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Leukemia Inhibitory Factor Decreases Neurogenesis and Angiogenesis in a Rat Model of Intracerebral Hemorrhage*

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Summary: Neurogenesis and angiogenesis can improve the neurologic function after intracerebral hemorrhage (ICH). Leukemia inhibitory factor (LIF) plays an important role in neurogenesis and angiogenesis. In this study, a rat model of autologous blood-induced ICH was used to evaluate the effect of LIF on the neurogenesis and angiogenesis following ICH. After ICH, LIF-positive neurons and dilated vessels were detected in the peri-hematomal region. It was found that LIF levels increased significantly and peaked 14 days after ICH induction. Double immunofluorescence confirmed that LIF was expressed in neurons and endothelial cells. ICH also led to increases of doublecortin (DCX)- and von Willebrand factor (vWF)-positive cells as well as proliferation of cell nuclear antigen (PCNA)+/DCX+ and PCNA+/vWF+ nuclei. All these ICH-induced increases were significantly attenuated by exogenous LIF infusion. These data suggested that LIF was a negative regulator of neurogenesis and angiogenesis after ICH.

Key words: intracerebral hemorrhage; neurogenesis; angiogenesis; leukemia inhibitory factor

Spontaneous intracerebral hemorrhage (ICH), induced by small vessel bleeding within brain parenchyma, is a fatal stroke subtype. A recent survey indicated that ICH accounted for 23.8% of all strokes in China[1]. Compelling evidence has reported that ICH frequently leads to severe and prolonged neurological deficits due to primary and secondary damage[2, 3].

It is well accepted that neurogenesis and angiogenesis play an pivotal role in facilitating the neurological recovery after stroke[4]. During neurogenesis, the newborn neurons from neural stem cells (NSCs) migrate to damaged brain regions and replace dead neurons. And the new blood vessels resulting from endothelial cells (ECs) proliferation could help new neurons migrate to damaged brain regions, and provide oxygen, glucose and nutrients supplement to new neurons[5]. Accumulating evidence showed that promoting post-hemorrhagic neurogenesis and angiogenesis could ameliorate functional outcome[6–9], which indicated that neurogenesis coupled with angiogenesis was a promising therapeutic strategy for ICH.

Increasing evidence showed that inflammation could block ICH-related neurogenesis and angiogenesis[7, 10]. Leukemia inhibitory factor (LIF), a member of the interleukin-6 cytokine family, has pleiotropic effects on cell function in different biological contexts[11]. Several studies have demonstrated that LIF could promote astrocytic differentiation of NSCs[12], inhibit ECs proliferation[13], and reduce neurogenesis[14]. However, LIF had been reported to promote neurogenesis following spinal cord injury (SCI)[15]. Therefore, the aim of this study was to further investigate the effect of endogenous LIF on ICH-induced neurogenesis and angiogenesis.

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1 MATERIALS AND METHODS

1.1 Animal Groups and ICH Model

A total of 120 adult male Sprague-Dawley rats weighing 250–300 g were purchased from the Laboratory Animal Center of China Three Gorges University and housed with free access to food and water. All animals were randomly allocated to 4 groups: sham group (*n*=40), ICH group (*n*=40), ICH + phosphate buffered saline (PBS) group (vehicle group, *n*=20), and ICH + LIF group (*n*=20). The rat ICH model was established as previously described[8]. Rats were anesthetized with chloral hydrate (400 mg/kg) and placed on a stereotaxic apparatus (STOELTING, USA). An approximately 1 cm midline incision was made to expose the skull, and a 1 mm burr hole was drilled, located on the right region 3.2 mm away from the bregma. One hundred μL non-heparinized autologous whole blood obtained from the femoral artery was infused into the right globus pallidus (3.2 mm lateral and 1.4 mm posterior to the bregma, 5.6 mm below the surface of the skull) using a 26-gauge needle. The sham control group received only a needle insertion. All experimental procedures in this study were approved by the Ethics Committee of China Three Gorges University and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

1.2 Exogenous LIF Administration

LIF (100 ng, Chemicon, Temecula, USA) was dissolved in PBS to a total volume of 10 μL, and was administered into the right lateral ventricles (0.8 mm posterior to the bregma, 4.8 mm ventral to the surface of the skull, 1.5 mm lateral to the midline) under stereotactic guidance immediately after ICH. An equal volume of vehicle (PBS) was given to the control group in the same way[16].

1.3 Immunohistochemistry

After anesthesia with chloral hydrate (400 mg/kg), rats were intra-cardially perfused with 0.9% saline and subsequently fixed with 4% paraformaldehyde. The whole brains were removed and kept in 4% paraformaldehyde for 4 to 6 h, then transferred into 30% sucrose solution for cryoprotection. Thirty μm coronal sections were obtained using a cryostat (CM1900, Leica, Germany).

1.4 Spatial Profile of LIF

Immunohistochemistry was used to observe spatial profile of LIF, and the method was described in detail in our previous publications[8, 9]. The primary antibody was rabbit anti-LIF (1:100, Santa Cruz Biotech, USA), and the secondary antibody was biotinylated anti-rabbit immunoglobulin G (1:100, Vector Laboratories, USA). Double immunofluorescence was used to determine whether LIF was expressed in neurons and ECs. The sections were incubated overnight with primary antibody rabbit anti-LIF (1:50) and different markers were used as follows: mouse anti-neuronal nuclei (NeuN, 1:200, Abcam, USA), and goat anti-von Willebrand factor (vWF, 1:200, Chemicon International, USA). Secondary antibodies used were AF488-conjugated goat anti-rabbit antibody (1:100) and Cy3-conjugated rabbit anti-mouse or goat antibody (1:100, Jackson Immuno Research Laboratories, USA).

1.5 Evaluation of Neurogenesis and Angiogenesis

Doublecortin (DCX) and vWF were used to label neurons and vessels, respectively. After pretreated with 3% H_2O_2, and blocked with 5% bovine serum albumin (BSA, Sigma) to hinder nonspecific binding, the sections were incubated overnight with goat anti-DCX (1:200, Santa Cruz Biotechnology, USA) or goat anti-vWF (1:400), which was followed by incubation in anti-goat immunoglobulin G (1:100, Vector Laboratories). Staining was visualized with diaminobenzidine (Vector Laboratories, USA).

Double immunofluorescence was performed to analyze proliferating cell nuclear antigen (PCNA)+/DCX+ and PCNA+/vWF+ nuclei. The sections were incubated overnight with mouse anti-PCNA (1:200, Santa Cruz Biotech, USA), goat anti-DCX (1:100) and anti-vWF (1:200) at 4°C, and a mixture of a AF488-conjugated rabbit anti-mouse antibody (1:100, Jackson Immuno Research Laboratories, USA) and a Cy3-conjugated rabbit anti-goat antibody (1:100, Jackson Immuno Research Laboratories, USA) for 2 h at room temperature. The stained sections were examined with a laser scanning confocal microscope (LSM-510, Zeiss, Germany). For a negative control, the primary antibody was replaced with 1% BSA in each experiment.

1.6 Quantification for the Staining

Five sections per rat were randomly selected, and four fields of the view in each section were randomly selected for cell counts at ×40 objective magnification with Motic Images Advance 3.2 image analysis software by an investigator blinded to the experimental groups[17]. The data were presented as the number of positive cells or nuclei per mm^2 (N/mm^2).

1.7 Real Time RT-PCR Analysis of LIF mRNA

The total RNA was extracted from 100 mg tissue near the hematoma in each group using TRIZOL Reagent according to the manufacturer's instructions (Invitrogen, USA). The integrity of total RNA was detected by agarose gel electrophoresis; the purity and concentration were detected by a spectrophotometer (UV-1201, Shimadzu, Japan). First-strand cDNA synthesis was performed with 2 μg of total RNA using a PrimeScript™ RT reagent kit with gDNA Eraser (Fermentas, USA). PCR amplification was performed using SYBR Premix ExTaq™ PCR kit (4 μL of 1:2 cDNA dilution was used. Takara Biotechnology, Japan) in a LightCycler Real-Time Detection System (Roche Diagnostics

Limited, Germany). The PCR reaction was performed as follows: initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 45 s. Oligonucleotide primers for LIF and β-actin were as follows: LIF, sense 5′-ACGGCAACCTCATGAACCA-3′ and antisense 5′-GGAAACGGCTCCCCTRGA-3′; β-actin, sense 5′-CGTTGACATCCGTAAAGAC-3′ and antisense 5′-TGGAAGGTGGACAGTGAG-3′. The PCR results were quantified using the threshold cycle (Ct) method, and the relative mRNA expression of target gene was normalized to that of the housekeeping gene β-actin[18].

1.8 Statistical Analysis

Data were presented as mean±standard deviation. Differences between groups were evaluated statistically by using Student's *t* test, and differences in the same group at different time points were evaluated statistically by using one-way analysis of variance (ANOVA) followed by Scheffe's *post hoc* test. Differences were considered to be significant at $P<0.05$.

2 RESULTS

2.1 LIF Expression around the Hematoma

Immunohistochemistry showed some LIF-positive neurons and dilated vessels in the peri-hematomal region. And these positive cells increased significantly and peaked 14 days after ICH induction ($P<0.05$). Double immunofluorescence confirmed that LIF immunoreactivity was co-localized with NeuN-positive neurons and vWF-positive ECs. However, LIF-positive neurons and LIF-positive dilated vessels were barely detectable in the sham group (fig. 1).

Consistent with the results of immunohistochemistry, real-time RT-PCR demonstrated that the notable up-regulation of LIF mRNA in the ipsilateral basal ganglion began at day 3, and it reached a maximal level at day 14 post-ICH ($P<0.05$) (fig. 1).

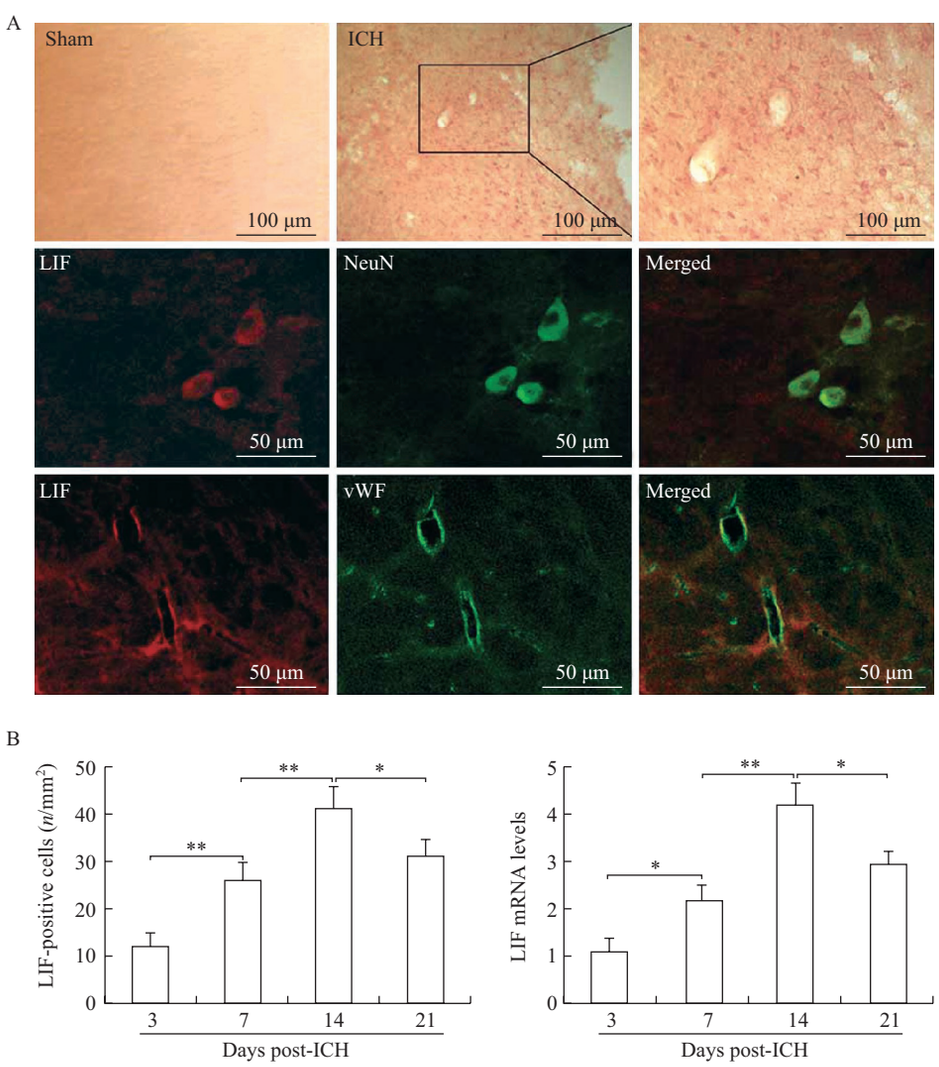

Fig. 1 LIF expression around the hematoma

A: LIF immunoreactivity in the peri-hematomal region or in the sham tissue. LIF immunoreactivity was co-localized with NeuN-positive neurons and vWF-positive ECs. B: Levels of the LIF staining and mRNA after ICH. $^{*}P<0.05$, $^{**}P<0.01$, $n=5$

2.2 Exogenous LIF Decreases Neurogenesis and Angiogenesis in Peri-hematomal Region

Immunohistochemistry was performed to quantify DCX- and vWF-positive cells, markers for neurogenesis and angiogenesis. In the sham control group, only a few DCX- and vWF-positive cells were found scattered. However, a markedly increase in DCX- and vWF-positive cells was shown in the peri-hematomal regions until day 14 (P<0.05). After exogenous LIF treatment, DCX- and vWF-positive cells decreased notably (P<0.01) (fig. 2).

PCNA+/DCX+ and PCNA+/vWF+ double immunofluorescence was used to analyze the number of newly formed neurons and microvessels. Results showed that PCNA+/DCX+ and PCNA+/vWF+ nuclei could be observed in the ipsilateral basal ganglion at day 3, and peaked at day 14 (P<0.01), which could be significantly attenuated by exogenous LIF infusion (P<0.01) (fig. 3).

3 DISCUSSION

In the past decade, the neurovascular unit (NVU), which consists of neurons, endothelial cells, astrocytes and basal lamina, is a key structural basis to enable proper brain homeostasis and has been presented as a new paradigm to understand the pathology of stroke[19, 20]. After hemorrhagic stroke, the homeostasis of the

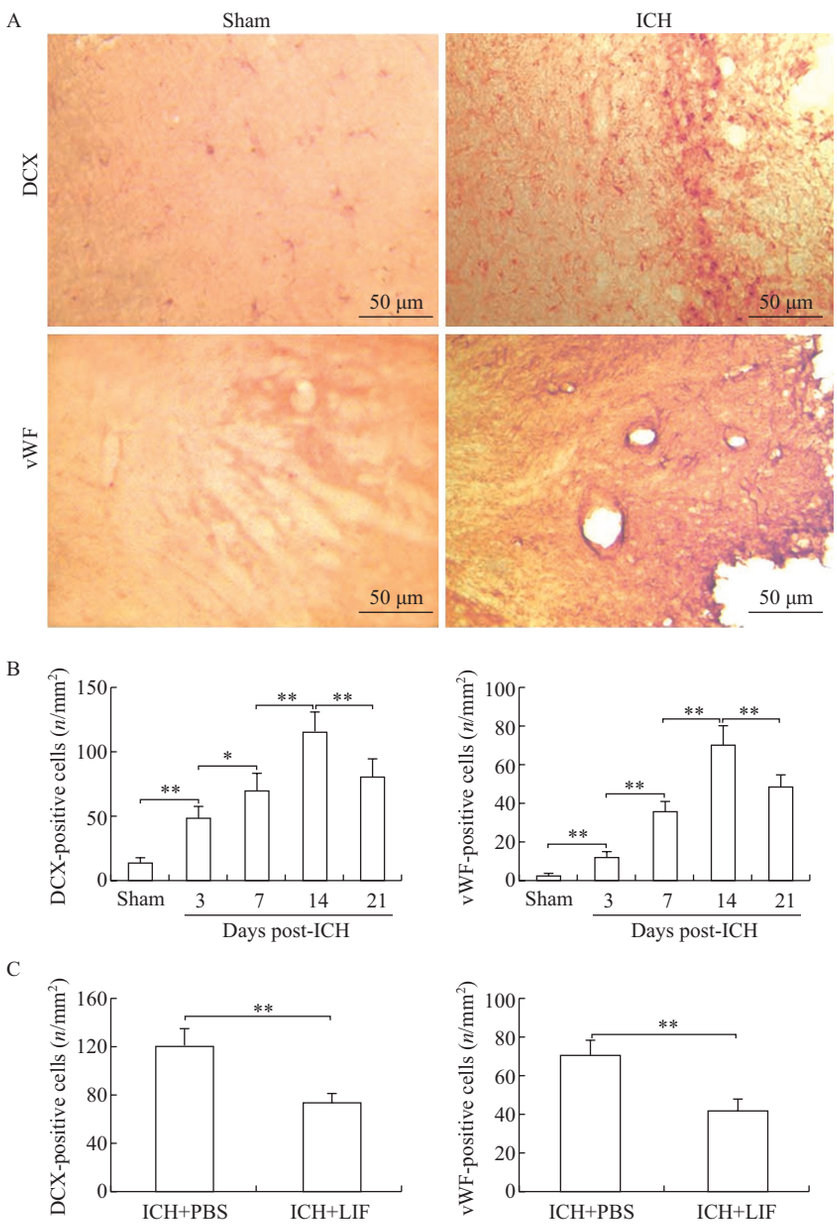

Fig. 2 DCX- and vWF-positive cells around the hematoma

A: immunohistochemistry for detection of DCX and vWF in sham group and ICH group; B: quantitation of the number of DCX- and vWF-positive cells after ICH; C: Exogenous LIF decreased the number of DCX- and vWF-positive cells 14 days after ICH. $^{*}P$<0.05, $^{**}P$<0.01, n=5

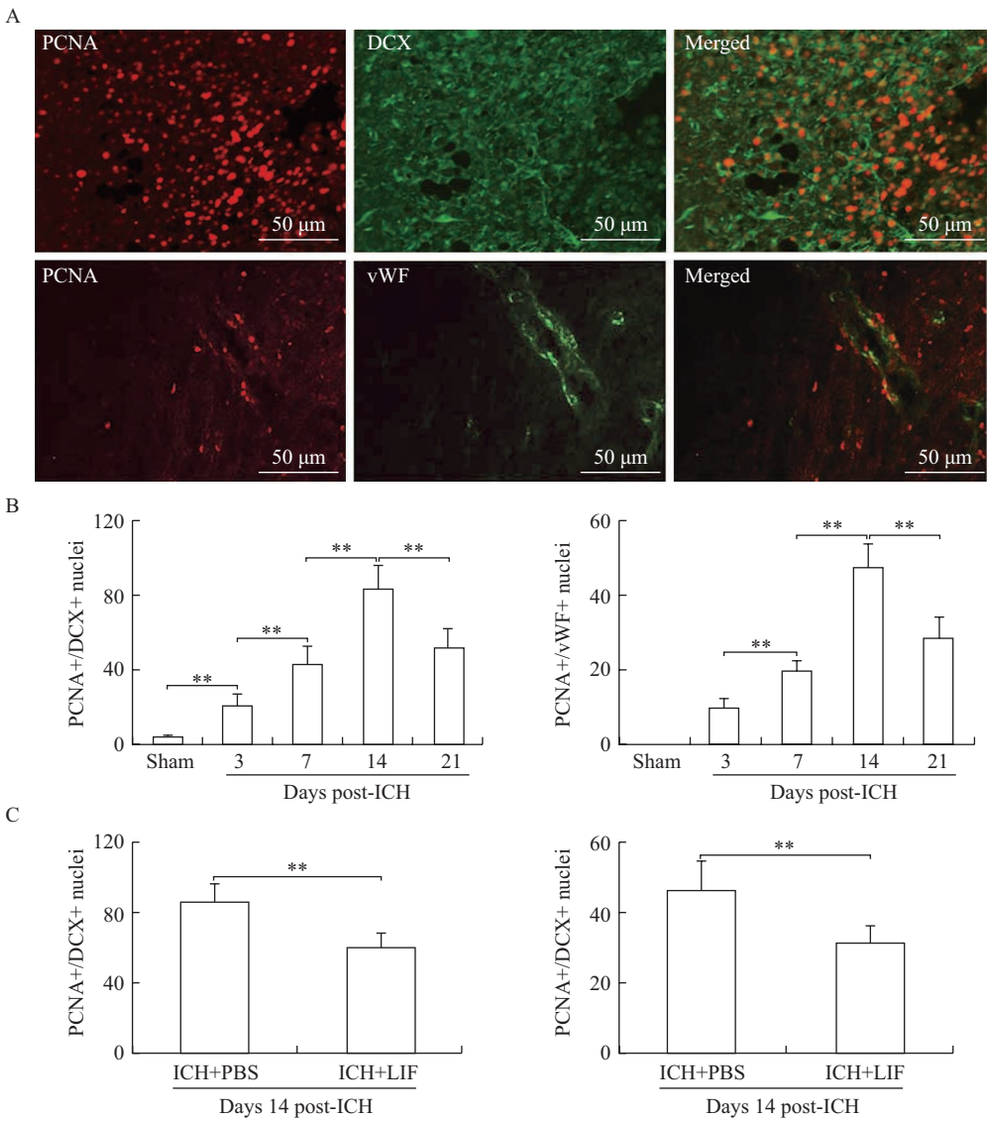

Fig. 3 Proliferated neurons and ECs after ICH
A: PCNA+/DCX+ and PCNA+/vWF+ nuclei were detected 14 days after ICH. B: quantitative analysis of PCNA+/DCX+ and PCNA+/vWF+ nuclei after ICH. C: Exogenous LIF significantly reduced PCNA+/DCX+ and PCNA+/vWF+ nuclei induced by ICH. $^{*}P<0.05$, $^{**}P<0.01$, $n=5$

NVU is broken[21]. To reconstruct the homeostasis of the NVU, endogenous neuroprotective programs such as neurogenesis, angiogenesis and astrocyte activation are activated[6, 7, 22]. Several studies have demonstrated that astrogliosis induced by astrocyte activation decreased neurogenesis and angiogenesis[23, 24]. Recently, the upregulation of LIF has been reported to contribute to astrogliosis and aggravate neurological deficits after ICH[25]. In the present study, we proved that LIF decreased ICH-induced neurogenesis and angiogenesis and well, and neurons and ECs proliferation could be inhibited by exogenous LIF infusion.

Neurogenesis and angiogenesis are linked together and coordinated after ischemic[5, 26] and hemorrhagic stroke[7, 27]. Neuroblasts migrate to the injured regions where angiogenesis occurs, and these neuroblasts were close to cerebral vessels[28]. Hence, not only newborn neurons but also new blood vessels could be detected around the hemorrhagic region.

LIF was expressed in a variety of cells including ECs, neurons, and astrocytes after central nervous system injury[29, 30]. Recently, LIF was reported to express in astrocytes after ICH[25]. Herein a strong up-regulation of LIF was observed and expressed in neurons and ECs after ICH as well, which indicated that the upregulation of LIF was related to neurogenesis and angiogenesis. LIF inhibited ECs proliferation and attenuated angiogenesis *in vivo*[13] and *in vitro*[31], and LIF-deficient mice exhibited increased microvessel density[32]. However, the role of LIF in neurogenesis is controversial. LIF was found to enable NSCs to differentiate into astrocytes rather than

neurons, and reduce neurogenesis in the adult brain[12], while endogenous LIF administration could exert neuroprotective effect by promoting neurogenesis after SCI[15]. Based on these research findings, endogenous LIF administration was given to investigate the effect of LIF on neurogenesis and angiogenesis after ICH in the current study. And the results showed that endogenous LIF led to a reduction of neurogenesis and angiogenesis induced by ICH. The possible explanation for these discrepancies, we speculate, could be due to different animal models used.

A multitude of cellular and molecular mechanisms may be associated with inhibitive effect of LIF on ICH-induced neurogenesis and angiogenesis. Firstly, LIF could activate Notch1 signaling[33]. Hairy/enhancer of split1, a classical target of Notch1, could reduce ECs proliferation[34] and NSCs differentiation into neurons[35]. Secondly, LIF could negatively regulate the expression of vascular endothelial growth factor (VEGF)[32, 36], which is critical for neurogenesis and angiogenesis[37]. A number of studies have demonstrated that Notch1 signaling is activated[38, 39] and expression of VEGF elevated[7, 40] after ICH. However, it is unclear whether or not LIF down-regulating neurogenesis and angiogenesis in ICH is partially Notch1/VEGF mediated.

In summary, our study demonstrated that the upregulation of LIF was a negative regulator of neurogenesis and angiogenesis in a rat model of ICH, suggesting a new potential therapeutic target for ICH treatment.

Conflict of Interest Statement

The authors declared that they have no conflicts of interest.

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