Early expressions of hypoxia-inducible factor 1 alpha and vascular endothelial growth factor increase the neuronal plasticity of activated endogenous neural stem cells after focal cerebral ischemia

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Abstract
Endogenous neural stem cells become “activated” after neuronal injury, but the activation sequence and fate of endogenous neural stem cells in focal cerebral ischemia model are little known. We evaluated the relationships between neural stem cells and hypoxia-inducible factor-1α and vascular endothelial growth factor expression in a photothrombotic rat stroke model using immunohistochemistry and western blot analysis. We also evaluated the chronological changes of neural stem cells by 5-bromo-2′-deoxyuridine (BrdU) incorporation. Hypoxia-inducible factor-1α expression was initially increased from 1 hour after ischemic injury, followed by vascular endothelial growth factor expression. Hypoxia-inducible factor-1α immunoreactivity was detected in the ipsilateral cortical neurons of the infarct core and peri-infarct area. Vascular endothelial growth factor immunoreactivity was detected in bilateral cortex, but ipsilateral cortex staining intensity and numbers were greater than the contralateral cortex. Vascular endothelial growth factor immunoreactive cells were easily found along the peri-infarct area 12 hours after focal cerebral ischemia. The expression of nestin increased throughout the microvasculature in the ischemic core and the peri-infarct area in all experimental rats after 24 hours of ischemic injury. Nestin immunoreactivity increased in the subventricular zone during 12 hours to 3 days, and prominently in the ipsilateral cortex between 3–7 days. Nestin-labeled cells showed dual differentiation with microvessels near the infarct core and reactive astrocytes in the peri-infarct area. BrdU-labeled cells were increased gradually from day 1 in the ipsilateral subventricular zone and cortex, and numerous BrdU-labeled cells were observed in the peri-infarct area and non-lesioned cortex at 3 days. BrdU-labeled cells rather than neurons, were mainly co-labeled with nestin and GFAP. Early expressions of hypoxia-inducible factor-1α and vascular endothelial growth factor after ischemia made up the microenvironment to increase the neuronal plasticity of activated endogenous neural stem cells. Moreover, neural precursor cells after large-scale cortical injury could be recruited from the cortex nearby infarct core and subventricular zone.

Key Words: nerve regeneration; brain ischemia; neural stem cell; neural precursor cell; hypoxia-inducible factor 1α; vascular endothelial growth factor; microenvironment; photothrombosis; neural regeneration

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Introduction
Blood vessels and synaptic connections arise in the developing brain after injury (Chopp et al., 2008). Stem cells have been proposed as a potential source of new cells to replace those lost due to central nervous system injury, as well as a source of trophic molecules to minimize damage and promote recovery (Burns et al., 2009).

Endogenous neural stem cells have the capacity to respond actively to their environment, and many brain injury model studies such as middle cerebral artery occlusion (Thored et al., 2006), cortical ischemic injury (Jin et al., 2003; Sundholm-Peters et al., 2005), and traumatic brain injury (Ghosh et al., 2003; Salman et al., 2004), have demonstrated that neuroblasts migrate from the subventricular zone into...
the cortical injury region. A question continuously raised is whether neural stem cells are sufficiently activated in a pathological condition (Fagel et al., 2006; Koketsu et al., 2007; Zhang et al., 2007). Furthermore, the precise mechanism underlying endogenous neural stem cell activation in the ischemic brain has not been thoroughly investigated.

Hypoxia-inducible factor 1 (HIF-1) is a basic helix-loop-helix Per-Arnt-Sim transcription factor consisting of two protein subunits called HIF-1α and HIF-1β (Semenza, 2000; Sharp and Bernaudin, 2004). This factor plays a crucial role in the cellular responses to hypoxia in normal and pathological tissues, including stroke. More than 100 putative target genes for HIF-1α have been identified, including erythropoietin and vascular endothelial growth factor (VEGF) (Semenza, 2000; Zhao et al., 2008; Ramos-Cejudo et al., 2012).

Endogenous expression of VEGF mRNA and VEGF have been observed in neuronal cells in postnatal and adult rat brains, and expression is upregulated following stroke (Hayashi et al., 1997; Hai et al., 2003). VEGF has direct neuroprotective effects in cerebral ischemic damage, which may be independent of angiogenesis (Mu et al., 2003). One of the main neuroprotective mechanisms of VEGF is enhanced proliferation and migration of neural progenitors in the subventricular zone, improvement of striatal neurogenesis, proliferation and migration of neural progenitors in the main neuroprotective mechanisms of VEGF is enhanced (Mu et al., 2003). One of the protective effects in cerebral ischemic damage, which may be independent of angiogenesis (Mu et al., 2003). One of the main neuroprotective mechanisms of VEGF is enhanced proliferation and migration of neural progenitors in the subventricular zone, improvement of striatal neurogenesis, and maturation of newborn neurons in adult rat brains after stroke (Schanzer et al., 2004; Sun et al., 2006).

Although activating HIF-1α promotes a widespread adaptive response via overexpression of important downstream genes such as VEGF in some cerebral ischemia or traumatic brain injury models, different expression patterns occur in different experimental models (Anderson et al., 2009). Although a few HIF-1α studies have been reported (Demougeot et al., 2004; Van Hoecke et al., 2007a), VEGF expression profiles (Gu et al., 2001) and endogenous neural stem cell activation in a photothrombotic focal ischemia model (Kang et al., 2008) have not been reported.

This study aimed to investigate the expression of HIF-1α, VEGF, and nestin using a photothrombotic rat stroke model. This study also evaluated the chronological changes of endogenous neural stem cells by 5-bromo-2'-deoxyuridine (BrdU) incorporation and compared HIF-1α, VEGF, and nestin expression in this model.

Materials and Methods

Animals and induction of focal cerebral ischemia

All surgical procedures and postoperative care were performed in accordance with the guidelines of the Chonnam National University Animal Care and Usage Committee, Korea. Sixty male Sprague-Dawley rats, weighing 230–300 g, were used. The animals were maintained on a 12 hour light/dark cycle and were allowed free access to food and water. The rats were anesthetized with 5% isoflurane and maintained with 2% isoflurane in an oxygen/air mixture using a gas anesthesia mask in a stereotactic frame (Stoeling, Wood Dale, IL, USA). Focal cortical ischemia was induced by photothrombosis of the cortical microvessels using Rose Bengal (Sigma Chemical Co., St. Louis, MO, USA) with cold light (Zeiss KL1500 LCD, Oberkochen, Germany), as described previously (Watson et al., 1985). Measurements were made 1 hour, 12 hours, 1 day, 3 days, and 7 days after the onset of ischemia (n = 5 rats per group). The scalp was sutured and the rats were allowed to awaken before being returned to their home cages. Five animals received illumination after infusion of normal saline instead of Rose Bengal for the sham surgery.

BrdU labeling

The S-phase marker 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg body weight; Sigma) was intraperitoneally injected twice a day for 5 consecutive days before ischemic injury. Sham surgery animals received the same BrdU pulse and were euthanized at the same time points.

Western blot analysis

The rat ischemic cerebral hemisphere was dissected, removed at each time point (sham surgery, 1 hour, 12 hours, 1 day, 3 days, 7 days; n = 5 rats per group), immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation. Bilateral cerebral cortex was dissected. Brain tissues were homogenized, and proteins were extracted using complete, Mini, EDTA-free (Roche Applied Science, Waltham, MA, USA) protease cocktail and RIFA lysis buffer (Millipore, Milford, MA, USA). Protein concentration was determined with the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Equal amounts of protein for HIF-1α (100 μg) or for nestin and VEGF (50 μg respectively) were separated on 6–12% SDS-polyacrylamide gels (USB Fueling Innovation, Cleveland, OH, USA). The proteins were transferred to pure nitrocellulose membranes (Bio-Rad, Richmond CA, USA). The membranes were incubated in blocking buffer, 5% non-fat dry milk at room temperature for 1 hour with rotation. The membranes were then incubated with mouse HIF-1α monoclonal antibody (1:500; Millipore), mouse VEGF monoclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse nestin monoclonal antibody (1:1,000; Millipore) overnight at 4°C. Following washes, the membranes were incubated with peroxidase-conjugated anti-mouse immunoglobulin (1:4,000; Santa Cruz Biotechnology) for 1 hour. After rinsing in washing buffer, the bound antibody signal was developed by enhanced Immobilon Western Chemiluminescent horseradish peroxidase (HRP) substrate (Millipore). Quantification of each band was performed by densitometry analysis (Scion software; NIH software, Bethesda, MD, USA) after a protein signal scan (Fuji LAS 3000 image analysis system; Fujifilm Medical Systems, Stanford, CT, USA). Mouse anti-β-actin monoclonal antibody conjugated with HRP (Sigma; 1:25,000) was detected on immunoblots as a loading control for protein quantitation.

Immunohistochemistry and immunofluorescence labeling

Immunohistochemistry was performed on 4-μm-thick paraffin-embedded brain sections as described previously (Kim et al., 2002) (sham surgery, 1 hour, 12 hours, 1 day, 3 days,
Figure 1 Western blot analysis for hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF) and nestin expression after focal cerebral ischemia.
(A) Representative time course of nestin, HIF-1α, VEGF, and nestin expression in control (sham) and ipsilateral cerebral hemisphere extracts from rats at baseline, 1 h, 12 h, 1 d, 3 d, and 7 d. (B) Time courses of normalized optical density of nestin immunoblots in ipsilateral hemispheres (*P < 0.05, **P < 0.01, vs. sham). Western blot protein levels were normalized to β-actin as a loading control. Relative optical density of protein bands was measured following subtraction of the film background. Experiments were repeated three times. Data are shown as mean ± SE. One-way analysis of variance with post-hoc test was used. h: Hour; d: day.

Figure 2 Immunohistochemical detection of hypoxia-inducible factor-1α (HIF-1α) expression and double immunofluorescence staining with HIF-1α and neurofilament (NF) antibodies after focal cerebral ischemia.
Immunohistochemical detection of HIF-1α expression in the ipsilateral cerebral hemisphere at 1 hour (A), 12 hours (B), and 1 day (C) in the infarct core of rats with focal cerebral ischemia. HIF-1α immunoreactive cells mainly co-localized with neurons along the peri-infarct area (D, red: HIF1α, green: neurofilament, blue: 4′,6-diamidino-2-phenylindole (DAPI)). Scale bars: Black is 50 μm and white is 100 μm.

Figure 3 Immunohistochemical detection of vascular endothelial growth factor (VEGF) and double immunofluorescence staining with VEGF and neurofilament (NF) antibodies after focal cerebral ischemia.
Vascular endothelial growth factor (VEGF) immunoreactivity was noted in neurons of the peri-infarct region at different time points. VEGF expression was noted in a few cortical neurons in control rats (A). VEGF positive cells after ischemia were noted in the infarct core and the peri-infarct lesions at 12 hours (B), and 3 days (C). VEGF (red) was colocalized with neurofilaments (green) (D, blue arrow). Scale bars: 100 μm.

Figure 4 Immunohistochemistry for nestin expression after focal cerebral ischemia.
Nestin expression appeared at 1 hour after focal cerebral ischemia in the capillary structures of the infarct core (A), and increased in the infarct core and peri-infarct area at 12 hours (B). Nestin immunoreactive cells near the infarct core are shown as capillary structure (C), and those near the non-lesioned cortex as glial differentiation (D). Scale bars: 100 μm.
7 days, *n* = 5 rats per group). Single-label immunohistochemistry was conducted using the Avidin-Biotin Complex (ABC) method. Briefly, the tissue sections were collected on 3-aminopropyltriethoxysilane-coated slides and immunostained using the ABC method with a Sequenza Immunostaining Rack (Thermo Fisher Scientific Inc., Waltham, MA, USA). Pretreatment of tissues with heat-induced epitope retrieval was required for 5 minutes at 125°C in a pressure cooker with 10 mmol/L citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked by incubation in PBS (pH 7.4) containing 1.5% H$_2$O$_2$. The primary antibodies used for immunohistochemistry were mouse anti-BrdU (1:100; Millipore), mouse anti-nestin (1:200; Millipore), mouse anti-VEGF (1:200; Santa Cruz Biotechnology), and mouse anti-HIF-1a (1:100, Sigma). The slides were incubated with each primary antibody overnight at 4°C. Antibody diluent (Dako, Copenhagen, Denmark) was applied as a negative control stain.

The following primary antibodies were added to the above HIF-1a, VEGF, and BrdU for double immunolabeling: rabbit anti-nestin (1:800; Abcam, Cambridge, MA, USA), and rabbit anti-neurofilament (1:200; Millipore). Cy3 conjugated goat anti-rabbit (1:500; Millipore) or Alexa Fluor488 conjugated chicken anti-rabbit (1:300; Invitrogen, Carlsbad, CA, USA) used as link antibodies emitted red and green fluorescence, respectively, after a fluorescently labeled secondary antibody, was mounted with DAPI/antifade solution (Millipore). Images of immunohistochemically labeled sections and fluorescently immunolabeled sections were acquired using an Olympus BX 51 microscope (Olympus, Tokyo, Japan). Images were captured using Cell-P imaging software (Olympus) in both the FITC and TRITC channels. The cells were yellow when the two images were merged.

Statistical analysis
Statistical analysis was performed using SPSS for Windows (Ver. 14.0; SPSS Inc., Chicago, IL, USA). Western blot protein levels were normalized to β-actin as a loading control. Relative optical density of protein bands was measured following subtraction of the film background. Experiments were repeated three times. Data are shown as mean ± SE. One-

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Figure 5  Immunohistochemical detection of 5-bromo-2′-deoxyuridine (BrdU) after focal cerebral ischemia.
BrdU-labeled cells increased in ipsilateral subventricular zone (A) and ipsilateral cortex (C) at 7 d. A few BrdU-immunoreactive cells were observed in the contralateral subventricular zone (B) and cortex (D). Scale bars: 100 μm. Quantitative analysis of time-dependent BrdU incorporation after focal cerebral ischemia by photothrombosis in the subventricular zone (E) and cortex (F) was presented. Data are expressed as mean ± SE of BrdU-labeled cells in the subventricular zone and cortex. *P* < 0.05, **P** < 0.01. Each experiment was performed three times. One-way analysis of variance with post-hoc test was used. HPF: High power field; h: hour; d: day.

Figure 6 Immunohistochemistry for BrdU and nestin co-labeling after focal cerebral ischemia.
Many ependymal lining cells were co-localized with nestin in the subventricular zone among the BrdU-labeled cells in the subventricular zone 7 days after focal cerebral ischemia. Red: BrdU; green: nestin. Scale bar: 50 μm. BrdU: 5-Bromo-2′-deoxyuridine.
way analysis of variance with post-hoc testing was used to compare multiple western blot and BrdU labeling datasets. A P-value < 0.05 was considered statistically significant.

Results

Western blot results

Western blot analysis with specific HIF-1α, VEGF, and nestin antibodies demonstrated induction of HIF-1α (120 kDa), VEGF (40 kDa), and nestin (210 kDa), respectively (Figure 1A). The HIF-1α protein level increased significantly at 1 hour, 12 hours (P < 0.01), and 1 day (P < 0.05) after ischemia. The transient increase in HIF-1α expression was barely detectable after 3 days. An immunoreactive band of approximately 40 kDa was detected using VEGF antibody, which increased significantly beginning at 1 hour (P < 0.05), and peaked at 12 hours (P < 0.01). Sustained VEGF expression increased to the end of the experiment (P < 0.01, respectively). The densitometry of nestin protein expression showed a gradual increase from 12 hours (P = 0.169), a significant increase at 1 day, and a peak at 7 days (P < 0.01, respectively) (Figure 1B). In summary, HIF-1α increased at a very early stage, and then VEGF increased dramatically at 12 hours following HIF-1α, and nestin increased after HIF-1α and VEGF.

Immunohistochemistry results

HIF-1α

HIF-1α positive cells were detected in the ipsilateral hemisphere beginning 1 hour after ischemia. The number of HIF-1α immunoreactive cells increased at 12 hours (Figure 2A), peaked at 1 day (Figure 2B), and declined at 3 days (Figure 2C). The HIF-1α immunoreactive cells were located in the infarct core and peri-infarct area. No HIF-1α immunoreactive cells were detected in the contralateral hemisphere of the brain. Double immunofluorescence staining with HIF-1α and neurofilament antibodies showed that HIF-1α immunoreactive cells co-localized with neurons (Figure 2D; arrow), rather than other cell types, such as astrocytes.

VEGF

VEGF immunoreactivity was observed in the choroid plexus epithelial cells and cortical neurons (Figure 3A) in control rats. VEGF immunoreactivity was noted in neurons of the peri-infarct region (Figure 3B, C) and vascular endothelial cells (data not shown). We demonstrated that brain VEGF existed in neurons using double-labeling method because VEGF immunoreactive cells were co-labeled with neurofilament protein (Figure 3D). VEGF immunoreactive cells were noted at 1 hour in the infarct core and peri-infarct area. Furthermore, most VEGF immunoreactive cells were easily found in peri-infarct area at 12 hours, peaked at 3 days, and declined at 7 days. A few VEGF immunoreactive cells were scattered in the ipsilateral subventricular zone, corpus callosum, and ipsilateral cortex. Through microscopy, we observed that VEGF immunoreactivity was stronger in the neurons than in the endothelial cells. A few VEGF immunoreactive cells were observed in the contralateral cortex at 1 and 3 days after focal cerebral ischemia.

Nestin

Cell-cycle-specific nestin was expressed in a time-dependent manner. At 1 hour after focal cerebral ischemia, capillary structures in the infarct core were only positive for nestin (Figure 4A). Nestin was weakly expressed in the subventricular zone, infarct core, and peri-infarct area at 12 hours (Figure 4B). Nestin immunoreactive cells increased in the subventricular zone at 1 day, and were clearly identified at 3 days. At 3 days, a large number of nestin immunoreactive cells near the infarct core showed flattened nuclei with luminal structures, as in the capillaries (Figure 4C), but nestin immunoreactive cells near the lesioned cortex showed glial differentiation as a morphological feature (Figure 4D).

BrdU labeling

We also checked BrdU-labeled cells by immunohistochemistry in the subventricular zone and ipsilateral hemisphere of the ischemic brain. BrdU-labeled cells were identified in the subventricular zone, which was thought to be the source of neural precursor cells, and the ipsilateral cortex. Ischemic injury gave rise to a large number of BrdU-labeled cells in the ipsilateral subventricular zone beginning at 12 hours, which increased gradually and then peaked at 7 days (Figure 5A). BrdU-labeled cells were mainly located in the subventricular zone under the ependymal lining in the early phase, which was clarified by BrdU-DAPI co-staining, but overlaid ependymal cells at 1 day (data not shown). In contrast to the subventricular zone, BrdU-labeled cells increased markedly in the peri-infarct area and non-lesioned ipsilateral hemisphere at 3 days and peaked at 7 days (Figure 5C). Interestingly, there were a larger number of BrdU-labeled cells in the peri-infarct cortex than in the ipsilateral subventricular zone at each peak (Figure 5E, F). In the initial stage of infarct development, the number of BrdU and nestin co-labeled cells was very low in the subventricular zone and ipsilateral cortex. However, nestin and BrdU co-labeled cells at 3 days increased dramatically in the ipsilateral cortex rather than in the contralateral subventricular zone and peaked at 7 days (Figure 6).

Discussion

We demonstrated serial changes in HIF-1α, VEGF, and nestin expression during the early stage in a photothermotic rat stroke model. These changes played a dynamic role in repair of brain injury through activation of endogenous neural stem cells.

In this study, the HIF-1α protein increased at 1 hour, 12 hours, and 1 day after focal cerebral ischemia, and HIF-1α immunoreactive neurons were dominantly noted in the infarct core and peri-infarct area (e.g., penumbra) immediately after ischemia. No HIF-1α immunoreactivity was detected in the non-lesioned ipsilateral hemisphere or the subventricular zone. A few reports have described HIF-1α expression in a photothermotic model (Demougeot et al., 2004; Van Hoecke et al., 2007). Although van Hoecke et al. (2007) reported a reverse regional distribution of HIF-1α mRNA expression to the finding by Demougeot et al. (2004), HIF-
HIF-1α was mainly located in the peri-infarct area (Stowe et al., 2008). Considering our results and those of previous reports, HIF-1α was immediately activated in the infarct core and peri-infarct area after hypoxia by decreasing oxygen concentration (Semenza, 2002), yet very little HIF-1α protein expression was probably due to the severity of the insult in the photothrombotic model.

Upregulation of VEGF has been reported after transient global or focal ischemia (Wan et al., 2007; Wang et al., 2007), and infusion of exogenous VEGF could produce an outcome in a rodent stroke model (Lee et al., 2007; Hansen et al., 2008). One report described the VEGF expression pattern by immunohistochemistry in a photothrombotic model (Gu et al., 2001). VEGF immunoactivity was usually noted in neurons rather than endothelial cells, which increased at 24 hours after ischemia. In our study, VEGF immunoreactivity was mainly identified in the neurons of the peri-infarct ipsilateral cortex. However, VEGF expression in the subventricular zone was scarcely identified during the experiment. VEGF is a well-known transcriptional target of post-stroke HIF-1α and has been implicated in the post-stroke recovery through angiogenesis and neuroprotection. HIF-1α plays a regulatory role in both the pro-survival and pro-death responses in the central nervous system after stroke. HIF-1α induces pro-caspase-3 expression after focal ischemia (Van Hoecke et al., 2007), and promotes cellular apoptosis in the peri-infarct area. Interestingly, VEGF inhibits caspase-3 activity in neurons (Jin et al., 2001) and directly upregulates VEGF receptor-2 mRNA (Stowe et al., 2008), which does not have a promoter region to allow for upregulation by HIF-1α. To determine whether HIF-1α and VEGF are co-localized in neurons of the peri-infarct area after neonatal stroke (Mu et al., 2003), unique expression of VEGF in neurons might occur due to neuroprotection in the photothrombotic stroke model.

We examined BrdU-labeled cells by immunohistochemistry in the subventricular zone and ipsilateral hemisphere of the ischemic brain. BrdU-labeled cells were observed in both regions, but the proliferation sequence and absolute BrdU-labeled cell count were significantly different. The subventricular zone, particularly the lateral wall, was a potential source of neural precursors for neuronal injury. Compared with the subventricular zone, BrdU-labeled cells in the ipsilateral cortex increased at 1 day and reached a peak at 7 days compared with those on the contralateral side. The absolute number of BrdU-labeled cells in the ipsilateral cortex was five times higher than the peak cell count in the subventricular zone. We question whether the subventricular zone is the dominant neural stem cell niche during cerebral ischemia. Even though the subventricular zone was destroyed by extensive ischemic injury in our model, a large number of BrdU-labeled cells were observed in the lesioned hemisphere. In fact, the migration speed of subventricular zone in the rostral migration stream was slow to reach the lesion (Luskin and Boone, 1994). These findings suggest the possibility of intraparenchymal progenitors in the neocortex such as in the subventricular zone, and previous reports support our findings (Shimada et al., 2010; Shimada et al., 2012).

Nestin is a well-developed member of the intermediate filament protein family. It is abundantly expressed during early embryogenesis but is only present in endothelial and selected subventricular cells (Li and Chopp, 1999). Nestin has been used to analyze proliferation and differentiation from central nervous system precursor cells in a cerebral injury model (Shin et al., 2008; Shin et al., 2013). As previously reported, nestin expression increased significantly at 1 day and peaked at 7 days in the ipsilateral hemisphere after stroke. In a long-term follow up, nestin expression peaked at 7 days and declined gradually thereafter (Li and Chopp, 1999; Shin et al., 2008). Our morphological and western blot results were consistent with these previous reports. We found that nestin-positive cells contained capillary-like structures in the peri-infarct area that were identified as astrocyte-like morphology. This finding suggests that neuronal progenitor cells and endothelial cells couple for neuronal plasticity in the adult rat neocortex after ischemic injury and induce gliosis to reduce brain tissue damage.

We revealed the sequential increase of HIF-1α, VEGF, and nestin expression in the ischemic hemisphere of a photothrombotic rat stroke model. BrdU+/nestin+ neural precursor cells increased after HIF-1α and VEGF expression. Considering previous reports and our results, HIF-1α and VEGF may play an important role in recruiting endogenous neural stem cells in the subventricular zone or some cortex for neural repair.

In conclusion, we report that HIF-1α and VEGF were expressed in the early stage after ischemia and may play a local guidance role in neural stem cell activation. We propose the possibility of a neural stem cell niche after large cortical injury not only in the subventricular zone but also in the cortex near the ischemic hemisphere in the photothrombotic rat stroke model.

**Author contributions:** Song S and Park JT were responsible for study conception and design, data collection, assembly, analysis and interpretation, and paper writing. Kim HS was in charge of study conception and design, data collection, assembly, interpretation and fundraising for this work. Park MS, Lee JK, and Lee MC participated in study conception and design, and data collection and assembly. Na JY provided manuscript review and revised the manuscript. All authors approved the final version of this study.

**Conflicts of interest:** None declared.

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