis and astrogenesis respectively in the post-ischemic brains in vivo. Furthermore, *Astragaloside VI* improved the learning and memory and enhanced motor function in MCAO ischemia-reperfused rats. Those results suggest that *Astragaloside VI* could be a promising neurogenic natural compound for brain repair in post-stroke treatment. To our knowledge, this is the first report that *Astragaloside VI* has neuroprotective and neurogenic effects with the potentials for promoting post-stroke brain repair treatment.

EGF-mediated signaling cascades are critically involved in promoting neurogenesis including NSCs proliferation [21], cell-fate decision, and NSCs dedifferentiation [22, 23]. EGFR could stimulate G0 quiescent cells to enter into S phase [53]. EGFR transfers extracellular signals into cells and induces cell proliferation [54]. Activation of EGFR induced

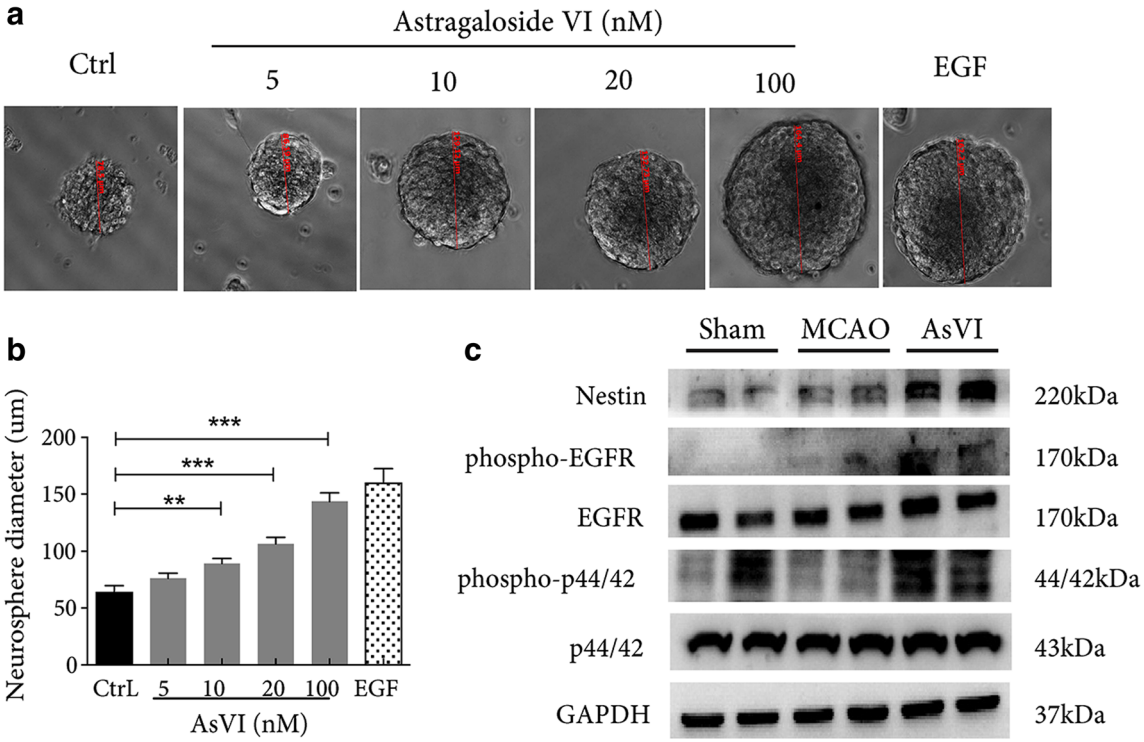

Fig. 6 *Astragaloside VI* has EGF-like effects on promoting neurosphere formation in primary cultured NSCs and activated EGFR/MAPK signaling in post ischemic brains in vivo. In the absence of EGF, primary NSCs were cultured with the medium and treated with PBS in control group (CtrL) or *Astragaloside VI* at the concentrations of 5, 10, 20, and 100 nM. **a** Representative neurophere images in the NSCs treated with PBS or *Astragaloside VI* treatment (5, 10, 20, 100 nM) (left) and that of the NSCs cultured with the medium containing EGF (10 ng/ml) (right). **b** Quantitative analysis on the neurosphere diameters in EGF deprivation control group (CtrL), EGF-positive control (EGF), and *Astragaloside VI* treatment groups with EGF deprivation (Mean ± S.D., $n = 8$ independent tests). Diameters of neurospheres were measured in a blinder manner with ImageJ software. $^{**}p < 0.01$; $^{***}p < 0.001$. **c** Western blot analysis for the effects of *Astragaloside VI* on EGFR-mediated MAPK pathway cascades in the hippocampus of transient ischemic rat brains. Rats were subjected to 1.5 h of MCAO ischemia plus 7 days of reperfusion. *Astragaloside VI* (2 μg/kg wt.) was intravenously injected into the rats once daily for 7 days. Representative immunoblot results of nestin, p-EGFR, EGFR, p-p44/42-MAPK, and p44/42-MAPK in the hippocampus of 1.5 h transient ischemic rat brains plus 7 day reperfusion

the proliferation of neural precursor cells in vivo [20, 24] and reduced brain damage in the experimental ischemic stroke animal model [55, 56]. EGF is also a cellular survival signaling [57, 58]. In the present study, EGF deprivation limited the NSCs growth and neurosphere formation. Interestingly, *Astragaloside VI* dose-dependently promoted NSCs growth and neurosphere formation in the NSCs with the EGF deprivation. It would be logical to confirm the physical interaction between *Astragaloside VI* and EGFR by using Co-IP experiment for pulling down assay. However, with the technical limitation, we can only use different biochemical methods to further confirm the EGFR-promoting effects of *Astragaloside VI* with both in vivo and in vitro experimental evidence. Western blot analysis revealed that *Astragaloside VI* treatment up-regulated phosphorylated EGFR level in both ischemic rat brains in vivo and cultured NSCs in vitro. *Astragaloside VI* treatment promoted the phosphorylated EGF receptor and phosphorylated MAPK in the hippocampus of the ischemic brains. Co-treatment of Gefitinib, a representative EGFR tyrosine kinase domain inhibitor, abolished the effects of *Astragaloside VI* on the NSCs proliferation. Furthermore, we investigated the transactivation of ERK, a downstream EGFR signaling responsible for cell proliferation [59]. PD98059 is a selective MAPK/ERK inhibitor binding to the ERK-specific MAP kinase MEK and prevents the phosphorylation of ERK (p44/p42 MAPK). Co-treatment of PD98059 inhibited the neurosphere formation in the cultured NSCs in presence of *Astragaloside VI*. Those results suggest that *Astragaloside VI* could activate EGFR/MAPK signaling pathway and promote neurogenesis. Nevertheless, even if labeled as specific inhibitors, Gefitinib and PD98059 have quite a few off-target inhibitory effects on other kinases [60, 61]. We should be careful to interpret the results obtained. We will consider using the genetic interventions to double confirm the effects in our future study.

We should note the inconsistencies of the differentiation-promoting effects obtained from the in vivo and in vitro studies. Our in vivo data showed that *Astragaloside VI* treatment remarkably increased neurogenesis and astrogenesis as evidenced by the increased BrdU/DCX and BrdU/GFAP-positive cells in the transient ischemic brains respectively. However, in the in vitro-cultured NSCs, *Astragaloside VI*

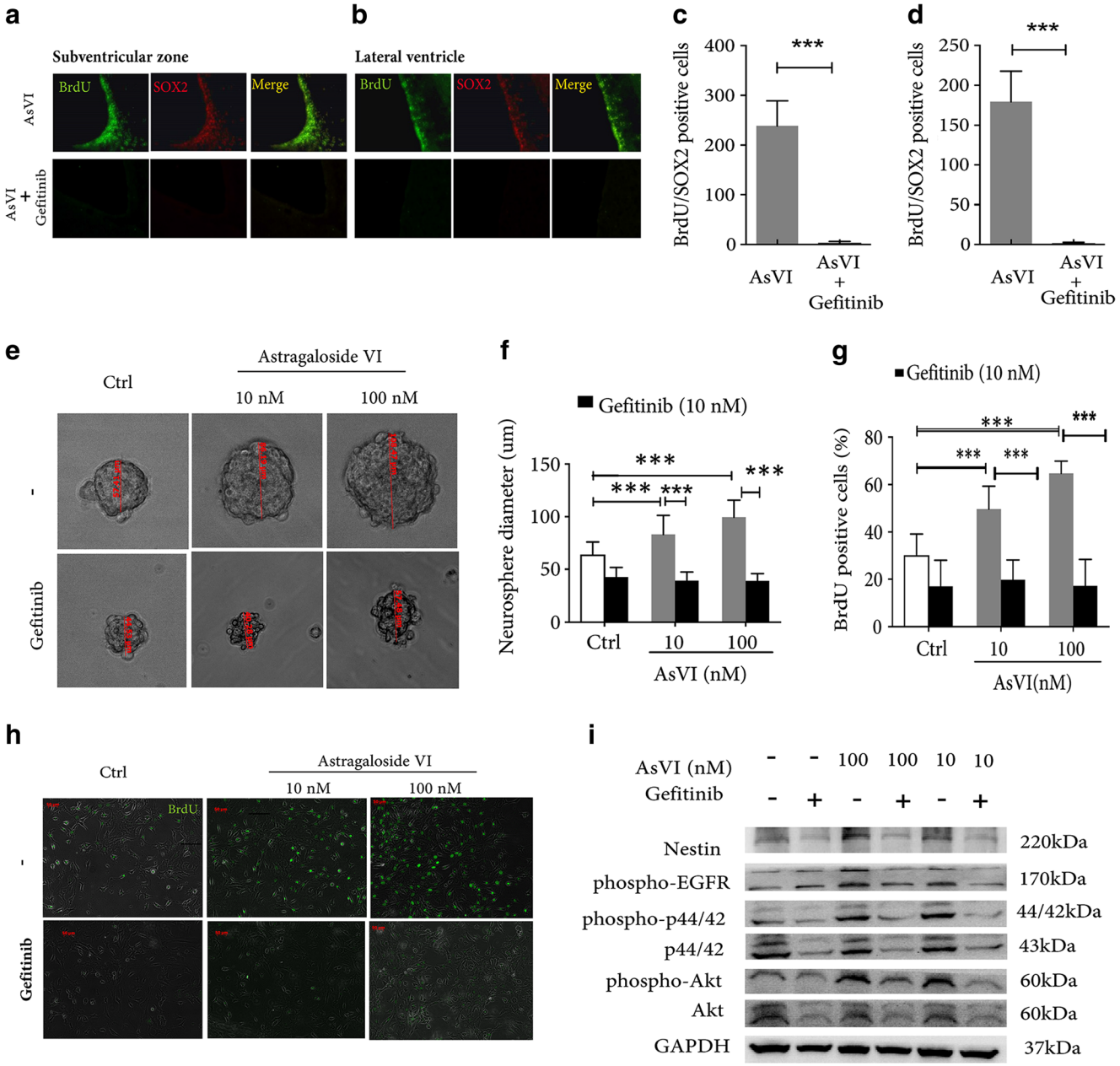

Fig. 7 EGFR inhibitor gefitinib blocks *Astragaloside VI* (AsVI)-mediated NSCs proliferation in transient MCAO ischemic brains in vivo and abolishes *Astragaloside VI*-mediated neurosphere formation and NSCs proliferation in primary cultured NSCs. Rats were subjected to 1.5 h of MCAO ischemia plus 7 days of reperfusion. *Astragaloside VI* (2 μg/kg wt.) was intravenously injected into the rats once daily for 7 days. Gefitinib (4 nM) was intravenously pre-treated to rats before MCAO ischemia and *Astragaloside VI* treatment. **a** Representative BrdU/Sox2 double immunostaining results at SVZ of ischemic rat brains; MCAO + AsVI, MCAO ischemia plus AsVI treatment group; MCAO + AsVI + gefitinib, MCAO ischemia plus AsVI treatment and gefitinib group. **b** Representative BrdU/Sox2 double immunostaining results at LV zone of ischemic brains. **c** Quantitative analysis on the BrdU/SOX2-positive staining cells at SVZ of transient MCAO ischemic rat brains treated with *Astragaloside VI* with or without gefitinib. ***$p < 0.001$. **d** Quantitative analysis on the BrdU/SOX2-positive staining cells at LV zone of transient MCAO ischemic rat brains treated with *Astragaloside VI* with or without gefitinib (Mean and S.D., $n = 3$ rats per group) ***$p < 0.001$. NSCs were pre-incubated with gefitinib (10 nM) for 2 h and then *Astragaloside VI* (10 nM, 100 nM) were added into the medium following with normal medium for another 48 h**. e** Representative images of the neurosphere incontrol groups and *Astragaloside VI* (AsVI) treatment groups (10, 100 nM) with or without Gefitinib treatment. **f** Quantitative analysis on the neurosphere diameters in respective groups (Mean ± S.D., $n = 9$ independent tests). ***$p < 0.001$. **g** Quantitative analysis on the rates of BrdU-positive cells in respective groups (Mean and S.D., $n = 9$ independent tests). *Scalar bar* 50 μm, ***$p < 0.001$. **h** Representative images of BrdU immunostaining in control groups and *Astragaloside VI* treatment groups (10, 100 nM) with or without gefitinib treatment. **i** Western blot analysis for effects of Gefitinib against *astragaloside VI*-mediated p-EGFR, p-p44/42-MAPK, and p-AKT expression in primary cultured NSCs

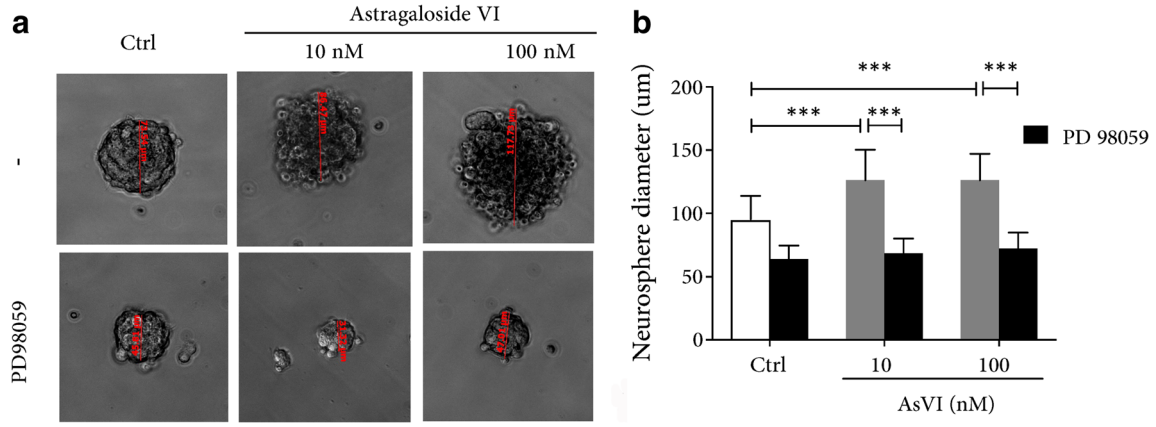

Fig. 8 ERK inhibitor PD98059 abolishes *Astragaloside VI*-mediated neurosphere formation in primary cultured NSCs. **a** Representative images to elucidate effects of ERK inhibitor PD98059 on the *Astragaloside VI*-induced neurosphere formation in primary cultured NSCs. NSCs were pre-incubated with PD98059 (10 μM) for 2 h and then treated with *Astragaloside VI* (10 nM, 100 nM) following with normal medium for another 48 h. **b** Quantitative analysis on the neuorsphere diameters in the respective groups (Mean ± S.D., $n = 9$ independent tests). ***$p < 0.001$

had no effect on inducing the neuronal or astrocyte differentiation as identified by the expressions of Tuj1 and GFAP respectively. The discrepancy might be explained that *Astragaloside VI* could only promote NSCs proliferation by activating EGFR-mediated MAPK/ERK signaling cascades without inducing NSCs differentiation in vitro. In the post-ischemic brain, with much complicated microenvironment, the different pharmacological effects of *Astragaloside VI* from the in vitro study are understandable. There are following potential explanations: (1) *Astragaloside VI* treatment promoted the NSCs proliferation, increased the pool of NSCs, and induced the newly formed neurons and astrocytes in the transient ischemic brain zones for brain repair, subsequently improving the neurological functions; (2) *Astragaloside VI* might affect the interactions of complex environmental factors in the in vivo system, subsequently inducing neurogenesis in

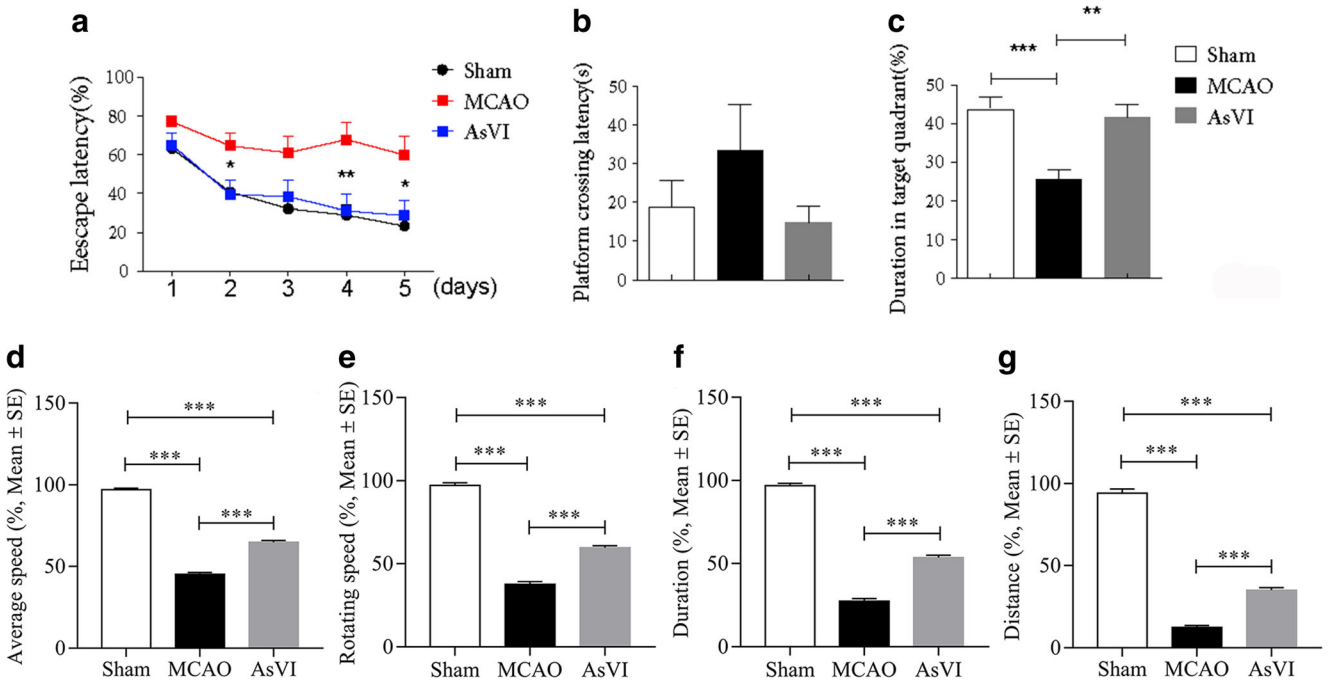

Fig. 9 *Astragaloside VI* restores learning ability, spatial memory, and motor functions in post MCAO ischemic rats. **a** Statistical analysis on the platform crossing latency during water maze test. **b** Statistical analysis on the duration in the quadrant during water maze test. **c** Representative escape latency in respective groups during water maze test. **d** Statistical analysis on the average speed in respective groups during motor detection. **e** Statistical analysis on the rotating speed in respective groups during motor detection. **f** Statistical analysis on the duration in the device in respective groups during motor detection. **g** Statistical analysis on the distance in respective groups during motor detection. Data are presented as Mean ± SEM. $n = 9$–13 rats in the groups. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

the post-ischemic brains. The exact mechanisms remain to be further investigated.

Astragalosides are a group of active compounds with similar parent structures from medicinal herb *Astragalus* [62]. Compared with other *Astragalosides*, *Astragaloside VI* has relatively low concentration in the ingredients of *Astragalus*. In our experiments, even used at low concentrations, *Astragaloside VI* had neuroprotective and neurogenic-promoting effects in both in vivo and in vitro experiments. The effects appear to be better than other *Astragalosides*. For example, *Astragaloside IV* was also reported to protect the blood-brain barrier (BBB) integrity and stimulate angiogenesis in cerebral ischemia-reperfusion injury [42–46]. However, *Astragaloside IV* used in those studies had much higher dosages than *Astragaloside VI* in our study. It is valuable to further elucidate the structure and function relationship between *Astragalosides* and neurogenesis-promoting effects. It would not only bring better understanding the underlying mechanisms of *Astragalus* for post-stroke treatments but also provide an opportunity for drug discovery to target neurogenesis and promote neurological recovery.

In conclusion, *Astragaloside VI* could effectively activate EGFR/MAPK signaling cascades, promote NSCs proliferation, enhance the motor function and improve learning and memory in transient cerebral ischemic rats. *Astragaloside VI* has the potentials to be a new therapeutic drug candidate for post-stroke treatment.

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Authors' Contribution The work was performed and accomplished by all authors. S-JG and XC received fund and designed the experiments. XC, HW, and C-HS conducted the experiments and statistical analyses. X-XJ and QW provided technical support for animal experiments, experimental design, data interpretation, and gave comments on the manuscript. XC and S-JG wrote the manuscript. All authors have read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval Animal experimental protocols were conducted in accordance with the national and institutional guidelines on ethics and biosafety, which were approved and regulated by the Committee on the Use of Live Animals in Teaching and Research (CULATR), HKU. This article does not contain any studies with human participants performed by any of the authors.

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