Hes1 Knockdown Exacerbates Ischemic Stroke Following tMCAO by Increasing ER Stress-Dependent Apoptosis *via* the PERK/eIF2 α /ATF4/CHOP Signaling Pathway

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Abstract Apoptosis induced by endoplasmic reticulum (ER) stress plays a crucial role in mediating brain damage after ischemic stroke. Recently, Hes1 (hairless and enhancer of split 1) has been implicated in the regulation of ER stress, but whether it plays a functional role after ischemic stroke and the underlying mechanism remain unclear. In this study, using a mouse model of ischemic stroke *via* transient middle cerebral artery occlusion (tMCAO), we found that Hes1 was induced following brain injury, and that siRNA-mediated knockdown of Hes1 increased the cerebral infarction and worsened the neurological outcome, suggesting that Hes1 knockdown exacerbates ischemic stroke. In addition, mechanistically, Hes1 knockdown

promoted apoptosis and activated the PERK/eIF2 α /ATF4/CHOP signaling pathway after tMCAO. These results suggest that Hes1 knockdown promotes ER stress-induced apoptosis. Furthermore, inhibition of PERK with the specific inhibitor GSK2606414 markedly attenuated the Hes1 knockdown-induced apoptosis and the increased cerebral infarction as well as the worsened neurological outcome following tMCAO, implying that the protection of Hes1 against ischemic stroke is associated with the amelioration of ER stress *via* modulating the PERK/eIF2 α /ATF4/CHOP signaling pathway. Taken together, these results unveil the detrimental role of Hes1 knockdown after ischemic stroke and further relate it to the regulation of ER stress-induced apoptosis, thus highlighting the importance of targeting ER stress in the treatment of ischemic stroke.

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Keywords Hes1 · Ischemic stroke · PERK/eIF2 α /ATF4/CHOP pathway · ER stress · Transient MCAO

Introduction

Ischemic stroke-induced brain damage is common, devastates the central nervous system, and causes death and disability across the world [1]. Currently, the first-choice therapeutic strategy applied to cope with the ischemic insult is timely reperfusion, which is aimed at restoring the cerebral blood supply [2]. However, reperfusion undesirably causes further damage to the vulnerable ischemic brain tissue, by ischemia/reperfusion (IR) injury, and eventually worsens the clinical outcome [3]. To date, there is overwhelming evidence that I/R injury causes brain damage by triggering a series of distinct but overlapping cell signaling pathways involved in such processes as

oxidative stress, inflammation, excitotoxicity, and acidosis [4]. Despite this knowledge, however, the mechanisms underlying I/R injury are still far from fully understood, and no effective therapy for preventing or limiting ischemic stroke-induced brain damage has been developed.

In the last decade, accumulating evidence has demonstrated that neuronal apoptosis induced by endoplasmic reticulum (ER) stress plays an important role in mediating brain damage after ischemic stroke [5, 6]. ER stress activates the unfolded protein response (UPR) pathways by inducing protein kinase RNA-like endoplasmic reticulum kinase (PERK), which increases the phosphorylation level of eukaryotic initiation factor 2 alpha (eIF2 α) and triggers a pro-adaptive response by inhibiting global protein synthesis and the selective translation of activating transcription factor 4 (ATF4). In contrast, prolonged ER stress results in apoptosis and damage, such as that reported in rat models of I/R injury [7–9]. The notion that ER stress may be a promising target in I/R injury is further supported by evidence that inhibiting ER stress protects neurons against ischemic damage [10]. Therefore, elucidating the regulatory mechanisms of ER stress during ischemic stroke holds the promise of discovering novel therapeutic targets for reducing brain damage.

Hes1 (hairy and enhancer of split 1) is a basic helix-loop-helix transcription factor that is important for several activities in the nervous system, such as development, differentiation, and proliferation [11]. Recently, Hes1 has been connected with the regulation of UPR pathways and ER stress-induced apoptosis [12]. However, whether it plays a role in regulating ER stress and subsequent brain damage during ischemic stroke is unknown. In this study, by using an animal model of ischemic stroke developed by transient middle cerebral artery occlusion (tMCAO), we report a detrimental role of Hes1 knockdown in ischemic stroke, which is dependent on increasing ER stress-dependent apoptosis *via* its modulatory function in the PERK/eIF2 α /ATF4/CHOP signaling pathway.

Materials and Methods

Antibodies and Reagents

The primary antibodies against Hes1, Bax, and Bcl-2 were from Abcam (Cambridge, MA, USA). The primary antibodies against p-PERK (Thr980, #3179), PERK (#3192), p-eIF2 α (Ser51, #9721), eIF2 α (#5324), and cleaved caspase-3 (#9661) were from Cell Signaling Technology (Beverly, MA, USA). The primary antibodies against ATF4 (sc-200), CHOP (sc-166682), and β -actin (sc-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies conjugated with

horseradish peroxidase were from Santa Cruz. The PERK inhibitor GSK2606414 was from Selleck Chemicals (Houston, TX, USA).

Animals and Cerebral Ischemic Stroke Model

Twelve-week-old wild-type male C57BL/6 mice were used in this study to establish the ischemic stroke model. Mice were housed in pathogen-free conditions throughout the study. Mice for all experiments were randomized and grouped prior to cerebral I/R induction by tMCAO [13, 14]. Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg) via intraperitoneal (i.p.) injection. A midline neck incision was made to isolate the left external carotid and pterygopalatine arteries, which were then ligated with silk. The internal carotid artery (ICA) at the peripheral site of its bifurcation and the pterygopalatine artery were occluded with a small clip and the common carotid artery (CCA) was ligated with silk. The external carotid was cut and a nylon monofilament with a blunted tip (0.20 mm) using a coagulator was inserted; a silk suture was tightened to prevent bleeding. The monofilament was advanced after removal of the clip on the ICA. The parietal bone turned pale on the occluded side during MCA occlusion, and a laser Doppler flowmeter was used to monitor the blood flow and assure successful occlusion [15]. The monofilament and the CCA ligature were removed after 1 h of occlusion and reperfused for 72 h with the release of blood flow from ICA. The mice in the sham group receiving surgery without ligation were used as controls. Each group included 12 mice. All animal experimental procedures were conducted in accordance with protocols approved by the Ethics Committee of The First Affiliated Hospital of Jinan University.

RNA Extraction and Real-time Quantitative RT-PCR

Total RNA was extracted from the ipsilateral cortex and striatum using TRIzol reagent (ThermoFisher Scientific, San Jose, CA, USA) and then complementary DNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed to quantify gene expression using the SYBR Green Realtime PCR Master Mix (ThermoFisher Scientific) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The mouse house-keeping *Actb* gene was used as an endogenous control. The primers were as follows:

Hes1 sense 5'-TCCCGGCATTCCAAGCTAG-3', anti-sense 5'-GTCACCTCGTTCATGCACTC-3'; *Actb* sense

5'-ACTGGGACGACATGGAGAAG-3', antisense 5'-GTCTCCGGAGTCCATCACAA-3'.

Western Blotting Analysis

Total protein was extracted from the ipsilateral cortex and striatum using the Tissue Protein Extraction Kit (Phygene Life Sciences, Shanghai, China). After denaturation and quantification, the protein samples were loaded onto 8%–10% gel for separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described [16]. Briefly, the separated proteins were transferred onto PVDF membranes (Millipore, Bedford, MA, USA), followed by blocking for 1 h at room temperature (RT) with 5% non-fat dry milk diluted in TBST. The membrane was cut based on the molecular weights of the protein targets and probed with the corresponding primary antibodies overnight at 4°C. After washing with TBST for 30 min, the membranes were incubated with the corresponding secondary antibodies for 1 h at RT. The blots were detected using the ECL Plus Detection System (GE Healthcare, Piscataway, NJ, USA). The band intensity was quantified using ImageJ software (<http://rsbweb.nih.gov/ij>).

In Vivo Administration and siRNA-mediated Knockdown

The administration of inhibitor and siRNA transfection in mice subjected to tMCAO or sham surgery was performed as adapted from a previous report [17]. A volume of 4 µL of the PERK inhibitor GSK2606414 (20 µmol/L) or 4 µL siRNA targeting mouse Hes1 or scrambled siRNA were injected through a Hamilton microsyringe (0.5 µL/min, 8-min duration) into the left cerebral ventricle at 1.0 mm posterior to bregma, 2.0 mm lateral to the midline, and 4.0 mm ventral to the skull surface 24 h before tMCAO or sham surgery. siRNAs were synthesized by GenePharma (Shanghai, China). The sequences were as follows: Hes1 siRNA: 5'-AAGGUUUUAUUAUGUCUUAGGG-3'; scrambled siRNA: 5'-CCUACGCCACCAAUUUCGU-3'.

Infarct Evaluation and Neurological Assessment

Brains harvested from euthanized (pentobarbital sodium, 50 mg/kg, i.p.) mice were cut into 2-mm coronal slices and stained for 0.5 h in the dark with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Aldrich, St. Louis, MO, USA) at 37°C. Infarct sizes were quantified from digitized images using ImageJ software and expressed as the damaged area normalized to the total area of the contralateral hemisphere. After 24, 48, and 72 h of reperfusion, each mouse was scored based on a five-point system to

assess the neurological outcome [18]. The scoring system was as follows: 0, without deficits; 1, unable to extend right paw; 2, circling to the right; 3, falling to the right; 4, unable to walk. The final score was expressed as the median value of 12 mice in each group.

TUNEL Staining

TUNEL staining was used to detect neuronal apoptosis following ischemic stroke [19]. Briefly, sections 10 µm thick were fixed in 4% paraformaldehyde for 1 h at RT and incubated with 0.2% H₂O₂ for 1 h to block endogenous peroxidase activity. The sections were then incubated for 1 h in the dark with TUNEL reaction mixture (Millipore) at 37°C. After the counterstaining with DAPI, the fluorescence signal was captured with a Nikon A1 laser confocal microscope. In each animal, the number of TUNEL-positive cells was counted across three random fields within the region of the penumbra, and its percentage among the total cells was calculated using ImageJ.

Statistical Analysis

All data are presented as the mean ± SD, except for the neurological scores which are expressed as median values. Statistical significance was calculated by one-way ANOVA followed by Dunnett's test using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). *P* < 0.05 were considered to be significant.

Results

Hes1 Expression is Induced in Mouse Brain After tMCAO

As a downstream effector gene of the Notch receptor, Hes1 expression varies during the differentiation of neurons [20]. However, how its expression changed following reperfusion in ischemic stroke was unknown. To address this, we used the tMCAO cerebral stroke model followed by reperfusion in wild-type C57BL/6 mice [21], and then compared the expression of Hes1 in brains with and without I/R injury. We found that, compared with sham surgery, the transcript level of Hes1 was significantly upregulated in the brain tissue with I/R injury followed by reperfusion for 12, 24, 48, and 72 h, as analyzed by qRT-PCR (Fig. 1A). Likewise, the results of Western blotting analysis showed that the protein expression of Hes1 was also upregulated after I/R injury (Fig. 1B). These findings showed that Hes1 expression is upregulated in the brain following I/R injury.

Fig. 1 Hes1 expression is induced in brain tissue following tMCAO. **A** The mRNA levels of Hes1 determined by qRT-PCR analysis. Data are presented relative to the sham group. **B** The protein levels of Hes1 determined by immunoblotting analysis. β -Actin was used as a

loading control. Left, representative images; right (band intensity analysis). Data are the mean $\pm$ SD. $**P < 0.01$, $*P < 0.05$, one-way ANOVA followed by Dunnett's test.

Hes1 Knockdown Exacerbates Cerebral Infarction and Neurological Deficit After tMCAO

The expression change of brain Hes1 following I/R injury led us to ask whether it plays a functional role during this pathological process. To address this, we depleted the expression of brain Hes1 by siRNA-mediated knockdown *in vivo*. Compared with sham surgery, tMCAO consistently resulted in an increased expression of brain Hes1, which was efficiently depleted when transfected with siRNA targeting Hes1 (siHes1) (Fig. 2A). Specifically, siHes1 transfection led to Hes1 knockdown in non-ischemic and

ischemic (core and penumbra) regions (Fig. 2B), indicating an efficient and broad knockdown effect. Next, the effect of Hes1 knockdown on I/R injury was examined by quantifying the cerebral infarct size delineated by TTC-stained brain slices [22]. As shown by the stained brain slices of tMCAO mice, the cerebral infarct size was increased when Hes1 was depleted by siHes1 as compared to the siRNA control (Fig. 2C, D). Moreover, at the same time, the extent of neurological deficit, as measured by a five-point system [18], was also significantly higher in tMCAO mice depleted of brain Hes1 (Fig. 2E). Together, these functional studies indicated that Hes1 knockdown increases the

Fig. 2 Hes1 knockdown increases the cerebral infarction and exacerbates the neurological outcome following tMCAO. **A, B** At 24 h of reperfusion, the ipsilateral brain tissues were harvested and the Hes1 protein level was measured by immunoblotting analysis. β -actin was used as a loading control. Left, representative images; right, band intensity analysis. Non, non-ischemic; PNB, penumbra. **C, D** Representative images of brain TTC staining from 3 representative mice in

each group (**C**) and the percentage infarct volume (**D**) (dashed outline, infarct area; scale bar, 2 mm). **E** Neurological scores after 24, 48, and 72 h of reperfusion. Data are mean $\pm$ SD, except for neurological scores which are expressed as median values. $**P < 0.01$; NS, not significant, one-way ANOVA followed by Dunnett's test.

cerebral infarction and worsens the neurological outcome after tMCAO, and thus suggested that Hes1 plays a protective role against I/R injury.

Hes1 Knockdown Promotes Neuronal Apoptosis After tMCAO

The apoptosis of neurons is a fundamental event mediating I/R injury-induced brain damage [23, 24]. To understand how Hes1 knockdown exaggerates I/R injury, we examined its effect on neuronal apoptosis following tMCAO. As shown by TUNEL staining of brain slices, tMCAO induced prominent apoptosis of neurons within the penumbra compared with sham surgery, and moreover, this extent of apoptosis induction was further significantly increased when Hes1 was depleted by siRNA transfection (Fig. 3A). Then, we confirmed this result by measuring the expression of apoptosis-related markers. As expected, Western blotting analysis showed that, in contrast to the sham group, tMCAO resulted in elevated expression of pro-apoptotic cleaved caspase-3 and Bax, and conversely decreased the expression of anti-apoptotic Bcl-2 (Fig. 3B, C). In addition, consistent with the TUNEL staining results (Fig. 3A), Hes1 knockdown markedly intensified the expression change of these apoptosis-related markers, compared with siCtrl group (Fig. 3B, C), showing that Hes1 knockdown promotes neuronal apoptosis after tMCAO. Taken together, given the important role of neuronal apoptosis in I/R injury,

these results suggested that the increased neuronal apoptosis at least partly explains the detrimental effect of Hes1 knockdown on exaggerating the cerebral infarction and the neurological deficit after tMCAO.

Hes1 Knockdown Promotes Activation of the PERK/eIF2 α /ATF4/CHOP Pathway After tMCAO

Recently, depletion of Hes1 in mouse and human cells has been reported to increase apoptosis in response to ER stress induced by dithiothreitol and thapsigargin; this is associated with its modulation of the PERK pathway [12]. Further, accumulating evidence has suggested that the dysregulation of ER stress plays a key role in inducing neuronal apoptosis and subsequent ischemic stroke-induced brain damage [25–27]. Hence, to elucidate how Hes1 knockdown promotes neuronal apoptosis following I/R injury, we next focused on examining the effect on ER stress. The results showed that the expression of p-PERK, p-eIF2 α , ATF4, and CHOP was increased in the I/R-injured brain (Fig. 4D), indicating activation of the PERK/eIF2 α /ATF4/CHOP signaling pathway and consistent with previous studies demonstrating that ER stress is induced in the rat model of stroke [9, 28]. Moreover, we found that the expression of p-PERK, p-eIF2 α , ATF4 and CHOP was further enhanced by Hes1 knockdown (Fig. 4A–D). These data suggested that activation of the PERK/eIF2 α /ATF4/CHOP pathway after tMCAO is enhanced in the absence of Hes1.

Fig. 3 Hes1 knockdown promotes apoptosis after tMCAO.

A At 24 h after reperfusion, the ipsilateral brain tissue was harvested and the apoptosis within the penumbra was assessed by TUNEL staining. Left, representative images; right, percentages of TUNEL-positive cells. **B, C** Protein expression of cleaved caspase-3, Bax, and Bcl-2 determined by immunoblotting analysis. β -Actin was used as a loading control. **B** Representative images. **C** Analysis of cleaved caspase-3 band intensity and the Bax/Bcl-2 ratio. Data are the mean $\pm$ SD. $^{**}P < 0.01$, one-way ANOVA followed by Dunnett's test.

Fig. 4 Hes1 knockdown promotes activation of the PERK/eIF2 α /ATF4/CHOP pathway. Protein expression of p-PERK, PERK, p-eIF2 α , eIF2 α , ATF4, and CHOP analyzed by immunoblotting. β -actin was used as a loading control. **A** Representative images. **B** Statistical analysis of p-PERK/PERK, and p-eIF2 α /eIF2 α ratios. **C**, **D** Band intensity of ATF4 (**C**) and CHOP (**D**). Data are the mean $\pm$ SD. $**P < 0.01$, $*P < 0.05$, one-way ANOVA followed by Dunnett's test.

PERK Inhibitor Attenuates Effects of Hes1 Knockdown After tMCAO

To establish a potential causal link between promotion of the PERK signaling pathway and exaggerated neuronal apoptosis, cerebral infarction, and neurological deficit by Hes1 knockdown after tMCAO, we gave mice GSK2606414, a selective inhibitor of PERK [29]. Western blotting analysis showed that treatment with 4 μ L GSK2606414 (20 μ mol/L) at 24 h prior to tMCAO surgery effectively inhibited the activation of PERK and the downstream signaling pathway in the I/R-injured brain, irrespective of Hes1 expression (Fig. 5A). More importantly, along with the abrogated activation of PERK/eIF2 α /ATF4/CHOP signaling pathway, Hes1 knockdown-induced expression changes of the apoptosis-related markers cleaved caspase-3, Bax, and Bcl-2, were all remarkably attenuated (Fig. 5A, B), suggesting that activation of PERK/eIF2 α /ATF4/CHOP pathway promoted by Hes1 knockdown is responsible for the increased neuronal apoptosis after cerebral I/R injury. Furthermore, consistent with the attenuated apoptosis, the exaggeration of infarct size (Fig. 5C) and neurological deficit by Hes1 knockdown (Fig. 5D) was also significantly diminished when PERK was inhibited by GSK2606414. Thus, these lines of evidence together suggest that Hes1 knockdown aggravates ischemic stroke following tMCAO at least through

promoting ER stress-dependent neuronal apoptosis mediated by the PERK/eIF2 α /ATF4/CHOP pathway.

Discussion

Since neuronal apoptosis is one of the major pathogenic factors that cause brain damage following ischemic stroke, reducing apoptosis during this condition is a potential therapeutic strategy to prevent the loss of neurons in the affected tissue and minimize the ischemic stroke-induced damage, holding promise to improve the clinical outcome of patients with acute ischemic stroke [3, 23]. Several signaling pathways lead to the initiation of apoptosis after cerebral ischemia, including the most studied mitochondrial apoptotic pathway [30, 31]. However, ER stress-induced apoptosis has emerged as another important factor during the pathophysiology of cerebral ischemia, as evidenced by investigations using rat and mouse models as well as *in vitro* systems [9, 32–34]. Accordingly, it has been proposed that drugs shown to attenuate ER stress after experimental cerebral ischemia may serve as strong candidates in the treatment of stroke patients [25], such as dantrolene [35], (-)-epigallocatechin-3-gallate [28], and sodium phenylbutyrate [36]. Nonetheless, ER stress signaling is initially aimed to restore ER homeostasis, and manipulating such stress may have deleterious side-effects

Fig. 5 PERK inhibitor attenuates Hes1 knockdown-induced effects following tMCAO. **A, B** At 24 h of reperfusion, ipsilateral brain tissue was harvested and the protein expression of targets as indicated was analyzed by immunoblotting. β -Actin was used as a loading control. **A** Representative images. **B** Analysis of band intensity of cleaved caspase-3 and of Bax/Bcl-2 ratio. **C** Percentages of infarct

volume from TTC staining. **D** Neurological scores after 24, 48, and 72 h of reperfusion. Data are the mean $\pm$ SD, except for neurological scores, which are expressed as median values. ** $P < 0.01$; in **D**, ** $P < 0.01$, siCtrl + Vehicle vs siHes1 + Vehicle; # $P < 0.01$, siHes1 + Vehicle vs siHes1 + GSK2656157, one-way ANOVA followed by Dunnett's test.

[37], so a comprehensive knowledge of the mechanisms underlying ER regulation following ischemic stroke is essential to better exploit the therapeutic potential.

It is known that UPR induced by severe or prolonged ER stress can eventually result in the activation of proapoptotic pathways through mediators such as CHOP, caspase-12, and JNK [38]. But the regulation of ER stress-induced apoptosis following ischemic stroke is still not completely understood. In this study, by using stroke mouse model established *via* tMCAO, we showed that Hes1 knockdown increased neuronal apoptosis and the cerebral infarction and exacerbated the neurological outcome partly by promoting the activation of the PERK/eIF2 α /ATF4/CHOP pathway, thus suggesting that Hes1 may function as a novel regulator of ER stress to affect the process of ER stress-induced neuronal apoptosis following cerebral I/R injury. Given the evidence from the animal model and mechanistic studies, we provide a molecular basis for the possible application of Hes1 as a target in stroke treatment.

The Notch pathway is an evolutionarily conserved cell-to-cell communication system that plays indispensable roles in a variety of cellular activities such as differentiation, proliferation, and apoptosis [39]. Hes1 is a target gene downstream of Notch signaling that is activated by the binding of a Notch ligand to its cognate receptor [40]. One previous study has reported that, after renal I/R injury,

I/R activates Notch2/Hes1 signaling, which plays an important role in the renal I/R injury-associated inflammation and apoptosis [41]. Besides, activation of Notch1/Hes1 signaling has cardioprotective effects and attenuate myocardial I/R injury [42, 43]. We found that Hes1 expression was upregulated in brain tissue following tMCAO, suggesting that this increased expression of Hes1 is derived from the activated Notch/Hes1 signaling in response to cerebral I/R injury. This is possible, since increased expression of Notch signaling molecules has been reported in the ischemic brain of animal models [44, 45]. In mammalian cells, four Notch receptors (Notch1–4) and five ligands (Delta-like1/3/4 and Jagged1/2) have been identified [46]. However, so far the upstream Notch receptor ligand that mediates the upregulation of Hes1 following tMCAO-induced cerebral I/R injury is unknown, and this needs to be resolved by future investigations.

The increased neuronal apoptosis, cerebral infarction, and neurological deficit by Hes1 knockdown following tMCAO was remarkably rescued by treatment with a selective PERK inhibitor; this not only suggests that the promoted activation of the PERK/eIF2 α /ATF4/CHOP pathway and the resulting elevated ER stress act as critical events to account for these effects, but also implies that other mechanisms may also exist. Coincidentally, the Notch1/Hes1 signaling pathway has also been associated

with the attenuation of ER stress-induced apoptosis and protects the heart against I/R injury [47]. On the other hand, it has been reported that a lack of Hes1 in human and mouse cell lines induces apoptosis during ER stress, and this is associated with the enhanced dephosphorylation of eIF2 α via upregulating the expression of GADD34, a pro-apoptotic protein [12]. Although whether this mechanism could also explain the Hes1-regulated ER stress following cerebral I/R injury is unclear, it provides a useful clue for further studies to uncover how Hes1 knockdown promotes activation of the PERK/eIF2 α /ATF4/CHOP pathway. Addressing this issue may help to advance our understanding of the mechanism by which Hes1 participates in the pathophysiology of ischemic stroke. Moreover, it should be noted that all the functional and mechanistic results presented in the current study were obtained from *in vivo* siRNA-mediated knockdown experiments. Further investigations using Hes1-deficient mice or gain-of-function tactics would be helpful to consolidate the protective role of Hes1 in cerebral ischemic stroke.

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

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