Original Article

TGR5 overexpression mediated by the inhibition of transcription factor SOX9 protects against hypoxia-/reoxygenation-induced injury in hippocampal neurons by activating *Nrf2/HO-1* signaling

Hong Jia[1], Yaoqi Chen[2], Yalin Liu[1]

[1]Department of Neurology, Xingtai People's Hospital, Xingtai, China; [2]Department of Cardiology, Xingtai Third Hospital, Xingtai, China

Contributions: (I) Conception and design: H Jia; (II) Administrative support: H Jia; (III) Provision of study materials or patients: H Jia; (IV) Collection and assembly of data: H Jia, Y Liu; (V) Data analysis and interpretation: H Jia, Y Chen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Hong Jia. Department of Neurology, Xingtai People's Hospital, 16 Hongxing Street, Xiangdu District, Xingtai 054001, China. Email: Jiahong_123456@163.com.

Background: Cerebral ischemia/reperfusion (CI/R) injury is a destructive cerebrovascular disease associated with long-term disability and high mortality rates. *TGR5* has been discovered in multiple human and animal tissues and to modulate a variety of physiological processes. The current study sought to reveal the function of *TGR5* in CI/R injury and uncover the latent regulatory mechanism.

Methods: A hypoxia/reoxygenation (H/R) model was established in mouse hippocampal HT22 cells. The *TGR5* expression in the H/R-treated HT22 cells was tested by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blots. After *TGR5* was overexpressed, Cell Counting Kit-8 assays were used to estimate cell viability, and lactate dehydrogenase (LDH) release was assessed by a LDH assay kit. Cell apoptosis was measured by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assays. Cytochrome c release was detected by immunofluorescence assays and western blots were used to analyze the protein levels of apoptosis-related factors. The oxidative stress levels were assessed by corresponding kits. Next, *SOX9* expression in the H/R-treated HT22 cells was tested by RT-qPCR and western blots. The interaction between the *TGR5* promoter and *SOX9* was verified by luciferase reporter and chromatin immunoprecipitation assays. Subsequently, after the H/R-treated HT22 cells had been co-transfected with *TGR5* overexpression and *SOX9* overexpression plasmids, *TGR5* expression was tested by RT-qPCR and western blots, and the above-mentioned functional experiments were repeated. Finally, the expression of *Nrf2/HO-1* signaling-related proteins was examined by western blots.

Results: *TGR5* expression was significantly decreased in the H/R-exposed HT22 cells. The elevation of TGR5 enhanced the viability, hindered the apoptosis, and alleviated the oxidative stress of the HT22 cells under H/R conditions. Additionally, *SOX9* had a strong affinity with *TGR5* promoter, and *TGR5* was transcriptionally inhibited by *SOX9*. Further, *SOX9* overexpression restored the protective role of *TGR5* upregulation in H/R-induced HT22 cell injury. Additionally, *TGR5* overexpression mediated by *SOX9* inhibition activated *Nrf2/HO-1* signaling.

Conclusions: *TGR5* was transcriptionally inhibited by *SOX9*, and the overexpression of *TGR5* played a protective role in CI/R injury.

Keywords: Cerebral ischemia/reperfusion (CI/R) injury; TGR5; SOX9; Nrf2/HO-1 signaling

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Introduction

Stroke is a major cause of disability and death worldwide (1). It has been referred to as the "incoming epidemic of the 21st century" by the World Health Organization (2). Generally, stroke is divided into the following 2 main types: (I) ischemic stroke due to lack of blood flow; (II) hemorrhagic stroke due to bleeding (3). Ischemic stroke is the most common type of stroke, and accounts for 80% of all strokes (4). During ischemic stroke, a series of harmful cascades occur, including oxidative stress and an inflammatory response, which may eventually result in apoptosis (5). To date, the restoration of blood reperfusion and the resupply of nutrients remain the most efficient treatments for stroke (6). However, cerebral ischemia/reperfusion (CI/R) injury presents a serious complication, which complicates therapy for ischemic stroke patients (7). Thus, novel therapeutic modalities need to be developed to reduce CI/R injury as a pre-requisite for protecting brain functions in ischemic stroke patients.

TGR5 is a G protein-coupled bile acid receptor belonging to the G-protein-coupled receptor (GPCR) superfamily (8). Numerous studies have shown that *TGR5* is expressed in various organs and widely participates in energy homeostasis and metabolism (9-11). More importantly, recent studies on *TGR5* have suggested that *TGR5* ameliorates inflammation in liver ischemia/reperfusion (I/R) injury (12,13). TGR5 could alleviated liver steatosis and inflammation in nonalcoholic steatohepatitis (14) and TGR5 antagonized kidney inflammation in lipopolysaccharide (LPS)-induced injury (15). It can be seen that TGR5 has a alleviating effect on inflammation. Li *et al.* conjectured that TGR5 mediates the protein kinase B/glycogen synthase kinase-3β pathway to prevent myocardial I/R injury (16). *TGR5* activated by Farnesiferol B was shown to alleviate I/R-triggered kidney damage (17). However, little is known about the effect of *TGR5* on CI/R injury.

JASPAR predicts binding of the transcription factor SRY-box transcription factor 9 (SOX9) to the TGR5 promoter. As a member of the *SOX* family, *SOX9* plays pivotal roles in multiple tumors and diseases by regulating the transcription of diverse genes (18). To date, research has primarily focused on the oncogenic roles of *SOX9* in malignant tumors (19,20). Notably, recent research on *SOX9* shows that *SOX9* expression is increased in CI/R rats and the inhibition of *SOX9* mitigates CI/R injury (21,22). Additionally, the elevation of *SOX9* is considered a predominant driver of ischemic brain injury (23). Elevated SOX9 promotes hepatic ischemia/reperfusion injury through activation of TGF-β1 (24). However, very little research has been conducted on the interaction between *SOX9* and *TGR5* in CI/R injury.

Nrf2/HO-1 signaling is known as primary antioxidant signaling. There is increasing evidence that *Nrf2/HO-1* signaling regulates oxidative stress and is involved in multiple human diseases, such as preeclampsia (25), acute lung injury (26), and myocardial I/R injury (27). Further, there is emerging evidence that the activation of *Nrf2/HO-1* signaling plays a protective role in CI/R injury (28-30). However, the specific association between *TGR5* and the *Nrf2/HO-1* signaling pathway in CI/R injury has not been examined.

In this study, a hypoxia/reoxygenation (H/R) model was constructed in HT22 cells to examine the effect of *TGR5* on CI/R injury and the relationship among *TGR5*, *SOX9*, and *Nrf2/HO-1* signaling in CI/R injury. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5225/rc).

Methods

Bioinformatics tools

The JASPAR database (https://jaspar.genereg.net/) was used to predict the binding relationship between *SOX9* and the *TGR5* promoter.

Cell culture and H/R

The mouse hippocampal HT22 cell line (cat. No. BNCC358041) acquired from BeNa Culture Collection (BNCC, Kunshan, China) was grown in Dulbecco's modified Eagle's medium (Saimike, Chongqing, China) with 10% fetal bovine serum (Lonza Group, Ltd.), 100 U/mL of penicillin, and 100 μg/mL of streptomycin as supplements at 37 ℃ in an atmosphere of 5% carbon dioxide (CO_2). For the H/R treatment, the HT22 cells were cultured under hypoxic conditions (5% CO_2 and 95% nitrogen) at 37 ℃ for 24 h, after which, the cells were transferred to a normal humidified incubator at 37 ℃ for 24 h under normal oxygen conditions (95% air and 5% CO_2) (31). The cells that were incubated at 37 ℃ with 95% air and 5% CO_2 were used as the control group.

Plasmid transfection

The PcDNA3.1 vector containing full-length open

reading frame (ORF) human *TGR5* (pcDNA3.1-TGR5) and corresponding negative control (pcDNA3.1), and the *SOX9*-specific overexpression vector (Oe-SOX9) and corresponding negative control (Oe-NC), were obtained from Shanghai GenePharma Co., Ltd. Plasmid transfection was conducted using Lipofectamine 3000 (Carlsbad Life Technologies) in accordance with the manufacturer's instructions. The cells were harvested, and the transfection efficiency was tested via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blots 48 h post-transfection. HT22 cells were transfected with recombined vectors before H/R treatment.

RT-qPCR

To obtain the complementary DNA, total RNA, which had been isolated from the HT22 cells using TRIzol Reagent (Sagon Biotech, Shanghai, China), was reverse transcribed using the QuantiTect RT kit (Qiagen GmbH). PCR was performed using the Fast Start Universal SYBR Green Master mix (Roche Applied Science) on a 7900HT system (Applied Biosystems, CA, USA). The PCR procedure was as follows: denaturation at 94 ℃ for 2 min, amplification at 94 ℃ for 30 sec (30 cycles), annealing at 58 ℃ for 30 sec, extension at 72 ℃ for 1 min, and terminal elongation at 72 ℃ for 10 min. Relative gene expression was estimated using the $2^{-\Delta\Delta Cq}$ method (32), which designated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference.

Western blot

For the detection of nuclear factor erythroid 2-related factor 2 (Nrf2), nucleoprotein and cytoplasmic protein extraction kit obtained from Beyotime (Cat. No. P0028) was used to extract the nucleoprotein and cytoplasmic proteins from the transfected HT22 cells. RIPA cell lysis buffer (Selleck Chemicals) was employed to lyse the transfected HT22 cells and protein quantification was conducted using a BCA protein assay kit (Vazyme). After being segregated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were loaded onto PVDF membranes, which were then blocked using 5% non-fat milk. The primary antibodies specific for TGR5 (Abcam, 1:1,000, ab72608), BCL-2 associated X (Bax; Abcam, 1:1,000, ab32503), B-cell lymphoma 2 (Bcl-2; Abcam, 1:1,000, ab194583), cleaved caspase-3 (Cell Signaling Technology, 1:1,000, #9664), cleaved-PARP (Cell Signaling Technology, 1:1,000, #94885), SOX9 (Abcam, 1:1,000, ab185966), Nrf2 (Abcam, 1:1,000, ab92946), HO-1 (Abcam, 1:2000, ab52947), GAPDH (Abcam, 1:10,000, ab181602) were added to the membranes to be incubated overnight at 4 ℃ and then the HRP-conjugated secondary antibody (Abcam, 1:1,000, ab133470) was added to the membranes to be incubated at room temperature for 1 h. The blots were visualized using the enhanced chemiluminescence system (Clinx Science Instruments Co., Ltd., Shanghai, China), and the signal intensity was measured using ImageJ software (version 1.48v; National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assays

The transfected HT22 cells (5×10^3/well) were seeded into 96-well plates and maintained at 37 ℃ overnight. The cells were cultivated for another 2 h at 37 ℃ after the addition of 10 μL of CCK-8 solution (Beijing Transgen Biotech Co., Ltd.). With the aid of a microplate reader (Dynex Technologies), the absorbance was read at 450 nm.

Detection of lactate dehydrogenase (LDH) release

Briefly, a LDH assay kit (C0016, Beyotime Institute of Biotechnology) was used to detect the LDH concentration in the HT22 cells. In accordance with the manufacturer's instructions, after centrifugation at 400 ×g for 10 min, the cell supernatant was collected. Next, 60 μL of the supernatant was mixed with 30 μL of the LDH substrate solution, and the cells were incubated for additional 30 min at 37 ℃. LDH activity was measured with a microplate reader (Dynex Technologies) at 450 nm.

TUNEL

The apoptotic rate of the HT22 cells was quantified by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) apoptosis kit (Nanjing Biobox Biotech Co., Ltd.) in accordance with the manufacturer's instructions. In brief, the transfected HT22 cells were immobilized with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 8 min. Next, the cells were incubated with 50 μL of TUNEL assay solution for 1 h, and the nuclei were labeled by DAPI (Koritai Biotechnology, Beijing, China) for 10 min. Finally, the images were captured under a fluorescence microscope

(magnification, ×200; UltraVIEW VoX; PerkinElmer, Inc.).

IF staining

In brief, 24 h post H/R treatment, the HT22 cells were immobilized with 4% paraformaldehyde (PFA) solution, and permeabilized with 0.2% Triton X-100 for 10 min. A primary antibody against cytochrome c (cyto-c; Abcam, 1:100, ab133504) was added to label the cells overnight at 4 ℃, and a goat secondary antibody to rabbit immunoglobulin G (IgG) (Alexa Fluor 488; Abcam, 1:1,000, ab150081) was used. DAPI (Koritai Biotechnology, Beijing, China) was used for the nuclear staining. The images were photographed with a fluorescence microscope (UltraVIEW VoX; PerkinElmer, Inc.).

Measurement of malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD)

The cells were decomposed in 300 μL of lysis buffer, and the total protein was quantified using a BCA protein assay kit (Vazyme). The corresponding kits from Nanjing Jiancheng Bioengineering Co. Ltd. were used to determine the levels of MDA (A003-1-2), GSH-Px (A005-1-2), and SOD (A001-1-2) in accordance with the manufacturer's instructions. The absorbance was determined at 450 nm using a microplate reader (Dynex Technologies).

ChIP

The EZ-Magna ChIP A/G kit (MilliporeSigma) was used for the chromatin immunoprecipitation (ChIP) assays. The HT22 cells were sonicated into 200–1,000 bp DNA fragments and centrifuged at 300 ×g for 3 min at 25 ℃. Next, the SOX9 antibody (Abcam, 1:60, ab185966) or the IgG antibody (Beyotime Institute of Biotechnology, 1 μg/μL, A7016) was used for immunoprecipitation.

Luciferase reporter assays

The sequences of *TGR5* promoter region were inserted into the pGL3 basic vector (Laboratory of Anhui Medical University) to construct *TGR5*-WT (CCATTGGTC) and *TGR5*-MUT (AACGGAAGA), after which, they were co-transfected with Oe-*SOX9* and Oe-NC into the HT22 cells via Lipofectamine 3000 (Carlsbad Life Technologies). After 48 h, the luciferase assay system (Ambion, Austin, TX, USA) was employed to estimate the luciferase activity.

Statistical analysis

All the statistical analyses were conducted using SPSS 22.0 (Chicago, Illinois, USA). The experimental data were presented as the mean ± standard deviation (SD) and were biologically repeated in triplicate. Differences between the groups were evaluated by the Student's *t*-test or a 1-way analysis of variance followed by a Tukey's post-hoc test. A P value <0.05 was considered statistically significant.

Results

Elevation of TGR5 contributes to HT22 cell viability after H/R

To assess the effect of *TGR5* on CI/R injury, *TGR5* expression was examined following H/R treatment in the HT22 cells. As *Figure 1A*,*1B* show, the RT-qPCR and western blots indicated that *TGR5* expression was sharply reduced in the H/R-induced HT22 cells compared to the control group. After the transfection of pcDNA3.1-*TGR5*, the overexpression efficiency was tested, and *TGR5* expression was found to be significantly increased (*Figure 1C*,*1D*). The experimental results of the CCK-8 assays revealed that H/R treatment significantly suppressed the viability of the HT22 cells, and when *TGR5* was upregulated, the viability of the H/R-induced HT22 cells was significantly increased (*Figure 1E*). Further, LDH release was enhanced in the HT22 cells after H/R treatment, after which, the overexpression of *TGR5* led to a decrease in LDH release (*Figure 1F*). Collectively, *TGR5* was downregulated in the H/R-induced HT22 cells and decreased the viability of H/R-induced HT22 cells.

TGR5 overexpression suppresses the apoptosis of HT22 cells under H/R conditions

Conversely, the TUNEL assays revealed that H/R treatment significantly increased the apoptotic rate of the HT22 cells, but this effect was decreased after *TGR5* was overexpressed (*Figure 2A*). The mitochondrial release of cyto-c has been confirmed as a hallmark of neuronal death (33). The immunofluorescence assays revealed that the increased cyto-c level was decreased by the upregulation of *TGR5* (*Figure 2B*). Additionally, the western blots showed that the Bcl-2 protein level declined, while the Bax, cleaved

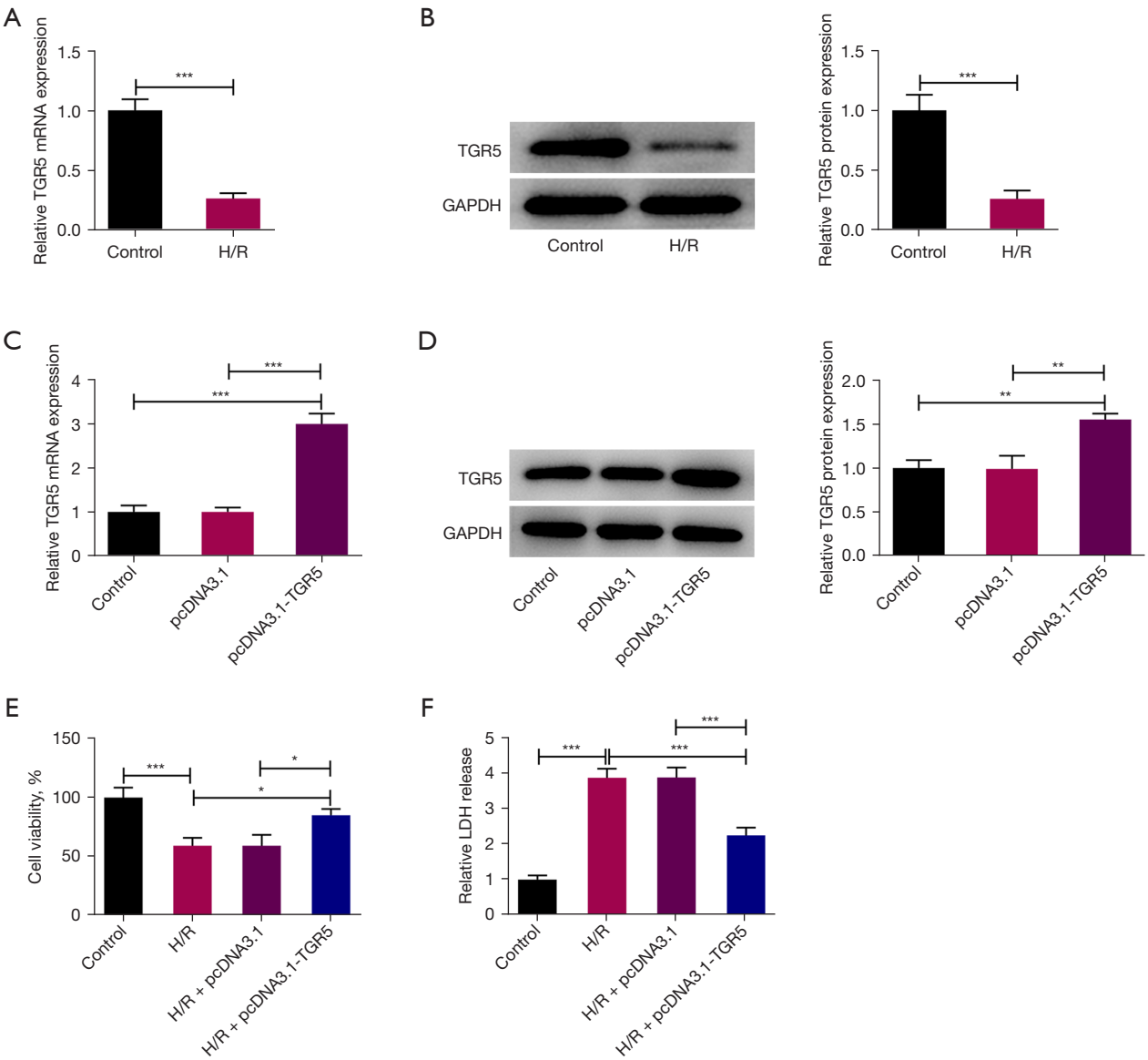

Figure 1 The elevation of TGR5 contributes to HT22 cell viability after H/R. (A) RT-qPCR and (B) western blots were used to determine TGR5 expression in the HT22 cells in the absence or presence of H/R treatment. (C) RT-qPCR and (D) western blot analyses of the overexpression efficacy of pcDNA3.1-TGR5 plasmid. The viability of the H/R-treated HT22 cells was assessed by (E) CCK-8 assays. (F) LDH release was measured by a LDH assay kit. *, P<0.05; **, P<0.01; ***, P<0.001. H/R, hypoxia/reoxygenation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LDH, lactate dehydrogenase.

caspase-3 and cleaved-PARP protein levels increased in the HT22 cells under H/R conditions, and *TGR5* elevation increased the protein level of Bcl-2 but further decreased the protein levels of Bax, cleaved caspase-3, and cleaved-PARP (*Figure 2C,2D*). Thus, these results suggest that *TGR5* played a suppressive role in the H/R-induced apoptosis in the HT22 cells.

TGR5 upregulation reduces H/R-evoked oxidative stress in HT22 cells

To explore the effect of *TGR5* on oxidative stress in the H/R-induced HT22 cells, the levels of oxidative stress markers, including SOD, GSH-Px, and MDA, were evaluated by corresponding kits, and we discovered that *TGR5* increased the downregulated SOD and GSH-Px levels and decreased

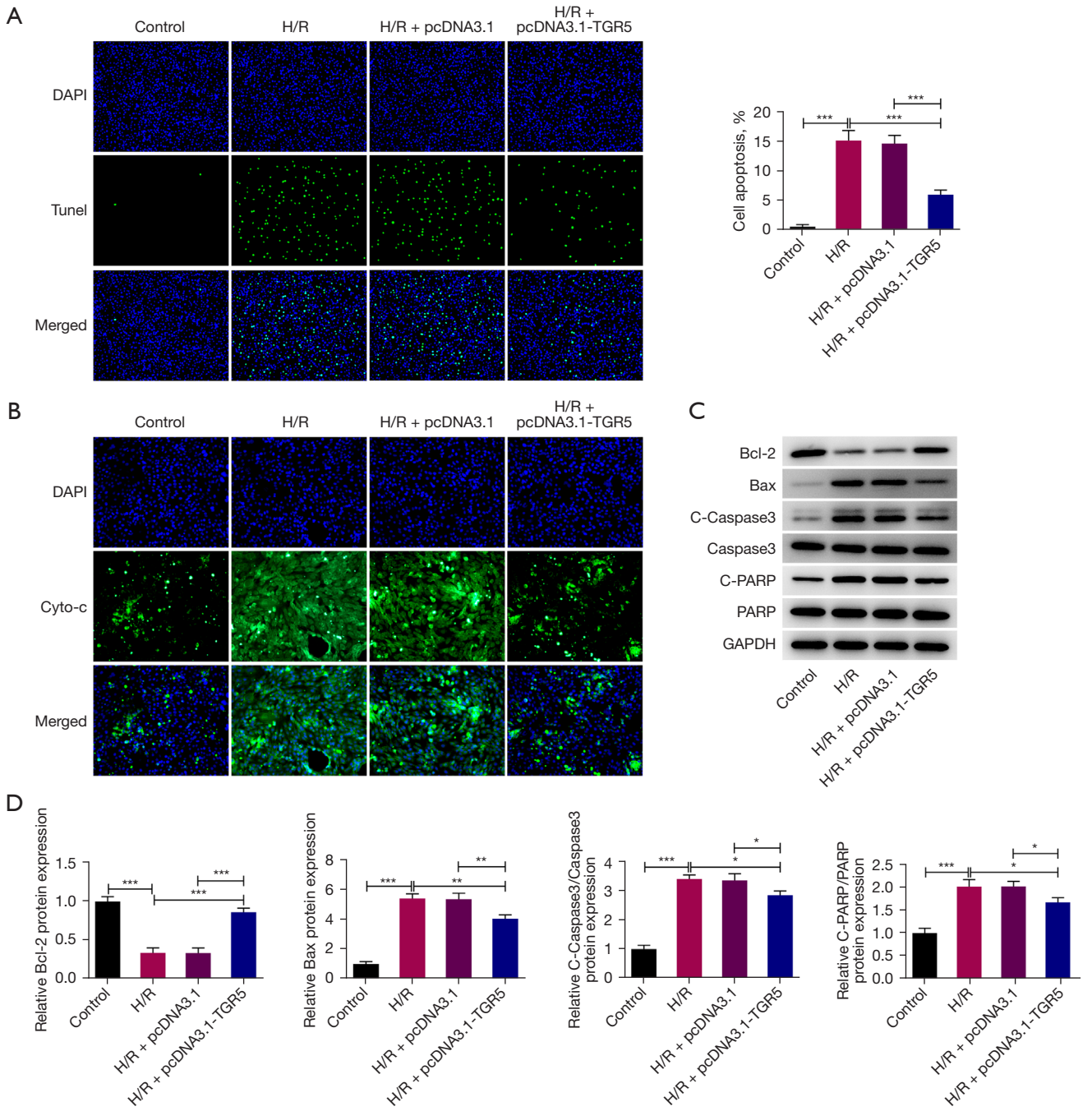

Figure 2 TGR5 overexpression suppresses the apoptosis of HT22 cells under H/R conditions. The apoptotic ability of the H/R-treated HT22 cells was evaluated by (A) TUNEL assays (magnification, 200×). (B) IF staining was used to detect cyto-c release (magnification, 200×). (C,D) Western blots were used to analyze the protein levels of Bcl-2, Bax, cleaved caspase-3, and cleaved-PARP. *, P<0.05; **, P<0.01; ***, P<0.001. H/R, hypoxia/reoxygenation; TGR5, Takeda G protein-coupled receptor 5; C-Caspase3, cleaved caspase3; C-PARP, cleaved-PARP.

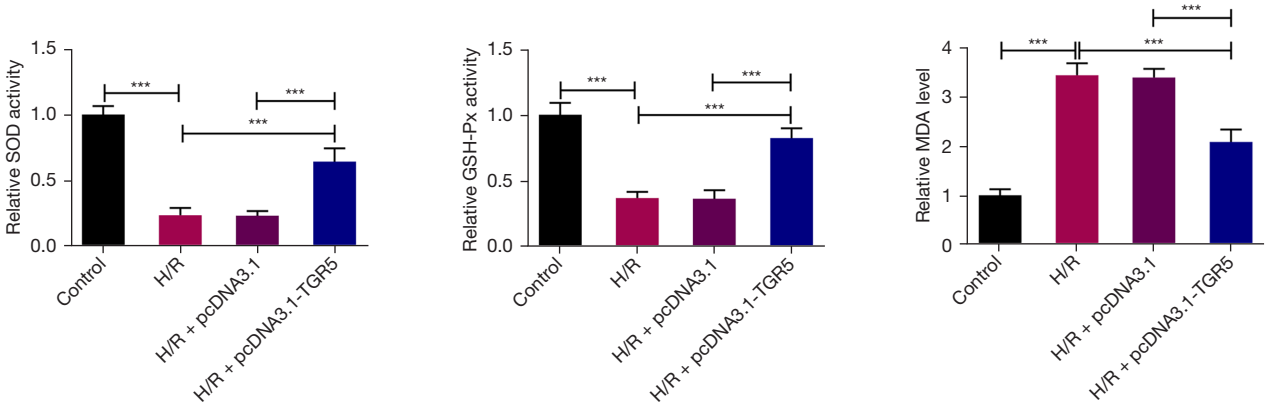

Figure 3 TGR5 upregulation decreases H/R-evoked oxidative stress in HT22 cells. Corresponding kits were used to examine the levels of SOD, GSH-Px, and MDA. ***, P<0.001. H/R, hypoxia/reoxygenation; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

the upregulated MDA level in the H/R-treated HT22 cells (*Figure 3*). In summary, the elevation of *TGR5* mitigated oxidative stress in HT22 cells under H/R conditions.

TGR5 is transcriptionally inhibited by SOX9

According to the JASPAR database, we hypothesized that *SOX9* would have a binding relationship with the *TGR5* promoter and predicted possible binding sites (*Figure 4A*). The RT-qPCR and western blots also showed that *SOX9* was highly expressed in the H/R-induced HT22 cells compared to the control group (*Figure 4B,4C*). Meanwhile, after *SOX9* overexpression following the transfection of the Oe-*SOX9* plasmid (*Figure 4D,4E*), the luciferase reporter assays showed that *SOX9* overexpression notably reduced the luciferase activity of *TGR5*-WT but not *TGR5*-MUT (*Figure 4F*). Additionally, the results of the ChIP assays revealed that the *TGR5* promoter was highly abundant in the SOX9 antibody, which suggested that the *TGR5* promoter had a strong affinity with *SOX9* (*Figure 4G*). Moreover, the increased expression of *TGR5* after the transfection of pcDNA3.1-*TGR5* plasmid was decreased in the H/R-induced HT22 cells when *SOX9* was upregulated (*Figure 4H,4I*). Overall, *SOX9* was an upstream transcription factor of *TGR5* and transcriptionally suppressed *TGR5* expression.

Upregulation of SOX9 reverses the inhibitory role of TGR5 in H/R-triggered HT22 cell injury

To further determine the effects of the *SOX9/TGR5* axis in the H/R-induced HT22 cells, pcDNA3.1-*TGR5* and Oe-*SOX9* plasmids were co-transfected into the H/R-induced HT22 cells and functional experiments were then conducted. The CCK-8 assays showed that the stimulated viability of the H/R-induced HT22 cells generated by TGR5 overexpression was restored when *SOX9* was upregulated (*Figure 5A*). In addition, the increased SOD and GSH-Px levels, the falling MDA level and LDH release resulting from *TGR5* were reversed by *SOX9* in the H/R-treated HT22 cells (*Figure 5B*). As *Figure 5C* shows, the TUNEL assays confirmed that the upregulation of *TGR5* impaired the apoptotic capacity of the HT22 cells under H/R conditions, but this effect was reversed by *SOX9*. Similarly, the decrease in cyto-c release due to *TGR5* was also reversed after *SOX9* was overexpressed (*Figure 5D*). Similarly, *SOX9* elevation offset the upregulation on Bcl-2 protein level and the downregulation on Bax, cleaved caspase-3 and cleaved-PARP protein levels (*Figure 5E*). Taken together, our results showed that the effects of *TGR5* on H/R-treated HT22 cells were all reversed by *SOX9*.

TGR5 elevation mediated by SOX9 interference activates Nrf2/HO-1 signaling

To examine the role of *Nrf2/HO-1* signaling in *SOX9/TGR5* axis-mediated HT22 cell injury under H/R conditions, the protein levels of related factors in *Nrf2/HO-1* signaling were detected by western blots. The H/R treatment increased the Nrf2 protein level in the cytoplasm but decreased the Nrf2 protein level in the nucleus and HO-1 protein level. After *TGR5* was overexpressed, Nrf2

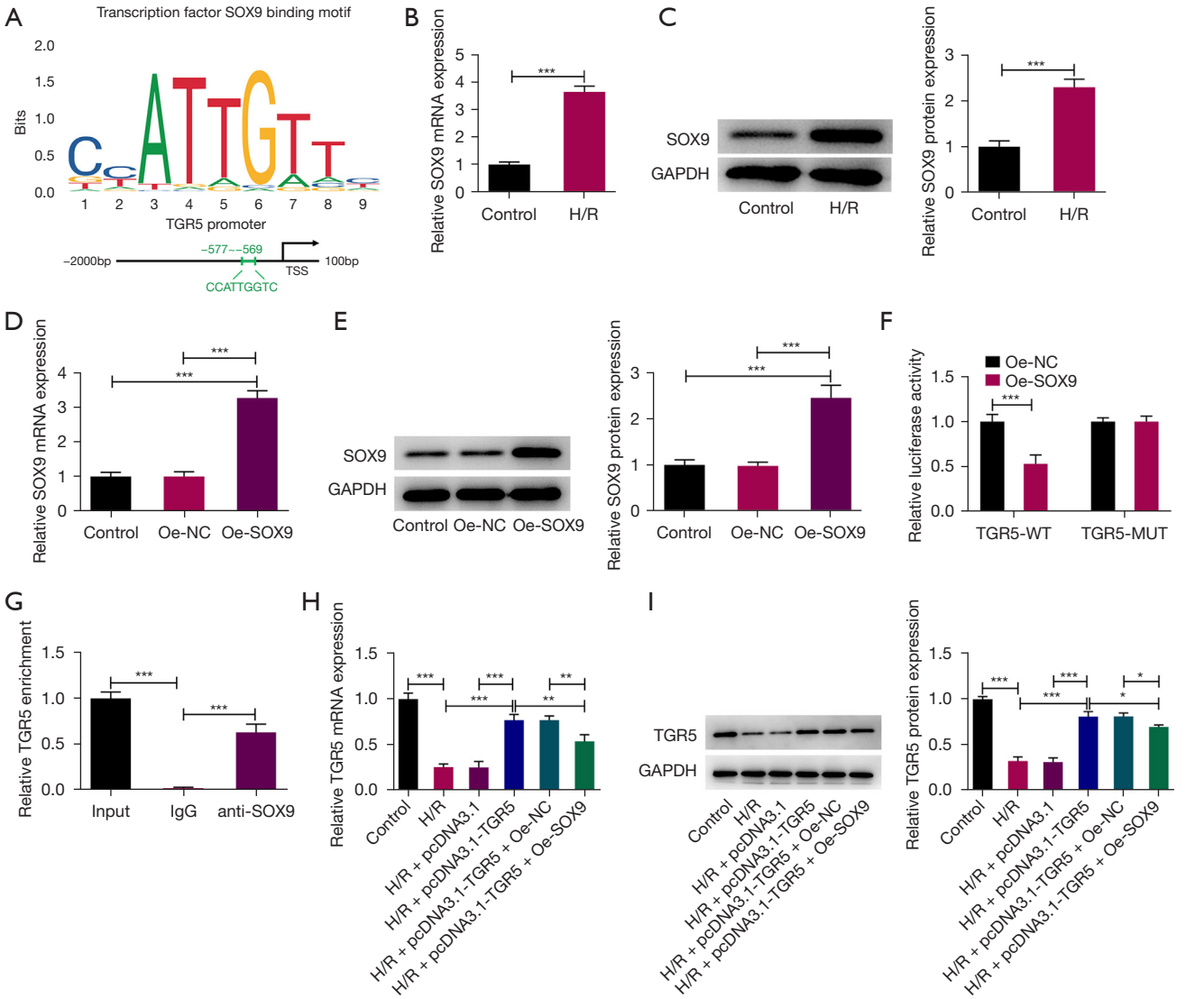

Figure 4 TGR5 is transcriptionally inhibited by SOX9. (A) The potential binding sites between TGR5 promoter and SOX9 were predicted by the JASPAR database. (B) RT-qPCR and (C) western blot were used to determine SOX9 expression in the HT22 cells in the absence or presence of H/R treatment. (D) RT-qPCR and (E) western blot assays were used to analyze the overexpression efficacy of the Oe-SOX9 plasmid. (F) Luciferase reporter assays were used to verify the luciferase activity of WT and MUT TGR5 promoter. (G) ChIP assays were used to identify the accumulation of the TGR5 promoter in the SOX9 antibody. (H) RT-qPCR and (I) western blot assays were used to ascertain TGR5 expression in H/R-induced HT22 cells co-transfected with pcDNA3.1-TGR5 and Oe-SOX9 plasmids. *, P<0.05; **, P<0.01; ***, P<0.001. H/R, hypoxia/reoxygenation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control.

expression was decreased in the cytoplasm but both Nrf2 expression in the nucleus and HO-1 expression were increased, and this effect was in turn reversed by *SOX9* (*Figure 6*). Thus, *SOX9* silencing-mediated *TGR5* elevation led to the activation of *Nrf2/HO-1* signaling.

Discussion

Cerebral ischemic stroke is generally acknowledged to be one of the most under-treated serious diseases and has high mortality and disability rates globally (1,34). Despite great advancements in therapy for ischemic stroke, the

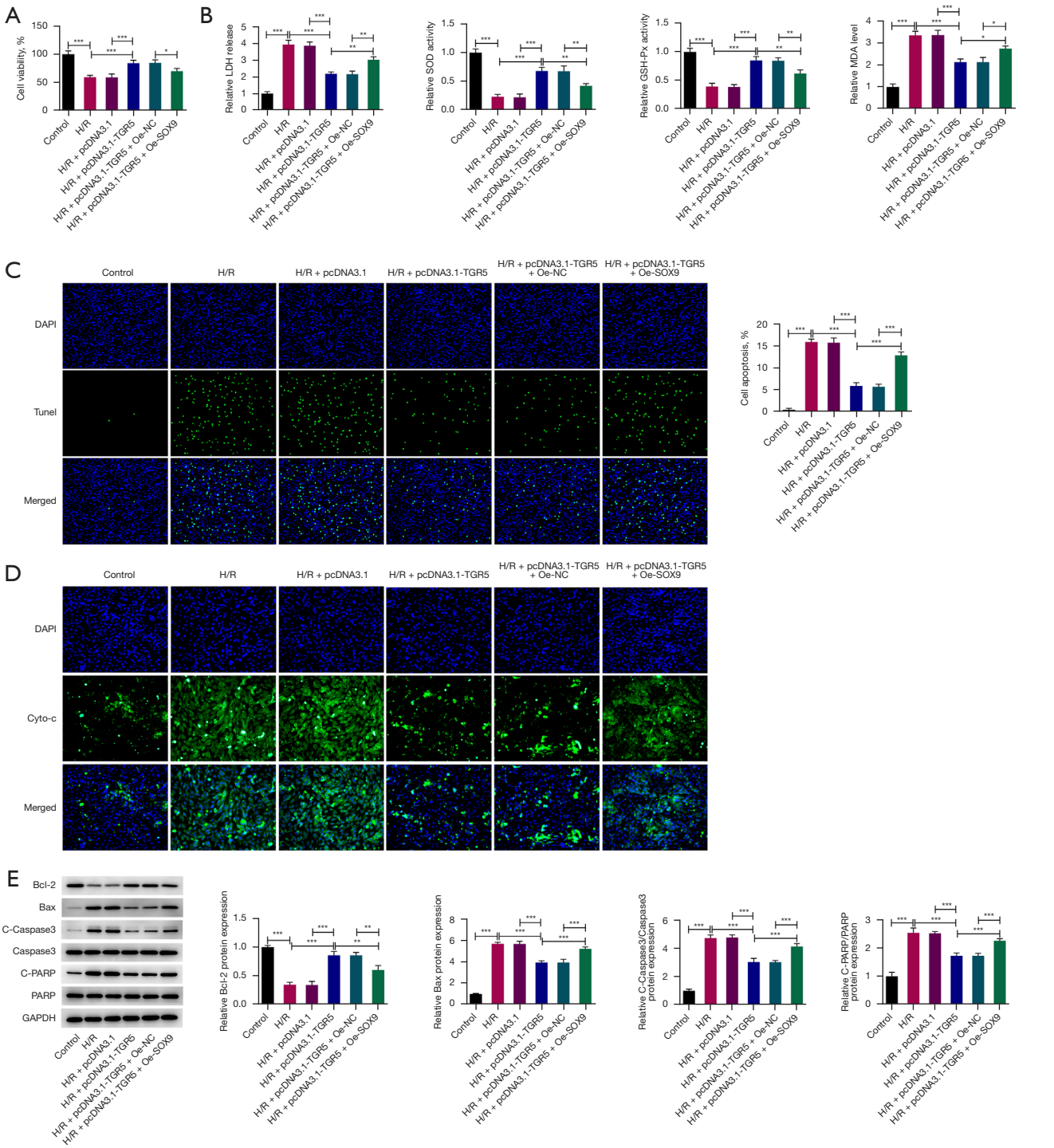

Figure 5 The upregulation of SOX9 reverses the inhibitory role of TGR5 in H/R-triggered HT22 cell injury. The viability of H/R-treated HT22 cells was assessed by (A) CCK-8 assays; (B) LDH release was measured by the LDH assay kit. Corresponding kits were used to examine the levels of SOD, GSH-Px, and MDA. The apoptotic ability of the H/R-treated HT22 cells was evaluated by (C) TUNEL assays (magnification, 200×). (D) IF staining was used to detect cyto-c release (magnification, 200×). (E) Western blots were used analyze the protein levels of Bcl-2, Bax, cleaved caspase-3, and cleaved-PARP. *, P<0.05; **, P<0.01; ***, P<0.001. H/R, hypoxia/reoxygenation; NC, negative control; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; C-Caspase3, cleaved caspase3; C-PARP, cleaved-PARP.

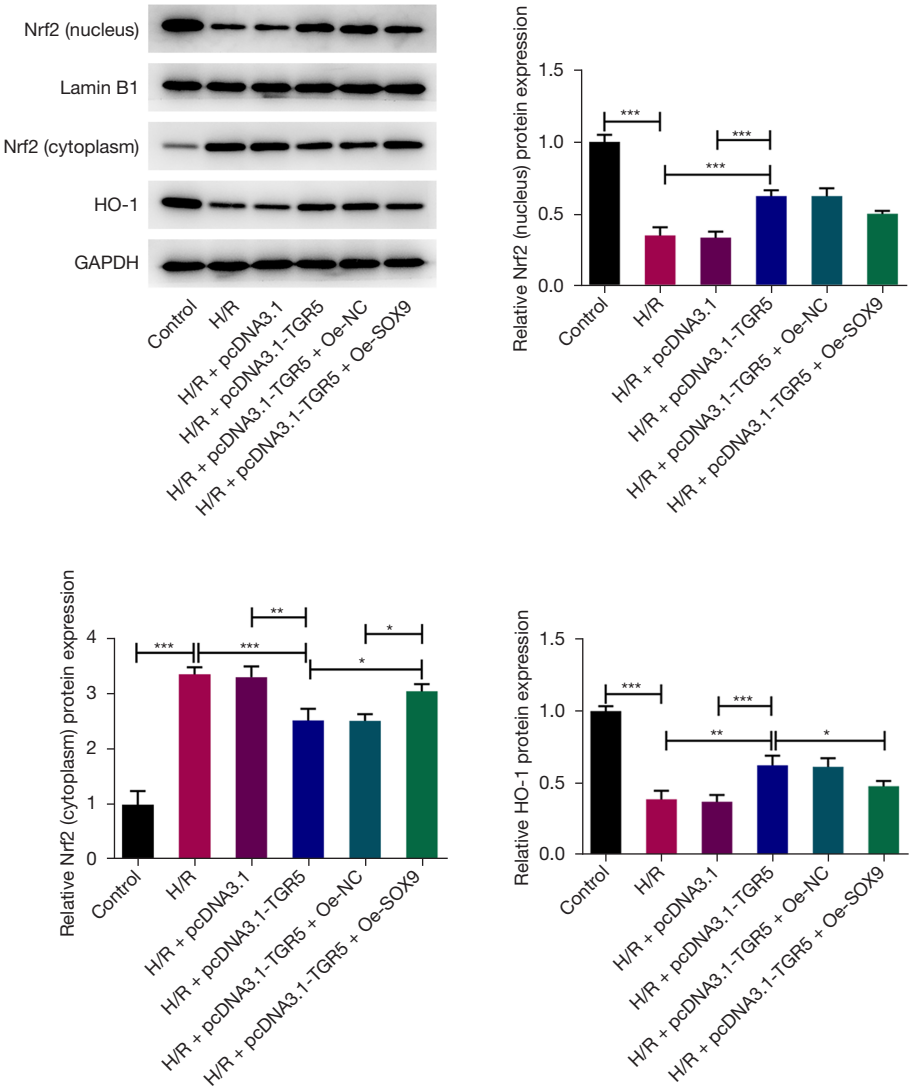

Figure 6 TGR5 elevation mediated by SOX9 interference activates Nrf2/HO-1 signaling. Western blots were used to analyze the protein levels of Nrf2 both in the cytoplasm and nucleus, and HO-1. *, P<0.05; **, P<0.01; ***, P<0.001. H/R, hypoxia/reoxygenation; NC, negative control.

therapeutic options remain limited (3,35). During the course of cerebral ischemic stroke, the existence of I/R injury may contribute to blood-brain-barrier disruption, neuron death, and the deterioration of cerebral infarction (36,37). Thus, effective therapeutic measures that protect the brain from CI/R injury need to be established. Oxidative stress, which frequently occurs following CI/R, may cause further damage to the brain tissue in ischemic stroke by stimulating cell death (38). In this study, a H/R model was first established in the HT22 cells. We then explored the proliferation, apoptosis, and oxidative stress of H/R-induced HT22 cells to gain further insights into the pathogenesis of CI/R injury.

In recent years, the role of *TGR5*, which was the first identified G-coupled protein receptor specific for bile acids, has received extensive attention in relation to the regulation of metabolic syndrome and related disorders (8,39,40). For example, Huang *et al.* showed that the *TGR5* agonist enhances glucose homeostasis in diabetes (41). While Guo *et al.* suggested that *TGR5* ameliorates gastric inflammation by suppressing the nuclear factor kappa B signaling pathway (42). Interestingly, there is extensive evidence that *TGR5* alleviates

I/R injury in the liver (12,43), myocardium (16), and kidney (17). Consistent with these findings, *TGR5* was found to be significantly downregulated in the HT22 cells under H/R conditions in the present study.

Functionally, the overexpression of *TGR5* facilitated the viability of the H/R-induced HT22 cells. LDH is a stable cytoplasmic enzyme, the activity of which can be determined to appraise cytotoxicity (44). The experimental results in this study showed that the stimulated LDH release decreased after *TGR5* was overexpressed. Additionally, *TGR5* elevation significantly reduced the apoptotic rate of the H/R-treated HT22 cells. Cyto-c is a multifunctional enzyme associated with cell apoptosis. As anticipated, TGR5 hampered the H/R-triggered cyto-c release in the HT22 cells. Further, *TGR5* upregulation increased the protein level of Bcl-2 and decreased the protein levels of Bax, cleaved caspase-3, and cleaved-PARP in the H/R-induced HT22 cells. Our study also showed that *TGR5* upregulated the levels of antioxidant stress-related enzymes, including SOD and GSH-Px, but downregulated the level of the oxidative damage marker MDA.

It has been reported that *SOX9* modulates the expression of some genes by functioning as a transcription factor (18). For example, *SOX9* is a transcriptional regulator of *TSPAN8* in pancreatic cancer (45). *FOXA1* is also dependent on the *SOX9* transcription factor in lung carcinoma (46). In this study, *SOX9* expression was found to be increased in the H/R-induced HT22 cells. The binding between *SOX9* and *TGR5* promoter was predicted using the JASPAR database and proven by mechanism assays. Additionally, *TGR5* expression in the HT22 cells transfected with pcDNA3.1-*TGR5* plasmid under H/R conditions was reduced when *SOX9* was upregulated, implying that *TGR5* was transcriptionally inhibited by *SOX9*. It should be noted that numerous studies have emphasized the significance of *SOX9* in CI/R injury, and all these studies have suggested that *SOX9* primarily serves as a promoter in CI/R injury (21-23). Our findings also suggested that the increased viability and the decreased LDH release, apoptosis, cyto-c release, and oxidative stress in the H/R-treated HT22 cells due to *TGR5* overexpression were all reversed by *SOX9*.

Transcription factor *Nrf2* provides adaptive protection against oxidative stress by activating the transcription of the antioxidant response element-driven gene HO-1 in the cell nucleus (47). More importantly, the dysregulation of *Nrf2/HO-1* signaling is associated with CI/R injury (28,29,48). TGR5 has also been shown to act as an activator of *Nrf2/HO-1* signaling in cholestatic liver disease (49) and high glucose-stimulated cardiomyocyte injury (50). Similarly, our results also indicated that *Nrf2* expression was increased in the cytoplasm but decreased in the nucleus and HO-1 expression was also decreased in the HT22 cells following H/R treatment. Additionally, the induced translocation of *Nrf2* from the cytoplasm to the nucleus and HO-1 expression due to *TGR5* were restored by *SOX9* overexpression.

Conclusions

In conclusion, *TGR5* overexpression mediated by the inhibition of *SOX9* increased the viability but decreased the apoptosis and oxidative stress of the H/R-treated HT22 cells by activating *Nrf2/HO-1* signaling. Our findings might lead to the identification of a novel molecular mechanism of *SOX9/TGR5/Nrf2/HO-1* signaling in CI/R injury. However, there was also existed limitation in this study. The effects of TGR5 on CI/R injury in animal models needed to be explored in future study.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5225/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5225/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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