TERT Promotes Neurogenesis and Neural Repair after Hypoxic Ischemic Brain Damage in Neonatal Rats

Jiao Li
Sichuan University
Hai-ting Liu
Sichuan University
Jing Zhao
Sichuan University
Zhi Fang (fangzhi@wchscu.cn)
Sichuan University https://orcid.org/0000-0002-0724-8485

Research Article

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Abstract

Neonatal hypoxic ischemic encephalopathy (HIE) endangers quality of life in children; but effective cure is rare. Neurogenesis plays an important role in neural repair following brain damage. Recent studies have demonstrated that telomerase reverse transcriptase (TERT) was involved in neurogenesis regulation. However, whether TERT participates in the regulation of neurogenesis after hypoxic-ischemic brain damage (HIBD) is unclear. Here, we established a model of HIBD in neonatal rats both *in vivo* and *in vitro*, and used lentivirus and adenovirus transfection for TERT overexpression to investigate its role in neurogenesis after HIBD in developmental stage. Using immunofluorescence staining, cell counting Kit-8 staining, TUNEL, and western blotting, we observed that TERT attenuated apoptosis and promoted proliferation, migration, and differentiation in neural stem cells (NSCs). Furthermore, TERT induced myelination in the brain of neonatal rats after HIBD. Neurobehavioral tests revealed that TERT could improve learning, memory, and neurological function after HIBD in neonatal rats, and thus promote the recovery of neurological function after HIBD. In addition, we investigated the regulatory mechanism of TERT during neurogenesis after HIBD in developmental stage. We found that TERT may regulate neurogenesis after HIBD through the Sonic Hedgehog/Gli1 signaling pathway. Our study demonstrated that TERT could promote neural repair and neurological function recovery after HIBD in neonatal rats. The new neuroprotective pathway regulated by TERT during HIBD described here could provide a basis for developing therapeutic strategy for neonatal HIE. Furthermore, TERT may be a potential target during neural repair and reconstruction in various diseases affecting nervous system.

1. Introduction

Hypoxic ischemic encephalopathy (HIE) is a type of neonatal encephalopathy caused by hypoxia and decreased blood flow resulting from perinatal asphyxia. The reported incidence of HIE is about 1.5 per 1000 live births (Kurinczuk et al. 2010), whereas it is much higher in China with a relatively high mortality rate. HIE is one of the major diseases that endanger the quality of life in children, as more than 25% of survivors have neurological sequelae (Magai et al. 2020). However, specific and effective treatments for HIE are rare.

The pathogenesis of hypoxic-ischemic brain damage (HIBD) during the developing stage comprises two phases: neural injury and repair. The outcome and prognosis of brain injury depend on the balance between these two phases. Early studies reported that a series of adaptive changes occurred in the brain that was recovering from brain injury, including neurogenesis, angiogenesis, and remyelination (Daadi and Steinberg 2009; Daadi et al. 2010). These endogenous repair processes play vital roles in improving the prognosis of brain injury and provide targets for treatment (Daadi et al. 2010). Therefore, research focused on understanding the repair mechanisms after HIBD may provide a new therapeutic strategy for neonatal HIE.

Telomerase reverse transcriptase (TERT) acts as a rate-limiting catalytic subunit and key regulator of telomerase activity (Wyatt et al. 2010). Previous studies on TERT function have been mainly focused on

tumors; however, recently, the role of TERT in the central nervous system has gradually become a research hotspot. In our previous studies, we reported the neuroprotective role of TERT in attenuating neural injury after HIBD in newborn rats (Li et al. 2011; Li et al. 2013). However, reports about the role of TERT in neural repair after HIBD are rare. This study aimed to investigate such role of TERT during HIBD in neonatal rats. To this end, a model of HIBD was established both *in vivo* and *in vitro* in neonatal rats. Furthermore, we developed a TERT overexpression model using lentivirus and adenovirus transfection to investigate the role and mechanism of TERT in neural repair during HIBD in developmental stage.

2. Experimental Procedures

2.1 Neural stem cells culture *in vitro*

Neural stem cells (NSCs) were purchased (Procell Life Science&Technology Co,.Ltd. Wuhan, China). Briefly, NSCs were cultured in serum-free DMEM: F12 medium (Hyclone, South Logan, UT, USA) supplemented with B27 (Gibco, Grand Island, NY, USA). Cells were maintained in a proliferative state by adding 20 μg/L bFGF (Gibco, Grand Island, NY, USA) and 20μg/L EGF (Gibco, Grand Island, NY, USA). Cells were cultured in a humidified atmosphere with 95% air and 5% CO2 at 37°C. After 6–7 days, a single cell suspension was prepared by centrifugal blow-beating of the formed neurospheres, and the cells were passaged at a ratio of 1:2.

2.2 Infection of NSCs using lentivirus

The lentivirus vector was constructed using the CDS sequence of the rat TERT. The constructed TERT overexpression vector or empty vector was transfected and packaged in human embryonic kidney (HEK) 293T cells using Lipofectamine 2000 Transfection (Invitrogen, Carlsbad, CA, USA). After 48 h, the medium containing viral particles was centrifuged and filtered through a 0.22 μm filter to produce lentivirus. NSCs were infected with packaged lentivirus. The optimal multiplicity of infection (MOI) of the lentivirus was determined (data not shown). NSCs were infected with TERT overexpression lentivirus and empty vector lentivirus at an MOI of 40. The expression of TERT mRNA in NSCs was detected using quantitative real-time PCR (QRT-PCR) after infection with lentivirus (data not shown). Four hours after infection, the NSCs were exposed to oxygen glucose deprivation (OGD).

2.3 Oxygen glucose deprivation treatment

The culture medium was replaced with glucose-free DMEM to mimic ischemia, and then the NSCs were transferred into a hypoxic airtight chamber (95% N_2 and 5% CO_2) at 37°C for 6 h to induce OGD. After OGD treatment, NSCs were cultured in a medium with glucose under normoxic conditions. The experimental groups included three groups: Non + empty (NSCs in normoxia transfected with empty vector lentivirus), OGD + empty (NSCs transfected with empty lentivirus vector then underwent OGD), and OGD + TERT (NSCs transfected with TERT overexpression lentivirus and underwent OGD).

2.4 Animal protocols: hypoxia–ischemia treatment and infection with adenovirus

All animal procedures and behavioral tests were performed in compliance with the protocols approved by the Animal Ethics Committee of West China Second University Hospital, Sichuan University.

Postnatal day 5 (P5) Sprague Dawley (SD) rats were obtained from the Animal Center of Sichuan Province with the approval of the Sichuan University Committee of Animal Research. After 2 days of adaptive feeding, all neonatal rats were randomly assigned to three experimental groups: sham, HIBD + vehicle, and HIBD + TERT. Adenovirus vectors, including the TERT overexpression adenovirus vector and empty adenovirus vector, were constructed and packaged by the ZHBY (ZHBY, Nanchang, China). QRT-PCR was conducted to identify adenovirus vectors (data not shown). In the HIBD + TERT group, the lateral cerebral ventricle of rats was injected with 2 μL of TERT-overexpressing adenovirus. In the HIBD + vehicle group, 2 μL of empty adenovirus vector was injected into the lateral cerebral ventricle of rats, and 2 μL saline was injected into the lateral cerebral ventricle of rats in the sham group. After 48 h, all neonatal rats underwent hypoxic-ischemic (HI) treatment or control (sham). Briefly, after anesthetization, the left carotid artery of rats was ligated for 2 h and then subjected to hypoxic treatment (8% oxygen in nitrogen) for 2.5 h. After hypoxia, the rats were returned to their cages. Sham control rats underwent surgical procedures but were not subjected to HI.

2.5 Quantitative real-time PCR

For detection of TERT expression, total RNA was extracted from NSCs using an Ultrapure RNA Kit (DNaseI) (CWBIO, Jiangsu, China) according to the manufacturer's instructions. First-strand cDNA was synthesized using a HiFiScript cDNA Synthesis Kit (CWBIO, Jiangsu, China) according to the manufacturer's instructions. The primers were designed (Generalbiol, Anhui, China). Amplifications were performed using the UltraSYBR Mixture (CWBIO, Jiangsu, China) at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

2.6 Western blot analysis

Western blot analysis was performed as described in our previous study (Li et al. 2013). Briefly, cell lysates were collected and centrifuged at 14,000 rpm for 30 min at 4°C. The protein concentration of the supernatant was measured (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples from different groups were denatured and loaded onto 8% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) gels for electrophoresis. The electrophoresed gel was electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). The membranes were incubated with primary antibodies against NeuN (Abways, Shanghai, China), GFAP (Abways, Shanghai, China), Galc (Bioss, Woburn, MA, USA), Gli1 (Bioss, Woburn, MA, USA), and β-actin (Bioss, Woburn, MA, USA), followed by incubation with secondary antibodies (Abcam, Cambridge, MA, USA). The immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL, Millipore, USA). β-actin was used as an internal reference to calculate the ratio of the optical density (OD). We repeated these experiments at least three times.

2.7 Immunofluorescence staining

The neonatal rats were anesthetized with 10% chloral hydrate, fixed, perfused with 30 mL saline, and slowly perfused with 10 mL of 4% paraformaldehyde (PFA). The brains of rats were removed and fixed with 4% PFA for 48 h, finally producing frozen sections of 50 μm thickness. Sections with consistent positions in each group were used for immunofluorescence staining. Brain sections were incubated with primary antibodies including TERT (Santa Cruz, CA, USA), nestin (Abcam, Cambridge, MA, USA), DCX (Abcam, Cambridge, MA, USA), NeuN (Abcam, Cambridge, MA, USA), GFAP (Abcam, Cambridge, MA, USA), O4 (R&D, Minneapolis, MN, USA), Iba-1 (Abcam, Cambridge, MA, USA), and MBP (Abcam, Cambridge, MA, USA). After incubation with the secondary antibodies conjugated with fluorescein, the sections were stained with DAPI. Fluorescent images were obtained using a fluorescence microscope (Nikon 80i).

2.8 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

Cell apoptosis in the brain was detected by TUNEL assay using an in-situ cell death detection kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Briefly, frozen brain sections were fixed and treated with 0.2% Triton X-100, incubated with biotinylated nucleotide and rTdT enzyme for 1 h at 37°C, and stained with DAPI for 5 min. Images were captured using a fluorescence microscope (Nikon 80i).

2.9 Cell proliferation detected by Cell Counting Kit-8

NSCs in each group were digested with trypsin and then diluted to $1–5 \times 10^4$ cells/mL. Cells were plated onto the 96 plates and cultured overnight at 37°C. Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) and serum-free DMEM were mixed and the mixture was added to the wells incubated at 37°C for 1 h in a 5% CO_2 incubator. The absorbance value (A) was measured at 450 nm using a microplate spectrophotometer. The proliferation rate was calculated using the A values in each group/A value in the control group × 100%.

2.10 Neurological function test

2.10.1 Morris water maze test

The Morris water maze (MWM) test was performed 14 days after the HIBD. All rats were trained in the test once daily for the first 4 days. At each training time, the rats entered water from the different quadrants to complete the training in the four quadrants. The time required to search for and climb the platform from different quadrants (escape latency) was recorded. If the rats did not find the platform within 120 s, they were placed on the platform and maintained for 10 s, and the escape latency was recorded as 120 s. The MWM test was performed on the fifth day. On the fifth day, the third quadrant was selected as the input point, and the rats were placed in water to record the escape latency. For rats that did not find the platform within 120 s, the escape latency was recorded as 120 s.

2.10.2 The modified neurological severity score

The modified Neurological Severity Score (mNSS) using the double-blind method was employed to evaluate the neurological function of neonatal rats after HIBD. The mNSS includes sensory, motor, reflex,

and beam balance tests. The neurological function of neonatal rats on 7th days and 14th day after HIBD was evaluated. The severity of neurological injury was proportional to the score (normal score, 0; maximal deficit score, 18).

2.11 Statistical analysis

Data are expressed as mean ± standard deviation (SD) and compared using analysis of variance (ANOVA) with Bonferroni/Dunnett post hoc test. Statistical significance was set at $P < 0.05$.

3. Results

3.1 TERT can promote NSCs proliferation and reduce NSCs apoptosis after HIBD

To increase the expression of TERT, we transfected cultured NSCs with TERT overexpression lentivirus and the empty control lentivirus vector prior to OGD *in vitro*. The experimental groups included Non + empty, OGD + empty, and OGD + TERT. TERT expression was detected using QRT-PCR analysis. CCK-8 staining was used to detect the proliferation of NSCs in each group. Figure 1a shows the expression of TERT in the different groups. Transfection with TERT overexpression lentivirus increased the expression level of TERT after OGD in cultured NSCs, compared with the Non + empty group and OGD + empty group, respectively ($P < 0.001$, Fig. 1a). Figure 1b shows the proliferation rate of NSCs detected using CCK-8. OGD significantly reduced cell proliferation in the OGD + empty group ($P < 0.001$, Fig. 1b); however, TERT overexpression reversed this effect in the OGD + TERT group ($P < 0.001$, Fig. 1b). These results suggest that the proliferation of NSCs was reduced after OGD, and that TERT could effectively promote proliferation of NSCs after OGD.

In vivo, a TERT-overexpressing model was constructed by transfection with adenovirus. The experimental groups included sham, HIBD + vehicle, and HIBD + TERT. NSCs were labeled with nestin, a characteristic marker of NSCs. Figure 1c shows the expression of TERT (green) and nestin (red) in the brain detected using immunofluorescence. TERT expression was significantly higher in NSCs in the HIBD + TERT group than in the other two groups. The TUNEL assay was conducted to detect the apoptosis in NSCs in each group (Fig. 1d). There was a significantly higher number of TUNEL-positive NSCs in the HIBD + vehicle group, whereas few TUNEL-positive cells were observed in the sham group. However, there was a lower number of TUNEL-positive NSCs in the HIBD + TERT group compared with the HIBD + vehicle group. This suggests that TERT can attenuate apoptosis in NSCs in the brain after HIBD.

3.2 TERT can promote the migration and differentiation of NSCs after HIBD

In vitro experiments included three groups: Non + empty, OGD + empty, and OGD + TERT. NSCs in OGD + empty and OGD + TERT after reoxygenation were induced to differentiate together with NSCs in Non +

empty for 7 days. Western blot analysis revealed the expression of NeuN (neuron marker), GFAP (astrocyte marker), and Galc (oligodendrocyte marker) in the three groups (Fig. 2a-c). OGD caused decrease in the expression of GFAP and Galc ($P < 0.001$, Fig. 2b, c), but did not change the expression level of NeuN compared with Non + empty ($P > 0.05$, Fig. 2a, c). Overexpression of TERT resulted in increased expression of NeuN, GFAP, and Galc in NSCs after OGD ($P < 0.001$, Fig. 2a-c) compared with the OGD + empty group. These results suggest that overexpression of TERT can increase the differentiation of neural stem cells into neurons, astrocytes, and oligodendrocytes after OGD.

Immunofluorescence staining was conducted *in vivo* to detect the expression of DCX (a marker reflecting the migration of NSCs), NeuN, GFAP, O4 (a specific marker of oligodendroglia), and Iba-1 (a specific marker of microglia) in the brain of neonatal rats. The experimental groups included sham, HIBD + vehicle, and HIBD + TERT. As shown in Fig. 2d, the expression of DCX, NeuN, GFAP, Iba-1 (green), and O4 (red) in the brain tissue of the HIBD + TERT group was higher than that in the sham and HIBD + vehicle groups. This suggests that TERT can improve the migration and differentiation of NSCs in the brain after HIBD.

3.3 TERT can promote the myelination in the brain after HIBD.

To further investigate the effect of TERT on myelination in the brain after HIBD, we constructed a TERT-overexpressing model using *in vivo* transfection with adenovirus. The experimental group included sham, HIBD + vehicle, and HIBD + TERT. Myelination of the brain in neonatal rats after HIBD was detected using immunofluorescence staining of myelin basic protein (MBP). As shown in Fig. 3, compared with the sham group, HI decreased the expression of MBP (green) in the brain of neonatal rats, while MBP expression was increased in the brain of the HIBD + TERT group compared with the HIBD + vehicle group. This indicated that HIBD reduced the myelination in the neonatal rat brain, and that TERT can promote the myelination in the brain of neonatal rats after HIBD.

3.4 TERT improves the learning and memory ability and neurological function of neonatal rats after HIBD

We further investigated the role of TERT in the recovery of neurological function after HIBD. The experimental groups included sham, HIBD + vehicle, and HIBD + TERT. The MWM test was used to evaluate learning and memory abilities. The MWM test was performed on the 14th day after the operation in all newborn rats. All rats were trained in the MWM test on the first 4 days and tested on the 5th day. The escape latency in each rat was calculated to reflect the spatial learning and memory ability of rats. Figure 4.a shows the trajectory diagrams in the water maze in each group of neonatal rats during the first and fifth days. Figure 4.b shows a statistical analysis of the escape latency of the test in each group during the 5th day. As shown in Fig. 4.b, the escape latency in the HIBD + vehicle group (81.23 ± 8.46 s) was significantly longer than that in the sham group (10.96 ± 3.37 s) ($P < 0.001$, Fig. 4.b). The time for rats in the HIBD + vehicle group was longer to find the platform compared to the sham group. However, the escape latency was shorter in the HIBD + TERT group (62.63 ± 4.92 s) than in the HIBD + vehicle group

($P < 0.01$, Fig. 4.b). This suggests that the learning and memory abilities of newborn rats were decreased after HIBD. However, escape latency was shortened in the TERT overexpression group. This indicated that TERT could improve the learning and memory ability in neonatal rats after HIBD.

We further assessed the neurological function of neonatal rats after HIBD using the mNSS with a double-blind method. The neurological function of neonatal rats on 7th days and 14th day after HIBD was evaluated. The experimental groups were the same as those before. As shown in Fig. 4c, on the 7th day after HIBD, HI increased the mNSS scores of rats compared with the sham group ($P < 0.01$, Fig. 4c), suggesting that the neurological function in neonatal rats was affected after HIBD. Overexpression of TERT did not change the mNSS scores ($P > 0.05$ versus the HIBD + vehicle group, Fig. 4c). On the 14th day after HIBD, HI also significantly increased the mNSS scores in rats compared with the sham group ($P < 0.001$, Fig. 4c); however, overexpression of TERT reversed this effect and decreased the mNSS scores in rats compared with the HIBD + vehicle group ($P < 0.001$, Fig. 4c), suggesting that overexpression of TERT can reduce the mNSS scores and improve the injured neurological function in neonatal rats after HIBD. These results demonstrate that TERT can improve the learning ability, memory, and neurological function of neonatal rats after HIBD, and therefore promote the recovery of neurological function after HIBD.

3.5 The regulatory mechanism of TERT on neurogenesis after HIBD

Based on these results, we have further discussed the possible regulatory mechanism of TERT during neurogenesis after HIBD. With an in-depth review of related research and literature, we found that the Sonic Hedgehog (SHH)/Gli1 signaling pathway may participate in regulating neurogenesis of NSCs. Therefore, we detected the expression of Gli1 protein in NSCs using western blotting. The experimental groups included Non + empty, OGD + empty, and OGD + TERT. As shown in Fig. 5, Gli1 levels were significantly upregulated in the OGD + empty group ($P < 0.001$ versus the Non + empty group, Fig. 5). Overexpression of TERT further increased the expression of Gli1 ($P < 0.001$ versus the OGD + empty group, Fig. 5). This suggests that overexpression of TERT increases the expression of Gli1 in NSCs after OGD, which indicates that SHH/Gli1 signaling pathway may be involved in the regulating mechanism of TERT in neurogenesis after HIBD.

4. Discussion

The prognosis of HIBD in the developing stage mainly depends on neural injury and repair. Neural injury is difficult to reverse; thus, neural repair, including neurogenesis after injury, plays an important role in the recovery of neurological function. In this study, we reported that overexpression of TERT attenuated the apoptosis in NSCs, induced proliferation, migration, and differentiation of NSCs, and promoted myelination in the brain of neonatal rats after HIBD. Our results showed that TERT could improve the learning and memory ability and neurological function of neonatal rats after HIBD. Further research indicated that the mechanism of TERT function in neurogenesis after HIBD may be related to the SHH /Gli1 signaling pathway.

Neurogenesis of the central nervous system is a complex process that involves the proliferation of NSCs, their gradual migration to functional regions, continuous neoplastic changes, differentiation into nerve cells, and establishment of contact with other nerve cells to preserve neurological function. When neurogenesis is activated after brain injury, NSCs play a significant role in neural repair and can promote post-injury repair in the damaged brain (Yin et al. 2013). Previous studies have already reported that neurogenesis after hypoxic ischemic injury in the neonatal brain was closely related to brain repair and recovery after injury (Yang and Levison 2007; Miles and Kernie 2008).

TERT is widely expressed in embryonic and early phases of the postnatal brain and decreases with neuronal differentiation (Klapper et al. 2001). TERT expression is observed only in highly proliferative regions of the adult brain, such as the hippocampus and olfactory bulb, which can provide NSCs for neurogenesis (Lee et al. 2010). These lines of evidence indicate that TERT may play a role in neuronal survival and differentiation and support neurogenesis in the brain (Klapper et al. 2001; Lee et al. 2010). In a study on adult mice, the proliferation and self-renewal ability of neural stem cells in TERT-deficient mice were significantly reduced, together with their ability to differentiate into various functional nerve cells, leading to the damage of neurological function. In contrast, reactivation of TERT improved the proliferation and differentiation ability of NSCs, promoted remyelination, and improved neurological function (Jaskelioff et al. 2011). In another study on adult mice, TERT was reported to be involved in depression-related behavioral modification by promoting hippocampal neurogenesis (Zhou et al. 2011). Consistently, overexpression of TERT promoted the proliferation of NSCs in mouse brains both *in vivo* and *in vitro* (Liu et al. 2012). TERT is believed to serve as an effective springboard for promoting differentiation and immortalization of stem cells in stem cell therapies (Yalvaç et al. 2011). However, whether TERT is involved in the process of neurogenesis in the neonatal brain after HIBD remains unclear. In this study on neonatal HIBD, we found that TERT attenuated the apoptosis in NSCs and promoted the proliferation, migration, and differentiation of NSCs in the neonatal brain after HIBD.

Myelination is an important part of the neurodevelopment process because it allows axons to connect to neurons, accelerate nerve conduction, and strengthen circuity throughout the nervous system. During neonatal HI brain injury, myelination is disrupted, thereby impairing functional recovery from brain injury and resulting in static motor deficits (Koch et al. 2008; Kaminski et al. 2020). In the present study, myelination was evaluated by immunofluorescence of MBP, a primary protein component of the myelin sheath. Our results suggest that TERT improves the neurodevelopmental process after HIBD by promoting myelination. Research on the role of TERT in regulating myelination is rare, and further studies are required to clarify the mechanism by which TERT promotes myelination.

Neurogenesis and myelination, which are important parts of post-injury repair, are closely related to the recovery of neurological function. In this study, neurological function after injury was estimated using the MWM test and mNSS. The MWM was widely performed to evaluate learning and memory ability during the research of neonatal hypoxic ischemic brain injury (Gao et al. 2020). The MWM test showed that the escape latency was improved by overexpression of TERT, indicating that TERT can improve the dysfunction of learning and memory impairment caused by HIBD. The mNSS is a behavioral functional

experiment used to evaluate neurological function, which is also applied in the research of HI brain injury (Ge et al. 2018). The mNSS score in neonatal rats after HIBD increased at 14th day, while TERT overexpression reduced the mNSS scores. Together with the MWM test, our research indicated that TERT could improve the neurological function in neonatal rats after HIBD, and therefore promote the recovery of neurological function after HIBD.

Taken together, our results indicated that TERT could improve the learning and memory ability and neurological function in neonatal rats after HIBD by improving neurogenesis and myelination. Additionally, we investigated the underlying mechanism of TERT in neurogenesis. Gli1 is an important downstream transcriptional factor of the SHH signaling pathway and can accurately reflect the activity of this pathway. Previous studies have reported that the SHH/Gli signaling pathway was involved in neurological development and central nervous system diseases (Ruiz i Altaba et al. 2002). Activation of the SHH/Gli pathway could promote the proliferation and differentiation of neural precursor cells during brain growth (Ruiz i Altaba et al. 2002). In adult mice, Gli1 has been reported to participate in regulating the proliferation of NSCs and neurogenesis in the hippocampus (Sun et al. 2018). Tayyab M et al. have reported that the SHH/Gli1 signaling pathway was an effective regulator of neurogenesis during embryonic development and in the adult hippocampus (Tayyab et al. 2018). In addition, both *in vivo* and *in vitro* studies have confirmed the neuroprotective role of the SHH/Gli1 pathway in promoting neurogenesis and thus improving neurological function after ischemic brain injury (Yu et al. 2017). However, little is known about the role of the SHH/Gli1 pathway in neonatal HIBD. Here, we found that Gli1 expression was increased after neonatal HIBD, and overexpression of TERT further increased the expression level of Gli1, indicating that the SHH/Gli1 signaling pathway may be involved in the neuroprotective effect of TERT during neonatal HIBD. A previous study in cancer cells proposed TERT as a target of the Hedgehog (HH)/Gli signaling pathway. Furthermore, Gli1 suppression reduced TERT expression in cancer cells (Mazumdar et al. 2013). However, studies on the relationship between TERT and Gli1 are quite rare, and further research is needed to elucidate the interaction between TERT and Gli1, especially in neonatal HIBD.

In summary, the present study demonstrated that TERT attenuated the apoptosis in NSCs, promoted the proliferation, migration, and differentiation of NSCs, and induced myelination in neonatal rats after HIBD. TERT could improve the learning and memory ability and neurological function in neonatal rats after HIBD. Moreover, the SHH/Gli1 signaling pathway might be involved in the neuroprotective effect of TERT in neonatal HIBD. Since neurogenesis is closely related to neural repair and prognosis after brain injury, and the repair effect of endogenous neurogenesis is limited, we believe that therapies such as those enhancing endogenous stem cell activity will become a hot topic in the treatment of HIE. To date, the role of TERT in neurogenesis after HIBD is a new field. In this study, the neural repair effect and related molecular mechanism of TERT were discussed, and a new neuroprotective pathway regulated by TERT was found, which could provide novel insights into the therapeutic strategies for neonatal HIE. In addition, it suggests new possibilities for the neuronal repair and reconstruction in other diseases affecting nervous system using TERT as the target.

Declarations

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Conflict of interest:

The authors declare that they have no conflict of interest.

Availability of data and material:

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval:

Approval was obtained from the Animal Ethics Committee of West China Second University Hospital, Sichuan University.

Consent to participate:

Not applicable

Consent for publication:

Not applicable

References

1. Daadi MM, Steinberg GK (2009) Manufacturing neurons from human embryonic stem cells: biological and regulatory aspects to develop a safe cellular product for stroke cell therapy. Regen Med 4:251-263. https://doi.org/10.2217/17460751.4.2.251

2. Daadi MM, Davis AS, Arac A, Li Z, Maag AL, Bhatnagar R, Jiang K, Sun G, Wu JC, Steinberg GK (2010) Human neural stem cell grafts modify microglial response and enhance axonal sprouting in neonatal hypoxic-ischemic brain injury. Stroke 41:516-523. https://doi.org/10.1161/strokeaha.109.573691
3. Gao Y, Zhang Y, Dong Y, Wu X, Liu H (2020) Dexmedetomidine Mediates Neuroglobin Up-Regulation and Alleviates the Hypoxia/Reoxygenation Injury by Inhibiting Neuronal Apoptosis in Developing Rats. Front Pharmacol 11:555532. https://doi.org/10.3389/fphar.2020.555532
4. Ge XH, Shao L, Zhu GJ (2018) Oxymatrine attenuates brain hypoxic-ischemic injury from apoptosis and oxidative stress: role of p-Akt/GSK3β/HO-1/Nrf-2 signaling pathway. Metab Brain Dis 33:1869-1875. https://doi.org/10.1007/s11011-018-0293-4
5. Jaskelioff M, Muller FL, Paik JH, Thomas E, Jiang S, Adams AC, Sahin E, Kost-Alimova M, Protopopov A, Cadiñanos J, Horner JW, Maratos-Flier E, Depinho RA (2011) Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. Nature 469:102-106. https://doi.org/10.1038/nature09603
6. Kaminski N, Köster C, Mouloud Y, Börger V, Felderhoff-Müser U, Bendix I, Giebel B, Herz J (2020) Mesenchymal Stromal Cell-Derived Extracellular Vesicles Reduce Neuroinflammation, Promote Neural Cell Proliferation and Improve Oligodendrocyte Maturation in Neonatal Hypoxic-Ischemic Brain Injury. Front Cell Neurosci 14:601176. https://doi.org/10.3389/fncel.2020.601176
7. Klapper W, Shin T, Mattson MP (2001) Differential regulation of telomerase activity and TERT expression during brain development in mice. J Neurosci Res 64:252-260. https://doi.org/10.1002/jnr.1073
8. Koch JD, Miles DK, Gilley JA, Yang CP, Kernie SG (2008) Brief exposure to hyperoxia depletes the glial progenitor pool and impairs functional recovery after hypoxic-ischemic brain injury. J Cereb Blood Flow Metab 28:1294-1306. https://doi.org/10.1038/jcbfm.2008.15
9. Kurinczuk JJ, White-Koning M, Badawi N (2010) Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. Early Hum Dev 86:329-338. https://doi.org/10.1016/j.earlhumdev.2010.05.010
10. Lee J, Jo YS, Sung YH, Hwang IK, Kim H, Kim SY, Yi SS, Choi JS, Sun W, Seong JK, Lee HW (2010) Telomerase deficiency affects normal brain functions in mice. Neurochem Res 35:211-218. https://doi.org/10.1007/s11064-009-0044-3
11. Li J, Tang B, Qu Y, Mu D (2011) Telomerase reverse transcriptase: a novel neuroprotective mechanism involved in neonatal hypoxic-ischemic brain injury. Int J Dev Neurosci 29:867-872. https://doi.org/10.1016/j.ijdevneu.2011.07.010
12. Li J, Qu Y, Chen D, Zhang L, Zhao F, Luo L, Pan L, Hua J, Mu D (2013) The neuroprotective role and mechanisms of TERT in neurons with oxygen-glucose deprivation. Neuroscience 252:346-358. https://doi.org/10.1016/j.neuroscience.2013.08.015
13. Liu M, Hu Y, Zhu L, Chen C, Zhang Y, Sun W, Zhou Q (2012) Overexpression of the mTERT gene by adenoviral vectors promotes the proliferation of neuronal stem cells in vitro and stimulates

neurogenesis in the hippocampus of mice. J Biomed Res 26:381-388. https://doi.org/10.7555/jbr.26.20110078

14. Magai DN, Karyotaki E, Mutua AM, Chongwo E, Nasambu C, Ssewanyana D, Newton CR, Koot HM, Abubakar A (2020) Long-term outcomes of survivors of neonatal insults: A systematic review and meta-analysis. PLoS One 15:e0231947. https://doi.org/10.1371/journal.pone.0231947
15. Mazumdar T, Sandhu R, Qadan M, DeVecchio J, Magloire V, Agyeman A, Li B, Houghton JA (2013) Hedgehog signaling regulates telomerase reverse transcriptase in human cancer cells. PLoS One 8: e75253. https://doi.org/10.1371/journal.pone.0075253
16. Miles DK, Kernie SG (2008) Hypoxic-ischemic brain injury activates early hippocampal stem/progenitor cells to replace vulnerable neuroblasts. Hippocampus 18:793-806. https://doi.org/10.1002/hipo.20439
17. Ruiz i Altaba A, Palma V, Dahmane N (2002) Hedgehog-Gli signalling and the growth of the brain. Nat Rev Neurosci 3:24-33. https://doi.org/10.1038/nrn704
18. Sun D, Sun XD, Zhao L, Lee DH, Hu JX, Tang FL, Pan JX, Mei L, Zhu XJ, Xiong WC (2018) Neogenin, a regulator of adult hippocampal neurogenesis, prevents depressive-like behavior. Cell Death Dis 9:8. https://doi.org/10.1038/s41419-017-0019-2
19. Tayyab M, Shahi MH, Farheen S, Mariyath MPM, Khanam N, Castresana JS, Hossain MM (2018) Sonic hedgehog, Wnt, and brain-derived neurotrophic factor cell signaling pathway crosstalk: potential therapy for depression. J Neurosci Res 96:53-62. https://doi.org/10.1002/jnr.24104
20. Wyatt HD, West SC, Beattie TL (2010) InTERTpreting telomerase structure and function. Nucleic Acids Res 38:5609-5622. https://doi.org/10.1093/nar/gkq370
21. Yalvaç ME, Yilmaz A, Mercan D, Aydin S, Dogan A, Arslan A, Demir Z, Salafutdinov, II, Shafigullina AK, Sahin F, Rizvanov AA, Palotás A (2011) Differentiation and neuro-protective properties of immortalized human tooth germ stem cells. Neurochem Res 36:2227-2235. https://doi.org/10.1007/s11064-011-0546-7
22. Yang Z, Levison SW (2007) Perinatal hypoxic/ischemic brain injury induces persistent production of striatal neurons from subventricular zone progenitors. Dev Neurosci 29:331-340. https://doi.org/10.1159/000105474
23. Yin X, Meng F, Wei W, Li A, Wang Y, Chai Y, Feng Z (2013) Role of mouse nerve growth factor in neural recovery following hypoxic-ischemic brain damage. Int J Clin Exp Med 6:951-955. https://doi.org/10.1155/2013/318471
24. Yu P, Wang L, Tang F, Zeng L, Zhou L, Song X, Jia W, Chen J, Yang Q (2017) Resveratrol Pretreatment Decreases Ischemic Injury and Improves Neurological Function Via Sonic Hedgehog Signaling After Stroke in Rats. Mol Neurobiol 54:212-226. https://doi.org/10.1007/s12035-015-9639-7
25. Zhou QG, Hu Y, Wu DL, Zhu LJ, Chen C, Jin X, Luo CX, Wu HY, Zhang J, Zhu DY (2011) Hippocampal telomerase is involved in the modulation of depressive behaviors. J Neurosci 31:12258-12269. https://doi.org/10.1523/jneurosci.0805-11.2011

Figures

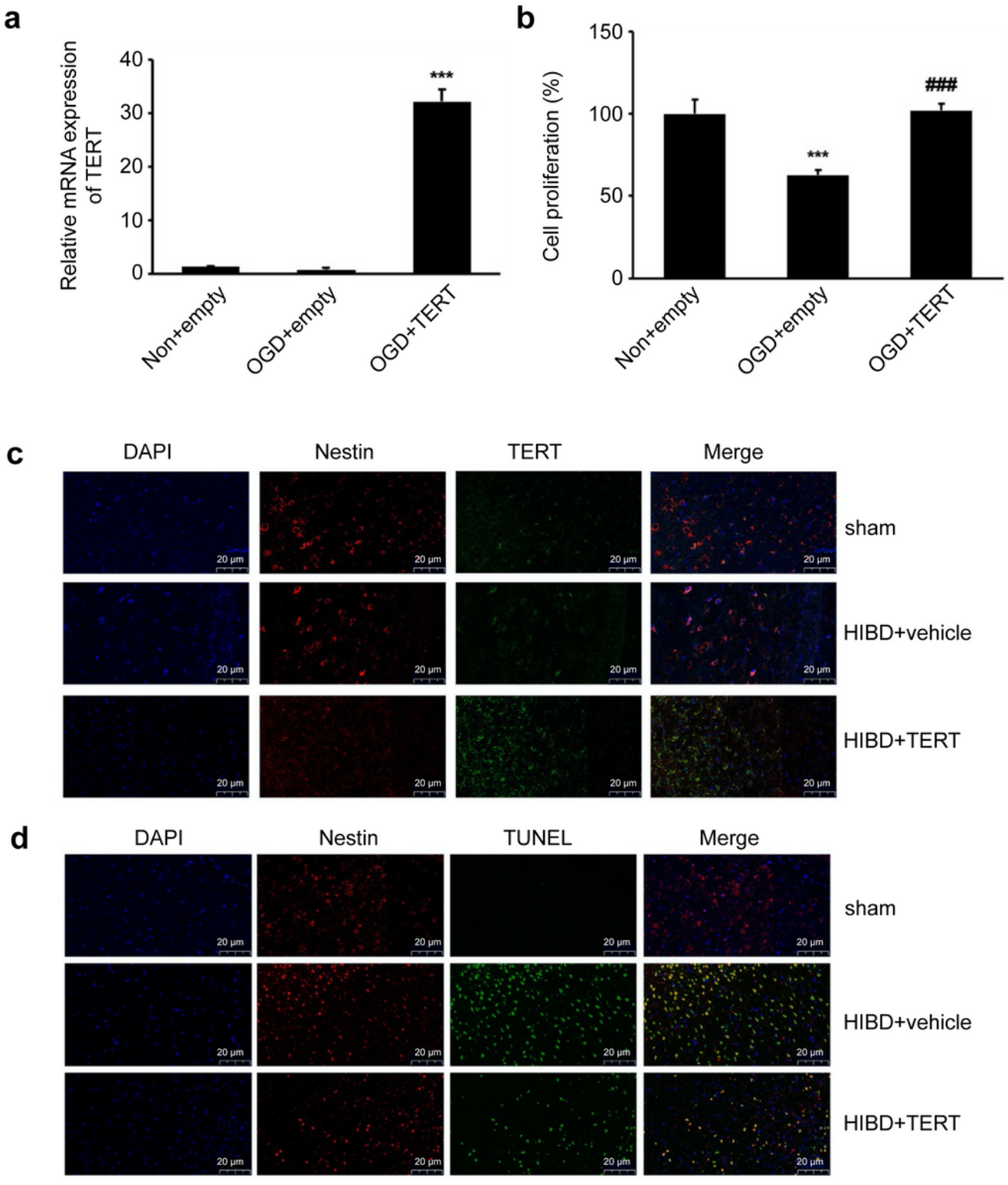

Figure 1

TERT promotes the proliferation of NSCs after OGD in vitro and attenuates the apoptosis of NSCs after HIBD in vivo (a) Q-PCR detects TERT expression level in three groups. ***: P<0.001 compared to Non+empty group and also OGD+empty group, n=3. (b) CCK8 staining detects cell proliferation rate in

NSCs in three groups. ***: P<0.001 compared to Non+empty group, ###: P<0.001 compared to OGD+empty group, n=4. (c) Immunofluorescence staining of TERT (green) and nestin (red) in the brain sections in each group. Nuclei are labeled with DAPI (blue). Scale bar: 20 μm. (d) TUNEL staining (green) and immunofluorescence staining of nestin (red) in the brain sections in each group. Nuclei are labeled with DAPI (blue). Scale bar: 20 μm. HIBD, hypoxic-ischemic brain damage; NSC, neural stem cells; OGD, oxygen glucose deprivation; TERT, telomerase reverse transcriptase

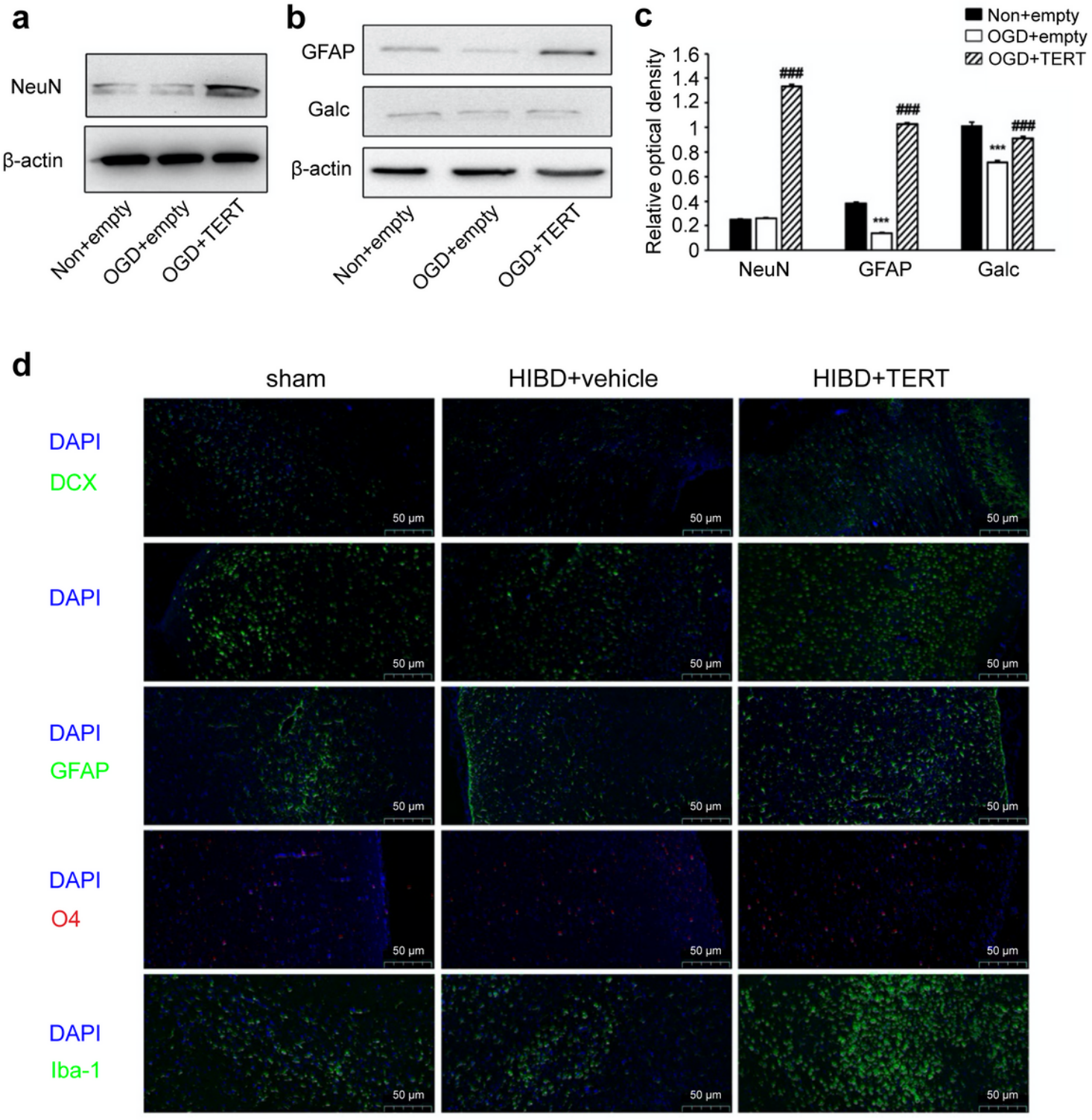

Figure 2

TERT promotes the migration and differentiation of NSCs after HIBD (a, b) Western blot reveals the expression NSCs markers NeuN, GFAP, Galc in Non+empty, OGD+empty and OGD+TERT groups. (c) Quantification of the expression levels of NeuN, GFAP, and Galc. b-actin is set as internal control. (***: P<0.001 compared to Non+empty, ###: P<0.001 compared to OGD+empty group, n=3). (d) Immunofluorescence staining of DCX (green), NeuN (green), GFAP (green), Iba-1 (green) and O4 (red) in the brain sections in each group. Nuclei are labeled with DAPI (blue). Scale bar: 50 μm. HIBD, hypoxic-ischemic brain damage; NSC, neural stem cells; OGD, oxygen glucose deprivation; TERT, telomerase reverse transcriptase

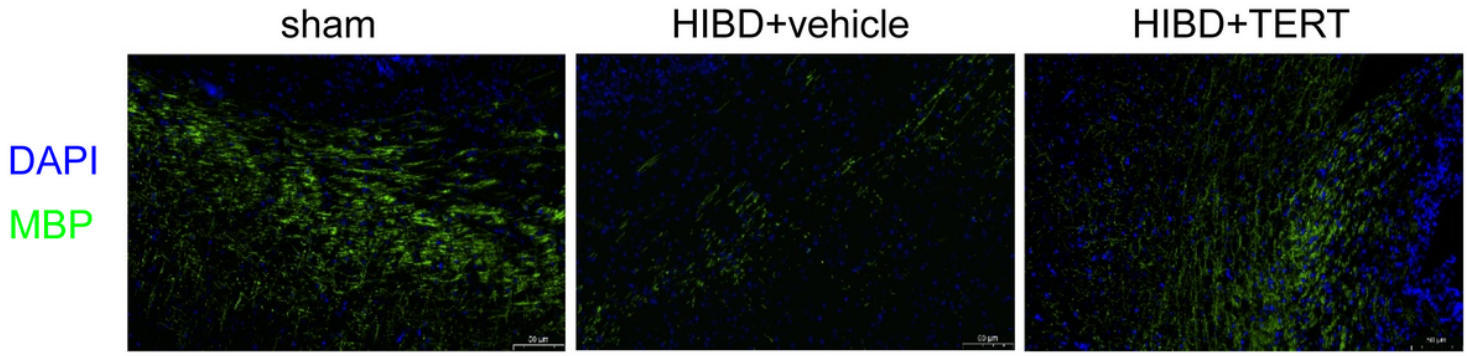

Figure 3

TERT promotes the myelination in the brain of neonatal rat after HIBD Immunofluorescence staining of MBP (green) in the brain section in each group. Scale bar: 50 μm. Nuclei are labeled with DAPI (blue). HIBD, hypoxic-ischemic brain damage, MBP, myelin basic protein; TERT, telomerase reverse transcriptase

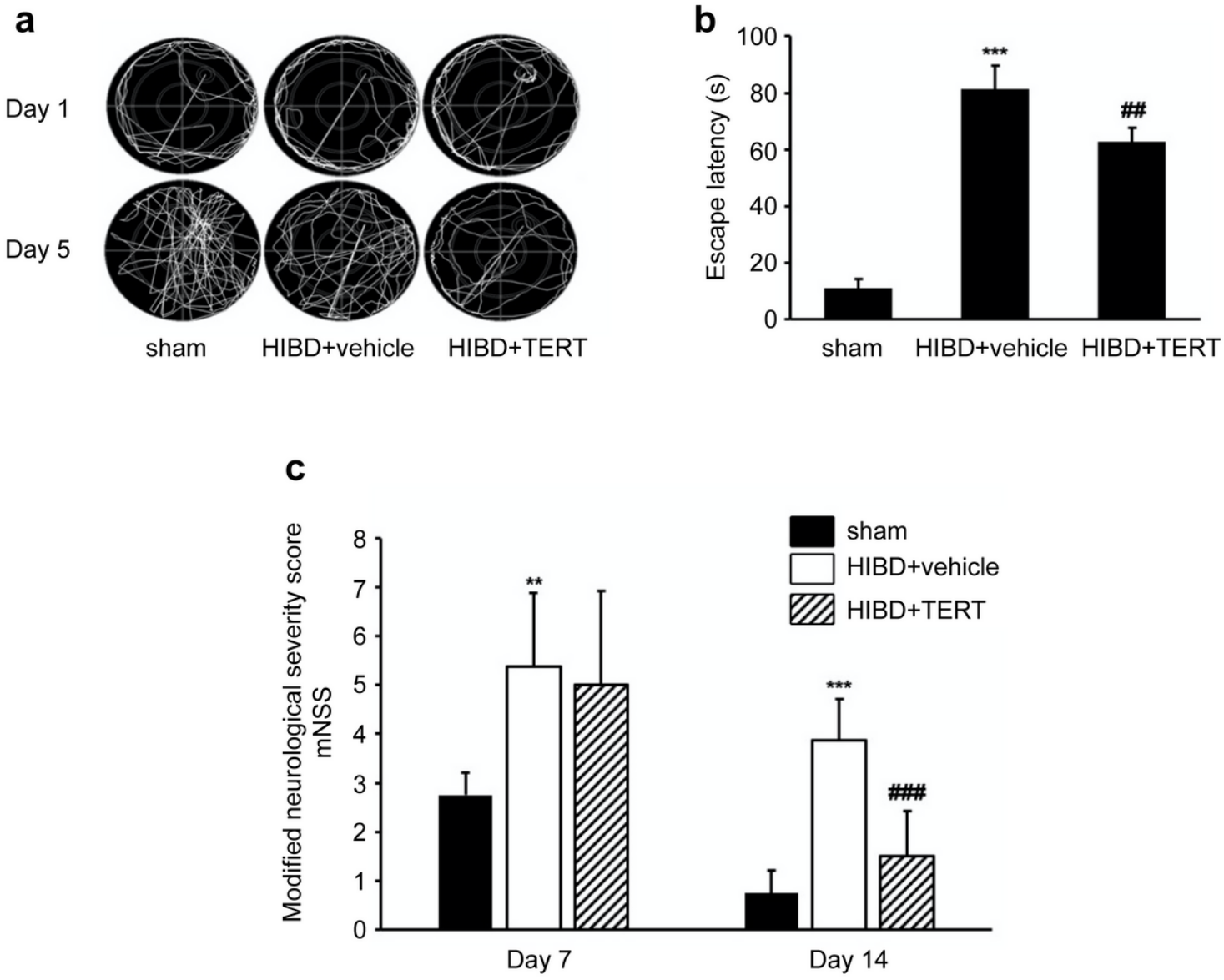

Figure 4

TERT improves the learning and memory abilities and neurological function in neonatal rats after HIBD (a) The trajectory diagrams in the water maze of each group during the first and fifth day. (b) Statistical analysis of the Escape Latency period of the test in each group during the 5th day. (***: P<0.001 compared to sham group, ##: P<0.01 compared to HIBD+vehicle group, n=4). (c) Analysis of the mNSS score in three groups on the 7th day and 14th day after HIBD. (**: P<0.01 compared to sham group, ***: P<0.001 compared to sham group, ###: P<0.001 compared to HIBD+vehicle group, n=8). HIBD, hypoxic-ischemic brain damage; mNSS, modified Neurological Severity Score; TERT, telomerase reverse transcriptase

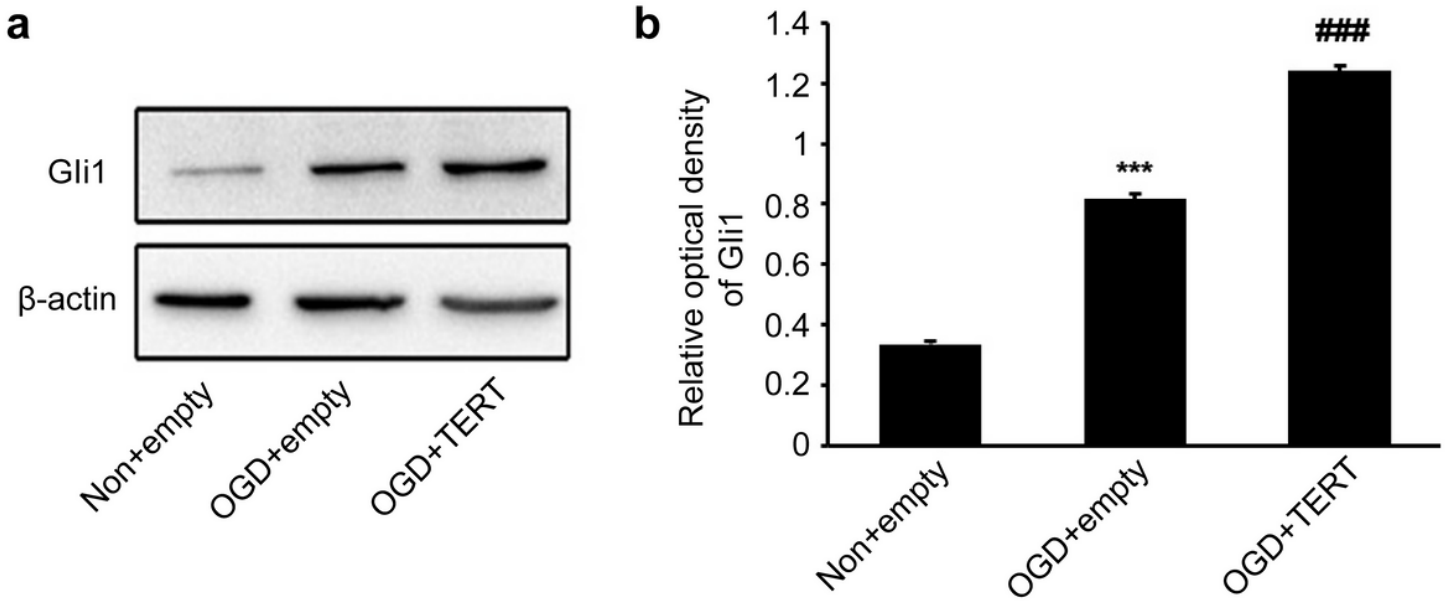

Figure 5

TERT increases the expression of Gli1 in NSCs after OGD (a) Western blot detection of Gli1 expression in three groups. (b) Quantification analysis of the Gli1 expression. b-actin is set as internal control (***: P<0.001 compared to Non+empty group, ###: P<0.001, compared to OGD+empty group, n=3). NSC, neural stem cells; OGD, oxygen glucose deprivation; TERT, telomerase reverse transcriptase