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Original article

Ischemia induced neural stem cell proliferation and differentiation in neonatal rat involved vascular endothelial growth factor and transforming growth factor-beta pathways

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Abstract

Brain ischemia is a leading cause of mortality and morbidity in premature infants. Knowing the fate of neural stem cells in the subventricular zone (SVZ) after ischemia and the mechanisms that determine this fate would be useful in manipulating neural stem cell proliferation and differentiation and possibly in reversing ischemic damage. We sought to identify the genes involved in the proliferation and differentiation of neural stem cells after exposure to ischemia in a 3-day-old rat model that approximates ischemia in premature infants. Proliferating cells were labeled by bromodeoxyuridine (BrdU) through intraperitoneal injection. Using immunfluorescence assays, we observed the proliferation and differentiation of neural stem cells. Genes were identified with GeneChip and real-time quantitative polymerase chain reaction analysis. Ischemic rats had more BrdU-positive cells in the SVZ at all four time points and more neural stem cells differentiation into neurons, astrocytes, and oligodendrocytes. GeneChip analysis showed a 3- to 10-fold increase in the mRNA expression of vascular endothelial growth factor, transforming growth factor-beta, and their receptors in the SVZ. PCR assays and Western blot analyses confirmed these results, indicating that vascular endothelial growth factor and transforming growth factor-beta might be two of the factors that involve post-ischemic neural stem cell proliferation and differentiation.

Keywords: Rat; Subventricular zone; Vascular endothelial growth factor; Transforming growth factor-beta; Neural stem cell

1. Introduction

Brain ischemia is a leading cause of mortality and morbidity in premature infants, and there are few therapeutic options once damage has occurred. Despite a large number of animal studies testing promising neuroprotective agents, no clinically successful strategy for neuroprotection has emerged [1]. The subventricular zone (SVZ) is a forebrain region in which neurogenesis continues postnatally. It contains stem cells and more mature progenitors of neurons, astrocytes, and oligodendrocytes [2]. The discovery that neurogenesis continues throughout life in the adult mammalian brain has brought hope that this endogenous mechanism of cell replacement might be enhanced to repair the damaged brain. Many studies have noted enhanced neurogenesis in the SVZ and the subgranular zone in adult rodent brains after focal ischemia [3,4], but the mechanisms of this process are not completely understood. Knowing the fate of neural stem cells after ischemia and the

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mechanisms that determine this fate will likely be useful in manipulating cell proliferation and differentiation.

Recently, several studies have shown that neurogenesis is regulated by stem cell niche [5]. However, little is known about the change of stem cell niche and fate of neural stem cells in the SVZ after cerebral ischemia in the premature brain. Therefore, the purpose of this study was to characterize, by GeneChip analysis, global gene expression after ischemic damage in a neonatal rat model that approximates the damage in a premature infant. The specific aim was to identify genes that may be involved in the proliferation and differentiation of neural stem cells in the SVZ after brain ischemia. This work will be helpful for knowing about the stem cell niche.

2. Materials and methods

2.1. Animal procedures

All procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Care of Experimental Animals Committee. All efforts were made to minimize both the suffering and the number of animals used. From the Experimental Animal Center, Shanghai Medical College, Fudan University, China, we obtained 96, 3-day-old Sprague–Dawley rats weighing between 8.2 and 10.5 g. Rats were evenly divided into two groups: ischemia group and control group, and then in each group 12 rats were killed at 24 h, day 4, day 7, and day 14 after operation, respectively.

As previously described [6], animals were anesthetized with chloral hydrate. Rectal temperature was maintained at 37 °C, plus or minus 1 °C, throughout the procedure using a feedback-regulated water heating system. Rats in the ischemia group were fully anesthetized, a midline neck incision was made, and the bilateral common carotid artery (BCCA) was identified. The BCCA was separated from the vagus nerve and then ligated using 6-0 silk. The incision was sutured and the animals were returned to dam after a recovery period of 1 h at 37 °C. Rats of the control group underwent the same surgical procedure, except that the BCCA was not occluded. To label proliferative cells, BrdU (50 mg/kg) was injected intraperitoneally once daily after the procedure until the rats were killed. Only half of the 12 rats in each group received BrdU at each time point.

2.2. Histologic analysis

Rats received BrdU were transcardially perfused with heparinized saline and 4% paraformaldehyde for 15 min before they were killed. Brains then were removed and immersion-fixed in 4% paraformaldehyde for 24 h at 4 °C. In the next day, the brain tissue samples were dehydrated through graded sucrose (each at 10%, 20%, and 30% for 24 h) at 4 °C and then embedded in OCT medium. Frozen sections of SVZ (10-μm thick) were used for the immunofluorescence assays and the Haematoxylin-osin staining.

The 6 rats at each time point that did not receive BrdU were anesthetized with chloral hydrate and then killed as scheduled. Brains were removed, rinsed with 0.1 M PBS, then SVZ was dissected, and preserved in liquid nitrogen. These SVZ tissues were used to GeneChip analysis, quantitative real-time polymerase chain reaction (PCR) assays and Western blot analysis.

2.3. Immunofluorescence assays

Using antibodies for BrdU, nestin, Neuronal Class III beta-Tubulin (Tuj1), glial fibrillary acidic protein (GFAP), and O4, we performed immunofluorescence assays to measure the proliferation and differentiation of neural stem cells in the SVZ (Table 1). Anti-BrdU antibody was used to label newborn cells. It was combined with an antibody for nestin to label newborn neural stem cells, with an antibody for Tuj1 to label newborn neurons, with an antibody for GFAP to label newborn astrocytes, and with an antibody for O4 to label newborn oligodendrocytes. All methods followed published protocols [7].

Table 1
List of primary and secondary antibodies used in the study.

Antibody against	Species	Dilution	Vendor
BrdU	Rat	1:200	Lab Vision, USA
Nestin	Mouse	1:200	R&D, USA
Tuj1	Mouse	1:200	Chemicon, USA
GFAP	Mouse	1:100	Neuromics, UK
NG2	Mouse	1:200	Chemicon, USA
TRITC-conjugated anti-rat IgG	Gat	1:100	Jackson ImmunoResearch, USA
FITC-conjugated anti-mouse IgM	Rabbit	1:100	Bethyl, USA
VEGF	Rabbit	1:1000	Abcam, UK
TGF-beta	Rabbit	1:2000	Abcam, UK
HRP-conjugated anti-rabbit IgM	Goat	1:2500	Beyotime, China

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For immunofluorescence assays, sections were washed (0.1 M Tris, pH 7.6, 15 min), denatured (2 N HCl, 37 °C, 30 min), rinsed (0.1 M PBS, 10 min), incubated with 1% H_2O_2 in 0.1 M Tris for 30 min, rinsed, blocked (10% normal goat serum, 37 °C, 30 min), and incubated with anti-BrdU primary antibody (4 °C over night). Sections were rinsed (0.1 M PBS, 10 min), incubated with tetraethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rat IgG (room temperature, 2 h), rinsed, then incubated with one of the other primary antibodies, respectively (4 °C, overnight). All sections were washed and incubated with secondary antibodies, and rinsed, mounted, dried, and coverslipped using DAPI. All secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (room temperature, 2 h).

2.4. Cell counting

The number of positive cells was counted with stereologic fluorescence microscopy (Leica, Germany). Systematic random sampling identified every 4th section in a series of 10-μm-thick sections through the SVZ (3 sections per brain, $n = 6$), according to the methods of Plane et al. [8]. Limits were set for counting positive cells in the SVZ in a randomly selected section of each brain. This section was used to determine the cell counting frame size, the number of samples per section, and the spacing of sample sites. All counts were performed using a 200× objective lens on a Leica fluorescence microscope and the StereoInvestigator v.5.0 computer program (MicroBrightField, Colchester, VT). The total number of cells per region was estimated using the Optical Fractionator technique [9]. Data are presented as the mean (standard deviation) of the total cells estimated for each region. Statistical analysis was conducted by one-way ANOVA followed by Bonferroni/Dunn post-hoc test with the SPSS 12.0 statistical software program.

2.5. GeneChip analysis

For this study, we used the SVZ dissected from 24 rat brains (3 from each group at each time point). None of the rats used for GeneChip analysis received BrdU injection. The methods used for RNA extraction, GeneChip sample preparation, hybridization, and data analysis have been described [10]. These methods were based on the Affymetrix GeneChip Expression Analysis Manual.

The RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA yield was measured spectrophotometrically, and the quality of the material was assessed with ethidium bromide staining on agarose gel. The extracted RNA was cleaned (RNeasy Mini Kit, Qiagen, Valencia, CA, USA), converted to double-stranded cDNA (Gibco BRL Superscript Choice System, Life Technologies, Rockville, MD, USA), and then to biotinylated cRNA (Bioarray High Yield RNA Transcription Labeling Kit, Enzo Diagnostics, Farmingdale, NY, USA). After fragmentation, 7 μg of the biotinylated cRNA was hybridized to an Affymetrix Rat 230 2.0 Array. The chips were washed, stained with streptavidin–phycoerythin, and scanned with a probe array scanner.

GeneChip data were analyzed by Affymetrix Microarray Suite MAS 5.0 software. A one-sided Wilcoxon's signed rank test generated a "detection *P*-value" (<0.05) to decide statistically whether a transcript was expressed on a chip.

2.6. Quantitative real-time PCR assays

Genes selected for this analysis exemplify functional categories relevant to our findings. Quantitative real-time PCR was conducted as described by Vemuganti et al. [11] on the same RNA samples used for the GeneChip analysis. The PCR analysis was conducted as described in the instruction manual of real-time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan). For each transcript, quantitative real-time PCR was conducted three times in duplicate using each of the cDNA samples. The amplified transcripts were quantified with the comparative C_T method using GAPDH as the internal control. The primers were designed using the Primer Express software (Primer premier 5.0) based on the GenBank accession numbers, and the sequences were as in Table 2.

2.7. Western blot analysis

Frozen tissues of SVZ (3 from each group at each time point) were cut into small pieces and homogenized in 0.5 mL of RIPA buffer (150 mmol/L NaCl, 1% N-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate, 50 mmol/L Tris–hydrochloric acid, 2 mmol/L phenylmethylsulfonyl fluoride, pH 7.4), then transferred into small tubes and rotated at 4 °C overnight. Solubilized protein was collected after centrifugation at 10000*g* for 30 min. The supernatant from each culture was collected, and protein concentrations were quantified with the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Haimen, China).

To detect VEGF, TGF-beta protein levels, tissue protein lysate from each group of rats were subjected to SDS–PAGE analysis with 10% (wt/vol) acrylamide gel and electrotransferred onto a PVDF membrane (Millipore Corporation). Immunoblotting was performed using 1 μg/mL of rabbit anti-rat VEGF polyclonal antibody, or 0.5 μg/mL of rabbit anti-rat TGF-beta polyclonal antibody, respectively. The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Antibodies are list in Table 1). Immunoreactive pro-

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Table 2
Primer sequence of 9 genes used for quantitative real-time PCR.

Gene name (Gene symbol)	Accession no. (GeneBank)	Primer sequence (5′-3′)
Nestin (Nes)	NM_012987	F: GGAGCAGGAGAAGCAAGGTC R: GGGTCCAGAAAGCCAAGAG
Tubulin, beta 3 (Tubb3)	NM_139254	F: CCCCAGCTTACCTTCCTACC R: GAGGACAGAGCCAAGTAAAC
Glial fibrillary acidic protein (GFAP)	NM_017009	F: AGAAAACCGCATCACCATTC R: GCATCTCCACCGTCTTTACC
Chondroitin sulfate proteoglycan 4 (NG2,Cspg4)	NM_031022	F: TGCAGCACAAAAGGATCTATG R: AGCACTGCCTCCTGGACTAC
Vascular endothelial growth factor A (Vegfa)	NM_031836	F: AGAAAGCCCATGAAGTGGTG R: ACTCCAGGGCTTCATCATTG
FMS-like tyrosine kinase 1 (Flt1)	NM_019306	F: GAGGATGAAGACGACCCTGA R: TGTCAGAGGCTGTTGTCTGG
Transforming growth factor, beta 1 (Tgfb1)	NM_021578	F: TGGAAATCAATGGGATCAGTC R: GGAGCTGTGCAGGTGTTGAG
MAD homolog 2 (Smad2)	NM_019191	F: GGGTTTTGAGGCCGTTTATC R: GCTTGAGCATCGCACTGAC
Thymoma viral proto-oncogene 1 (Akt1)	NM_033230	F: ACTCATTCCAGACCCACGAC R: CCGGTACACCACGTTCTTCT

teins were then visualized using ECL plus a Western blotting detection system (Beyotime Biotechnology, Haimen, China).

3. Results

3.1. Pathological changes following BCCA

Haematoxylin-eosin staining was used to observe the histological change following BCCA. There were no changes in the control group. The pathological changes were significant at the 4th day and then reduced from the 7th day after the operation in the rats of ischemia group. There were cerebral edema widely, neuronal cells necrosis and focal cerebromalacia formation (Fig. 1).

3.2. Neural stem cell proliferation in the SVZ after ischemia

To determine whether BrdU+ cells in the SVZ were neural stem cells, sections from control and ischemic rats at each time point were double-stained with nestin and BrdU antibodies. Representative images of the SVZ at day 7 are shown in Fig. 2A. A few BrdU–Nestin

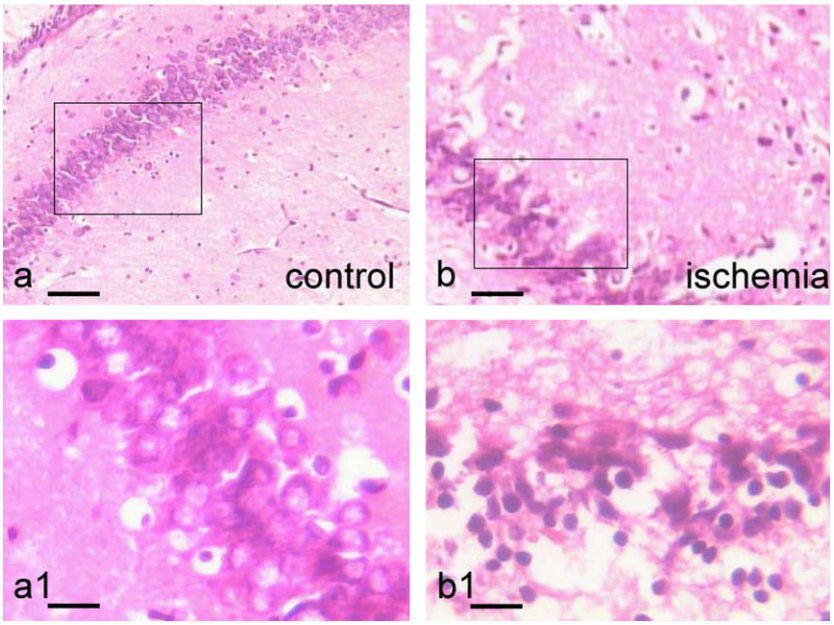

Fig. 1. Pathological changes in the SVZ in rats at day 4 after operation with Haematoxylin-eosin staining. The boxes in a and b indicate the areas magnified in a1 and b1. There were cerebral edema widely, neuronal cells necrosis and focal cerebromalacia formation in ischemia group. And there are no changes in the control group. Bar = 100 μm in a and b. Bar = 50 μm in a1 and b1.

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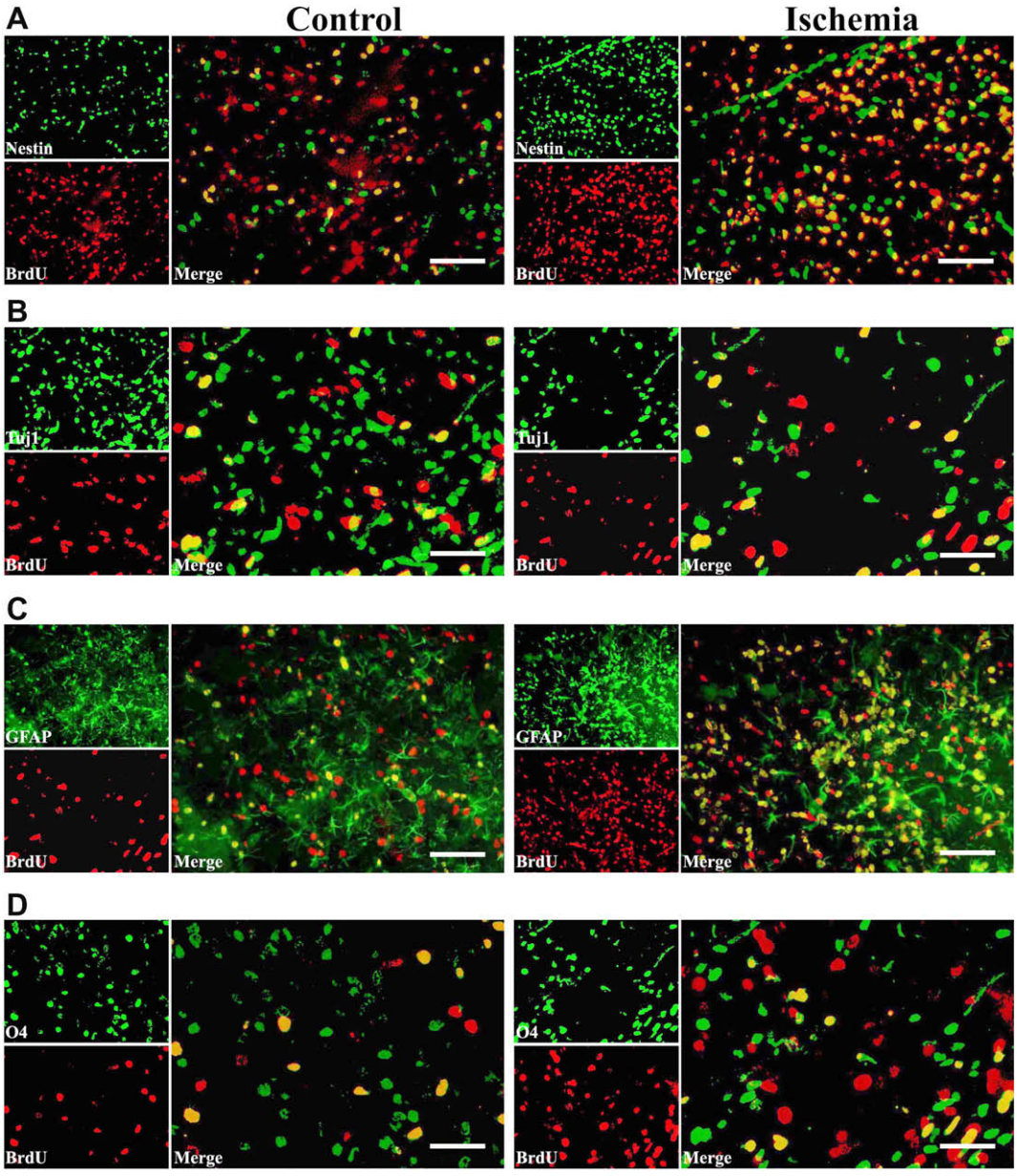

Fig. 2. The proliferation and differentiation of neural progenitor cells in the SVZ after ischemia 7 days. (A) Cells staining positive for BrdU and Nestin, a marker for neural progenitor cells. (B) Cells staining positive for BrdU and Tuj1, a marker for immature neurons. (C) Cells staining positive for BrdU and GFAP, a marker for astrocytes. (D) Cells staining positive for BrdU and O4, a marker for oligodendrocytes. Bar = 50 μm in A, B, C and D.

positive cells were seen in the SVZ of control animals, showing a basal level of neural stem cell proliferation. The number of BrdU–nestin positive cells was the highest at day 7 and was significantly higher in the SVZs of ischemic rats than in control rats ($P < 0.01$; Fig. 3A).

3.3. Neural stem cell differentiation in the SVZ after ischemia

To determine whether the BrdU-labeled cells expressed phenotypic neuronal features, we double-labeled frozen sections with antibodies against BrdU and against Tuj1, a marker for immature neurons; glial fibrillary acidic protein (GFAP), a marker for identifying astrocytes; and O4, a marker for oligodendrocytes (Fig. 2B–D). The number of BrdU–Tuj1 positive cells, BrdU–GFAP positive cells, and BrdU–O4 positive cells peaked 7 days after ischemia. The number of these three types of cells was higher in ischemic rats than in controls. Differences between the two groups at 24 h, day 4, day 7 and day 14 were significant ($P<0.01$; Fig. 3B–D).

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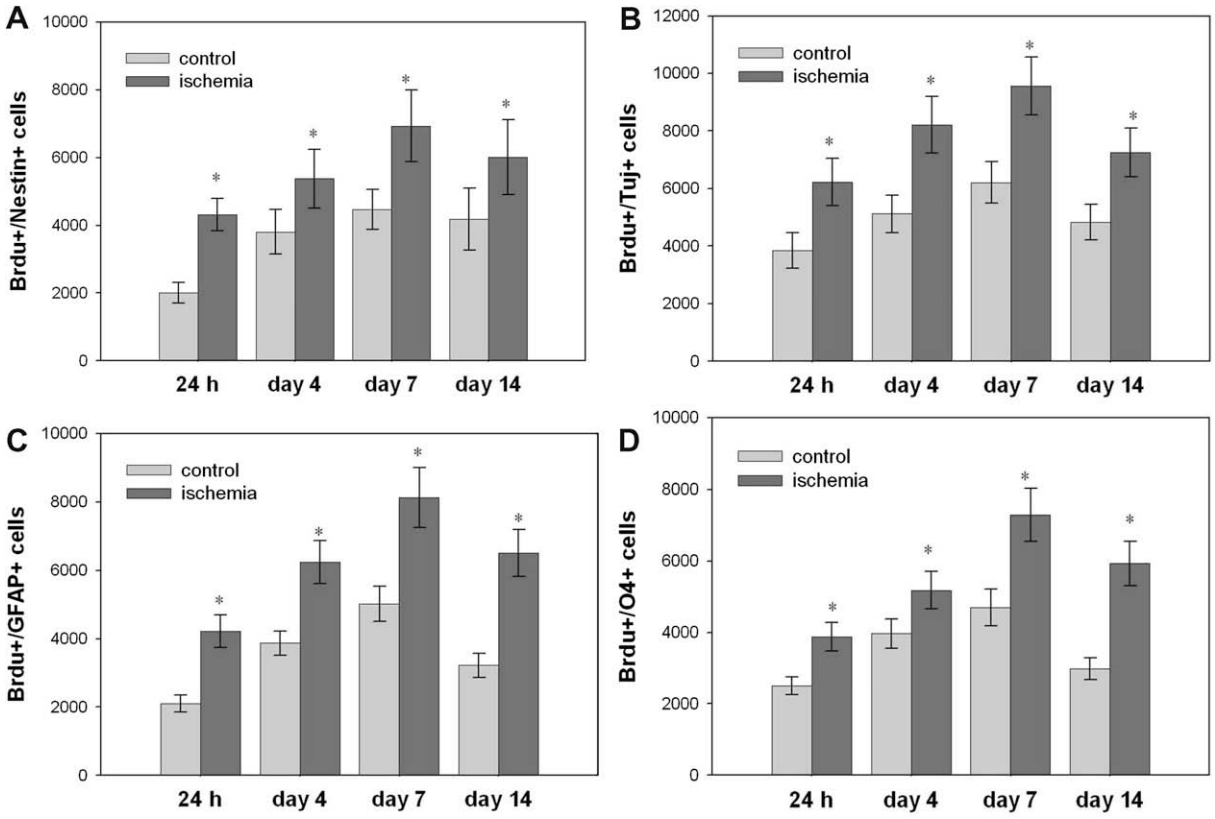

Fig. 3. Quantitfication of double-stained positive cells in the SVZ of control and ischemic neonatal rats at different time points after operation. (A) The number of BrdU+/Nestin+ cells, indicating newborn neural progenitor cells. (B) The number of BrdU+/Tuj1+ cells, indicating newborn neurons. (C) The number of BrdU+/GFAP+ cells, indicating newborn astrocytes. (D) The number of BrdU+/O4+ cells, indicating newborn oligodendrocytes. Values are means and SDs. Values were compared with ANOVA, $^{*}P < 0.01$.

3.4. Changes in gene expression in the SVZ after ischemia

To observe the changes of gene expression following ischemia and to find which one play a role in the increased neural stem proliferation in the ischemic area, we analyzed the mRNA expression profiles of the ischemic SVZ after BCCA was ligated. In both groups, 35–42% of the total transcripts analyzed (840 to 1008 of 2400) were expressed in the SVZ. Of the 2400 transcripts analysed using the Affymetrix Rat Genome GeneChip, 82 increased (by three-fold or higher) and 39 decreased (by three-fold or lower) in abundance in the ischemic SVZ compared with controls. The gene ontology analysis revealed that categories of protein modification and folding, immune and inflammatory response, programmed cell death, cell growth and/or maintenance and regulation of cellular processes were overrepresented after ischemia. Of the total, 21 transcripts were related to cell death, proliferation and differentiation. Nine transcripts were up-regulated and 12 transcripts were down-regulated more than 3-fold change as much in the ischemia group than in the control group. The full names of all the transcripts altered are given in Table 3. To avoid redundancy, only abbreviations are used in the text. Hierarchical clustering of the data from all the chips resulted in two clusters of transcripts (Vegfa, Tgfb1 and their receptors) showing increased abundance in the ischemic group than in the control group. The changes in the expression of these two clusters transcripts in the post-ischemic brain were confirmed by real-time PCR.

To confirm the changes observed by GeneChip analysis, we analyzed the mRNA expression profiles of the SVZs by quantitative real-time PCR. Consistented with the GeneChip results, real-time PCR also showed significant differences in mRNA levels among the groups. The mRNA expression of vascular endothelial growth factor (VEGF), flt1 (VEGF receptor 1), AKT1, transforming growth factor-beta (TGF-beta) and smad2 (a central component of the TGF-beta signaling pathway) was significantly greater in ischemic rats than in control rats ($P < 0.01$; Fig. 4).

3.5. Changes in protein expression in the SVZ after ischemia

In order to confirm histological expression of the gene products we found out from the data of GeneChip

Table 3
Major classes of transcripts altered in the neonatal rat subventricular zone after ischemia.

Gene name	Gene symbol	Accession NO. (GeneBank)	Probe set	Parametric *p*-value	Ratio (ischemia/control)
Up-regulated					
Vascular endothelial growth factor A	Vegfa	NM_031836	1387328_at	0.0024823	10.27
Gap junction membrane channel protein beta 1	Gjb1	NM_017251	1387145_at	0.003439	4.58
Dishevelled, dsh homolog 1 (Drosophila)	Dvl1	NM_031820	1369997_at	0.0017693	9.54
Platelet derived growth factor, alpha	Pdgfa	NM_012801	1393494_at	0.0175357	2.13
FMS-like tyrosine kinase1	Flt1	NM_019306	1395197_at	0.0036449	4.12
Thymoma viral proto-oncogene 1	Akt1	NM_033230	1367402_at	0.0057642	3.52
Fas ligand (TNF superfamily, member 6)	Tnfsf6	NM_012908	1377655_at	0.0496303	1.69
Transforming growth factor, beta 1	Tgfb1	NM_021578	1378652_at	0.0027843	9.75
Growth differentiation factor 11	Gdf11	XM_343148	1393697_at	0.0061968	2.86
Down-regulated					
Thymus cell antigen 1, theta	Thy1	NM_012673	1369651_at	0.0164697	0.33
Hepatocyte growth factor	Hgf	NM_017017	1387701_at	0.0437724	0.83
Serine (or cysteine) peptidase inhibitor, clade F, member 1	Serpinf1	NM_177927	1381012_at	0.0044109	0.11
Tumor necrosis factor receptor superfamily, member 8	Tnfrsf8	NM_019135	1368635_at	0.0132373	0.31
Interleukin 18	Il18	NM_019165	1378533_at	0.0307787	0.67
Insulin-like growth factor 1 receptor	Igf1r	NM_052807	1390671_at	0.007606	0.16
Fibroblast growth factor 10	Fgf10	NM_012951	1369604_at	0.0093072	0.18
Coagulation factor II (thrombin) receptor-like 1	F2rl1	NM_053897	1387596_at	0.0113626	0.27
Phosphatase and tensin homolog	Pten	NM_031606	1370112_at	0.0273252	0.54
Interleukin 10	Il10	NM_012854	1387711_at	0.0319167	0.69
Nudix (nucleoside diphosphate linked moiety X)-type motif 6	Nudt6	NM_181363	1370435_at	0.0057096	0.13
Interleukin 6 signal transducer	Il6st	NM_001008725	1373140_at	0.0359866	0.78

and real-time PCR analyses, we observed the protein expression of VEGF and TGF-beta by Western blot analysis. The results showed that the expression of VEGF and TGF-beta protein peaked on day 7 and it was higher in the ischemia group at all time points compared with those in control group ($P < 0.01$, Fig. 5).

4. Discussion

Neuronal loss is a major feature of numerous brain diseases, including ischemic brain injury in premature infants. Increases in neurogenesis after brain injury also correlate with improved outcomes [12]. The immature neonatal brain maintains a similar or perhaps even greater potential for neuronal repair after hypoxic–ischemic injury than does the adult brain. In 2001, Levison et al. found that hypoxic–ischemic injury results in the sustained depletion of neural stem cells [13]. Moreover, cells within the SVZ are particularly sensitive to insults [14]. Thus, the repair of neonatal brain injury through activating the endogenous neural stem cells in SVZ has received much attention.

Neurogenesis requires three major steps: neural stem cell proliferation, migration, maturation. Ischemic and traumatic insults in adult mammals increase endogenous neural stem cell proliferation and differentiation [4,15]. Neural stem cell proliferation and differentiation toward a specific phenotype are regulated by the characteristics of the environment or niche in which they reside [16]. However, the mechanisms underlying neurogenesis, with its tight regulation, specialized microenvironments, and susceptibility to multiple stimuli, are not completely understood [17].

In this work, we provide evidence that the VEGF and TGF-beta pathways mediate ischemia-induced proliferation and differentiation of neural stem cell in neonatal rats. First of all, we observed cellular changes in the SVZs of 3-day-old rats after ischemic injury using immunofluorescence assays. BrdU, an analog of thymidine, is incorporated into the DNA during the S-phase and has been used to investigate cell proliferation. Although BrdU labeling is not without pitfalls and limitations, it is still mainly strategy to study cell proliferation [18]. In the control group, the Brdu-positive cells were peaked at day 7. The data indicate that immature brain keeps on development after birth, suggesting the immature neonatal brain perhaps maintains greater potential for neuronal repair. Moreover, our BrdU labeling data revealed significant increases in the number of BrdU-labeled cells in the SVZ after BCCA occlusion, suggesting that cerebral ischemia induces cell proliferation.

To investigate whether the BrdU-labeled cells expressed phenotypic neuronal features, we used different markers (nestin for neural stem cells, Tuj1 for immature neurons, GFAP for astrocytes, and O4 for oligodentrocytes) to double-label the sections. Within 7 days after ischemia, most of BrdU-positive cells were

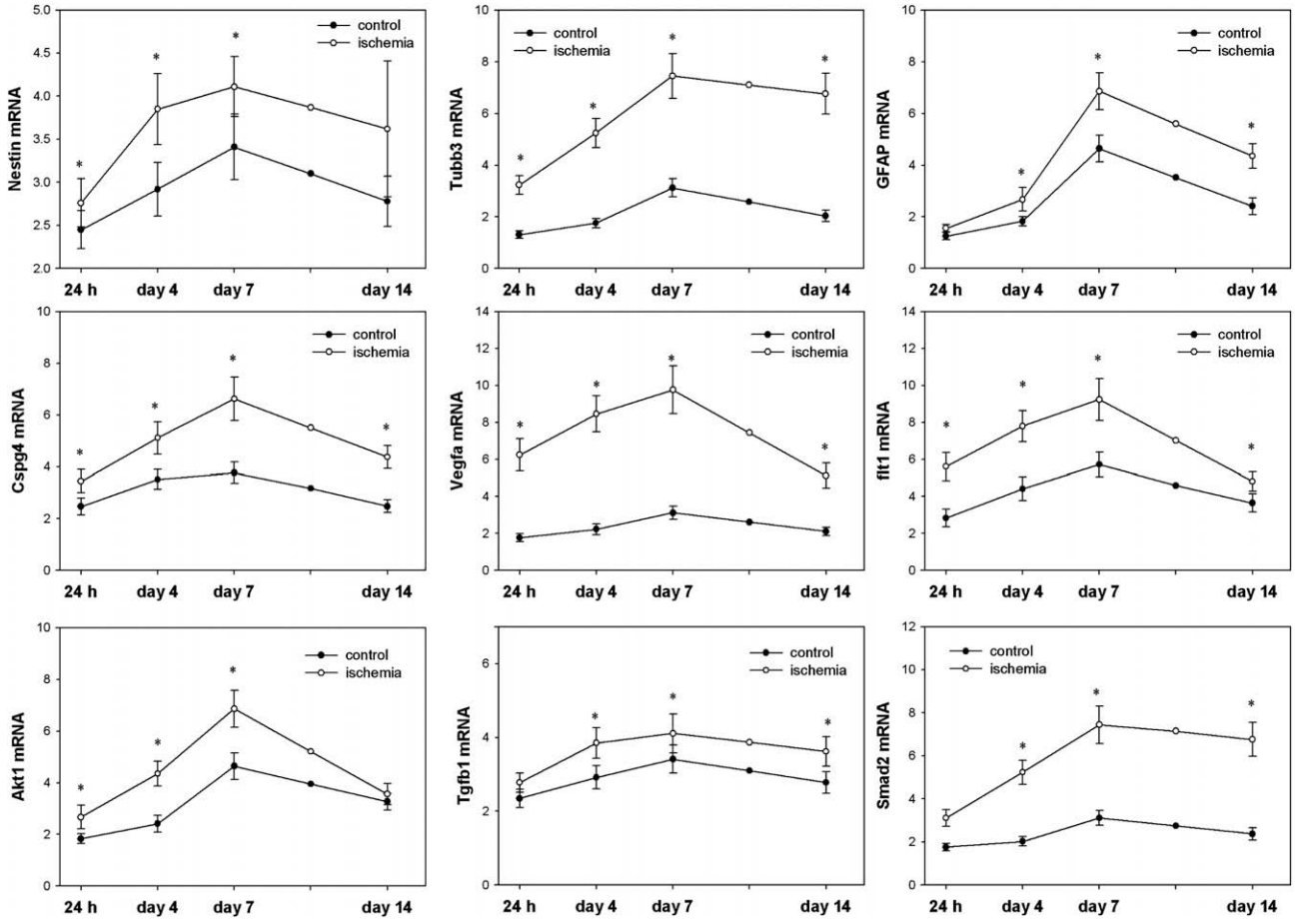

Fig. 4. Changes in the expression of genes related to cell proliferation and differentiation over time in SVZ of neonatal rats after brain ischemia. Values are means and SDs of log copies/2μg total RNA. Values were compared with ANOVA, $^{*}P < 0.01$.

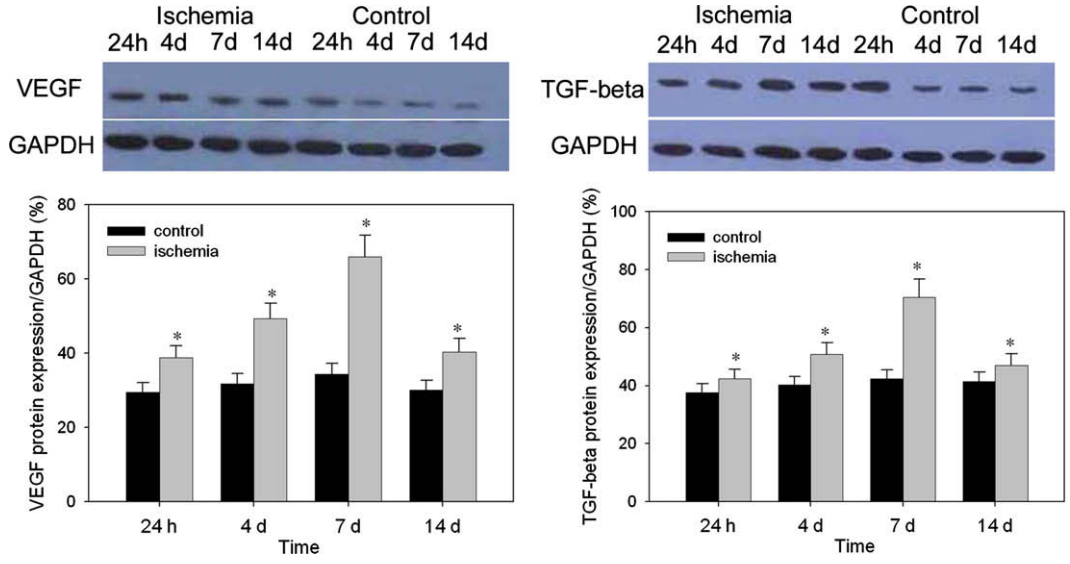

Fig. 5. Changes in the expression of VEGF and TGF-beta protein in SVZ of neonatal rats after brain ischemia. $^{*}P < 0.01$.

nestin-positive, and the number of these cells increased over time, suggesting that ischemia stimulates the self-renewal of neural stem cells. BrdU-positive cells expressing Tuj1, an immature neuron marker; GFAP, an astrocyte marker; and O4, an oligodendrocyte marker, also increased in the SVZ affected by ischemic injury. These

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data strongly indicate that ischemic insults in neonatal rats up-regulate the endogenous neural stem cell proliferation and differentiation.

To explore the characteristics of the environment or niche in which neural stem cells reside, we analyzed the changes of gene expression in the SVZ after ischemia by GeneChip analysis and quantitative real-time PCR assays. GeneChip analysis showed that 9 transcripts were up-regulated in the SVZ after ischemia over time. Hierarchical clustering of the data from all the chips resulted in two clusters of transcripts showing increased abundance in the ischemic group than in the control group, which were related to cell proliferation. Among these transcripts, VEGF and TGF-beta are two potent endogenous mitogens, both in development and in adult brain. VEGF, discovered as an angiogenic and vascular permeability factor, is now recognized as a neurotrophic factor [19]. Endogenous neuronal VEGF is up-regulated in rat brains after transient ischemia, and is neuroprotective [20]. VEGF acts as a chemoattractant for neuronal progenitors, increases axonal outgrowth, improves neuronal survival, and protects cells against ischemic in vitro [19,21,22]. A direct effect of VEGF in stimulating proliferation of neuronal precursors was reported by Zhu et al. [23]. These studies suggested that VEGF might be important in neurogenesis.

TGF-beta is a key multifunctional growth factor in development and tissue repair [24]. In the CNS, TGF-beta is an injury-related cytokine, especially associated with astrocyte-scar formation in response to brain injury [25]. Emerging data on TGF-beta and its signaling molecules suggest that TGF-beta might be a crucial regulator of CNS development [26,27]. TGF-beta receptor expression has been described in different brain regions and cell types, although their location is still a subject of discussion [28,29]. Importantly, TGF-beta has been implicated in the development of dopaminergic neurons in vitro and in vivo, in cooperation with sonic hedgehog [30]. Therefore, we observed the mRNA expression of VEGF, TGF-beta and components of their signaling pathway in the SVZ after ischemia by quantitative real-time PCR. We found that the mRNA expression of VEGF, flt1 (VEGF receptor 1), AKT1, TGF-beta and smad2 (a central component of the TGF-beta signaling pathway) increased after ischemia injury in accordance with the GeneChip results. Then, we observed the protein expression of VEGF and TGF-beta and got the identical results. These data further confirmed that VEGF and TGF-beta may be important in ischemia-induced neural stem cell proliferation and differentiation in neonatal rat, through activation of the flt1 and smad2 signaling pathway. Further study of VEGF and TGF-beta in neurogenesis will be beneficial in efforts to manipulate cell proliferation and differentiation.

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