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Original Article

Regulation of ZNF580 in pathogenesis of ischemic cerebrovascular disease accompanied with upregulation of *Smad2* and ADNP

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Abstract: Ischemic cerebrovascular disease is caused by the reduction or suspension of artery resulting in nerve cell degeneration or necrosis. We conducted the middle cerebral artery occlusion-reperfusion model (MACO) in rats. The reperfusion increased the mRNA and protein level of zinc finger protein 580 (ZNF580). Meanwhile, upregulation of transforming growth factor-β (TGF-β), SMAD family member 2 (*SMAD2*) and activity-dependent neuroprotective protein (ADNP) were also observed. ZNF580 siRNA treatment decreased the expression of *Smad2* and ADNP. Furthermore, we also found that a significant increase in the percentage of necrosis cells in MACO. In summary, the results indicated that ZNF580 may regulate ADNP and *Smad2* through TGF-β signaling pathway for protecting cerebrum from ischemic cerebrovascular disease.

Keywords: MACO, ZNF580, TGF-β, *Smad2*, ADNP

Introduction

Ischemic cerebrovascular disease (ICVD) remains the most prevalent causes of mortality and morbidity worldwide. It is a group of brain dysfunctions related to disease of the blood vessels supplying the brain such as hypertension and stroke. Ischemia or other blood vessel dysfunctions can affect the person during a cerebrovascular incident [1, 2]. The experiment studies revealed that it is caused by the reduction or suspension of artery resulting in nerve cell degeneration or necrosis [3, 4].

ZNF580 (zinc finger protein 580) was found involved in the regulation of endothelial cell proliferation and migration [5]. Animal studies have suggested a link between the ischemia injury caused by sclerosis or cerebral infarction and the progress of recovery [6, 7]. Study showed that TGF-β signaling pathway have been found to affect the brain after cerebral ischemia, which is a protective response to ischemic brain injury [8, 9]. Increased TGF-β1 can reduce the infarct area against cerebral ischemic injury [9].

Smad2, a member of the Smad family, is an important downstream factor in the TGF-β signaling pathway [10, 11]. It possibly related to the following molecular events, including phosphorylation by Smad4, translocation into nucleus and regulation of downstream genes transcription. Studies indicated that Smad4 was significantly increased after reperfusion in gerbils, which might indicate a sign of extent of ischemic injury [12, 13]. In addition, previous research showed that ZNF580 could interact with *Smad2* in the progress of ischemic injury [14]. ADNP (activity-dependent neuro-protector) was considered to play an important role in the process of cell damage and recovery. It was expressed mainly in astrocytes and neurons and identified as a mediator of neuronal survival induced by various stimuli causing cell death. ADNP can enhance the expression of genes

associated with organogenesis and neurogenesis. Although there have been studies foucus on the ischemia-reperfusion injury, the precise biological mechanisms of the relationship between ADNP and TGF-β pathway are still not well understood.

In order to investigate the function of ZNF580 in the process, we employed the middle cerebral artery occlusion-reperfusion model (MACO) for assessing the change of ZNF580, TGF-β, *Smad2* and ADNP with different treatments. The mRNA and protein levels were detected by Real time-PCR, Western blotting and Immunohistochemistry, and the cell apoptosis was also investigated.

Materials and methods

Animals and groups

SD male rats (280-300 g) were obtained from the Department of Laboratory Animal Science of Peking University, Health Science Center (Beijing, China). All the experiments were conducted under the guidelines of the Animal Care.

Experimental procedures were carried out with approval of the ethical committee guidelines of the School of Medicine of the Logistics University of Chinese People's Armed Police Force, which are in accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Rats were divided into five groups: the middle cerebral artery occlusion (MACO, n=5) group was set as a stands operating control; siRNA ZNF580 group (n=5) and siRNA control (n=5) group were transfected with siRNA ZNF580 or siRNA control respectively before operating; sham group (n=5) was just silted neck skin but not got middle cerebral artery occlusion; and the control group (n=5) was not treated at all.

The middle cerebral artery occlusion-reperfusion model (MACO)

Chloral hydrate (400 mg/kg) was used to anesthetize rats. Double flank holes of the first cervical vertebra were exposed under the operating microscope; inserting bipolar coagulation forceps into it to electro coagulate bilateral vertebral artery and making it permanent occlusion. Animal was insulated to let it awake and then anesthetized as described previously after housing for 24 h, exposing bilateral carotid artery and monitoring EEG (Nation 7128, Beijing, China). Occluding bilateral carotid artery quickly with artery clips to make global brain ischemia. Remove the clips after 15 min and reperfusion. Animal appearing resting brain waves is regarded as a successful model. Sham group is treated as the same except no coagulating or occluding of bilateral vertebral artery [15, 16].

SiRNA transfection

Short interfering RNA (siRNA) transfection in vivo was performed. Firstly, siRNA ZNF580 (guide: 5'AAAUAUAAAAUGAUAUUUCUC3', passenger: 5'GAAAUAUCAUUUUAUAUUUUC3') or siRNA control (Jima, Suzhou, China) was dissolved in RNase-free water to a final concentration of 1 μg/μL. Then, 5 μL ZNF580 siRNA or 5 μL control siRNA and 5 μL Entranster-in vivo transfection reagent (18668-11, Engreen, Co., Beijing, China) were respectively diluted with 5 μL 10% glucose. And then, 10 μL of diluted Entranster-in vivo transfection reagent was mixed with equal volume of above suspension for 15 min at room temperature. Finally, 20 μL of mixture were injected from the tail vein to Rats.

Real-time PCR

To analyze the mRNA level target genes, the real-time PCR was performed. Rats were euthanatized after being treated as previously described and the whole brains were removed out. Then, 0.5 gram of each brain tissue was weighed for RNA extraction using the Trizol RNA regent (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA was digested by the DNAse I at 37°C for 1 h and stop with the stop buffer at 65°C for 10 min. RT-PCR was performed to synthesis cDNA with the Reverse Transcriptase XL (TaKaRa, Co., Dalian, China) and oligo-d18T primer (Toyobo, Co., Ltd., Osaka, Japan). The synthesized cDNA was used as template for real-rime PCR using the ABI 7900 real-time PCR system (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Foster City, CA, USA). The program was: 95°C 10 min, 95°C 15 s, 60°C 1 min, 72°C 10 min, 40 cycles. The primers were given as follow. β-actin, forward: 5'-GGCTGTGTTGTCCCTGTATGC-3', reverse: 5'-GTCACGCACGATTTCCCTCTC-3'; ZNF580, forward: 5'-CGCCATCTGCTCATCGACG-3' reverse: 5'-AGCGGGCAGGTGTGAGGTG-3'; TGF-β, for-

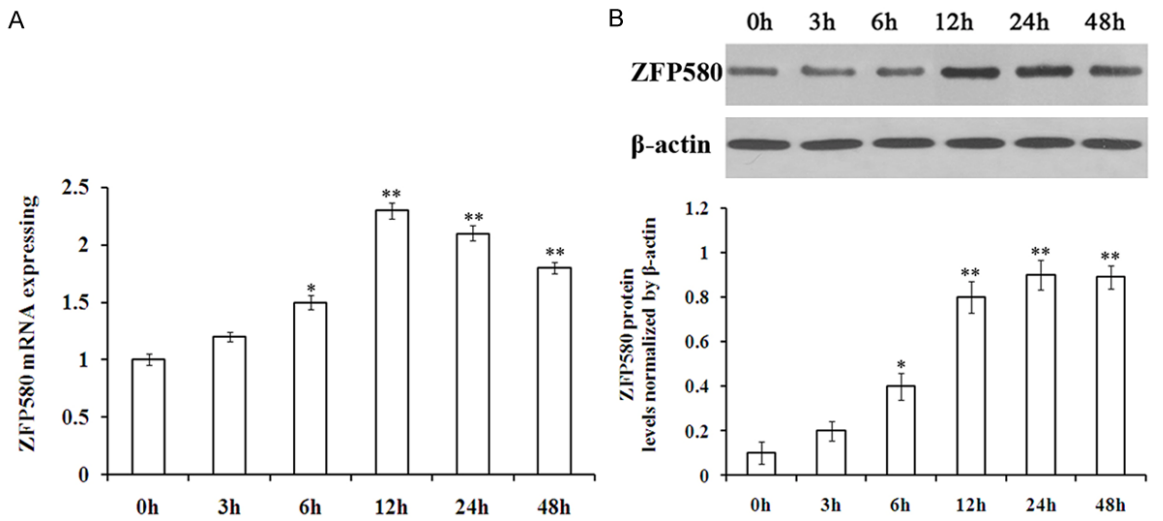

Figure 1. Detection of the mRNA and protein level of ZNF580. Rats (n=6) were euthanatized 0 h, 3 h, 6 h, 12 h, 24 h and 48 h after reperfusion respectively and brains were removed out to conduct real-time PCR (A) and western blotting (B). Data are expressed as mean ± standard error of six replicates in each group. **P* < 0.05, ***P* < 0.01, compared with solvent control group.

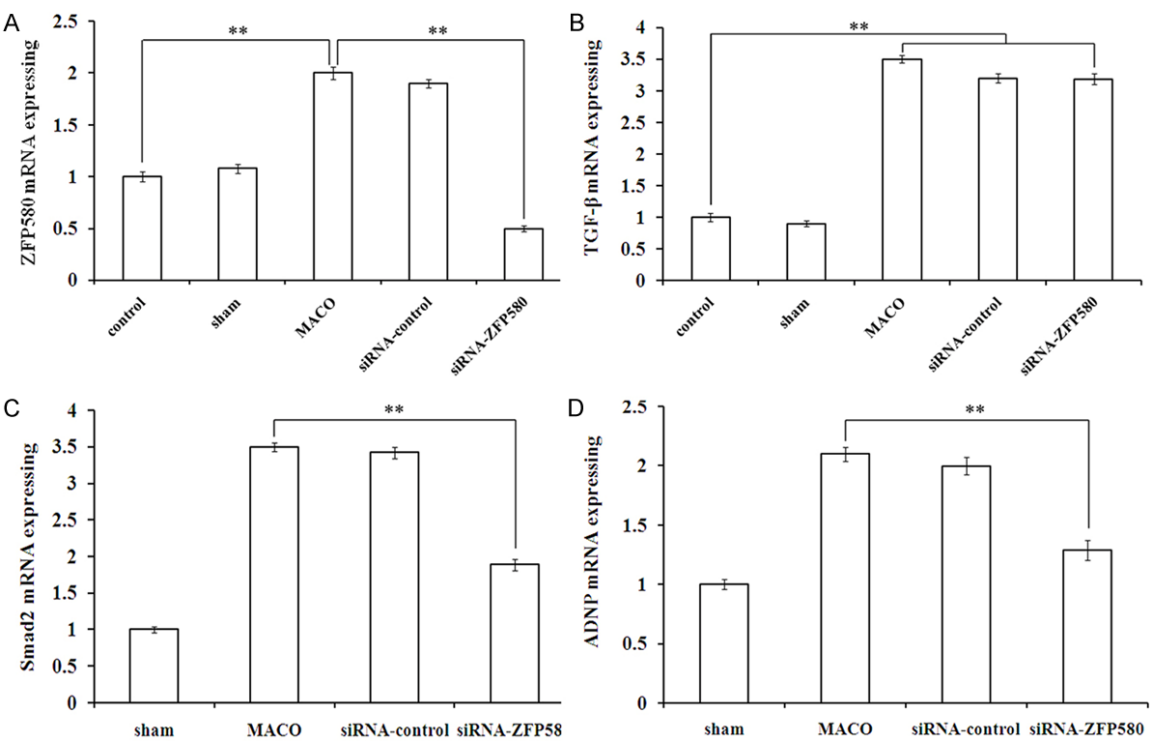

Figure 2. Detection of the mRNA level of ZNF580 (A), TGF-β (B), *Smad2* (C) and ADNP (D) by RT-qPCR on the condition of ZNF580 Silencing. Rats (n=5 each group) were transfected with siRNA ZNF580 or siRNA control respectively and euthanatized 12 h after reperfusion. Brains were removed out for real-time PCR. Data are expressed as mean ± standard error of five replicates in each group. **P* < 0.05, ***P* < 0.01, compared with solvent control group.

ward: 5'-GGACCGCAACAACGCCATC-3', reverse: 5'-GCCCTGTATTCCGTCTCCTTGG-3'; *Smad2*, forward: 5'-ATGCGTCACAGCCCTCG-3', reverse: 5'-AGGCACTCAGCAAACACTTCC-3'; ADNP, forward: 5'-CCGTTCCACCTTCAATGATGTAGA-3', reverse: 5'-TTTGGAGGAACTGGGGCATT-3'.

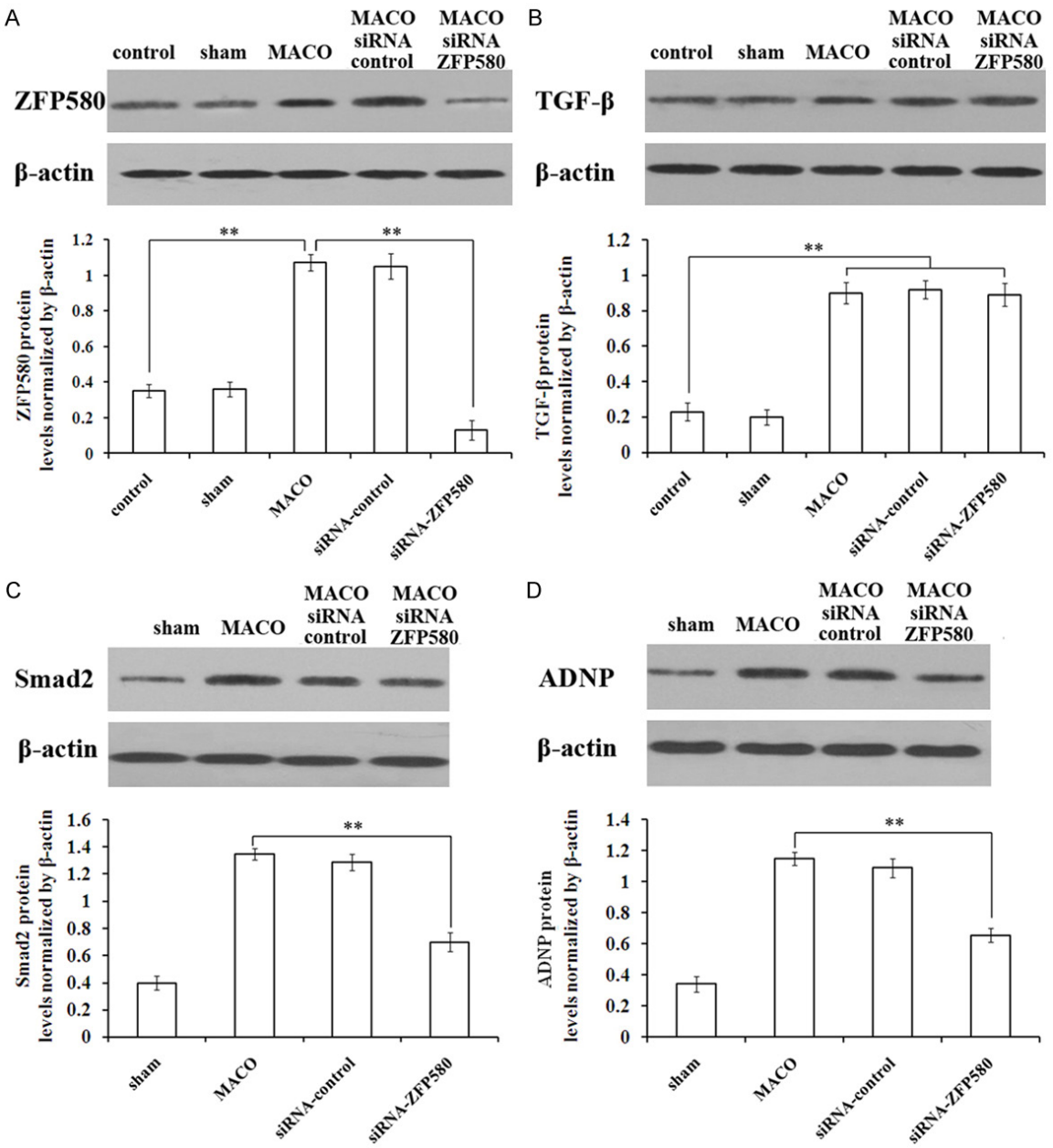

Figure 3. Detection of the protein level of ZNF580 (A), TGF-β (B), *Smad2* (C) and ADNP (D) by real-time-PCR on the condition of ZNF580 Silencing. Rats (n=5 each group) were transfected with siRNA ZNF580 or siRNA control respectively and euthanatized 12 h after reperfusion. Brains were removed out for western blotting. Data are expressed as mean ± standard error of five replicates in each group. **P* < 0.05, ***P* < 0.01, compared with solvent control group.

Western blotting

To verify the result of real-time PCR, western blotting was used to detect the expression of each gene in this model. Rats were treated as previously described and euthanatized. Brains were removed out and 0.5 gram of each was lysed with 1 ml mammalian tissue lysate (Sigma-Aldrich Co. USA) for 30 min on ice. Then the mixture were centrifuged with 12000 rpm/min for 10 min at 4°C and the supernatant was collected for quantizes with G250. 50 μg of each sample was used for the SDS-PAGE gel electrophoresis and the protein was transferred onto a nitrocellulose filter membrane. Then primary antibody (ZNF580: Santa Cruz, CA, USA

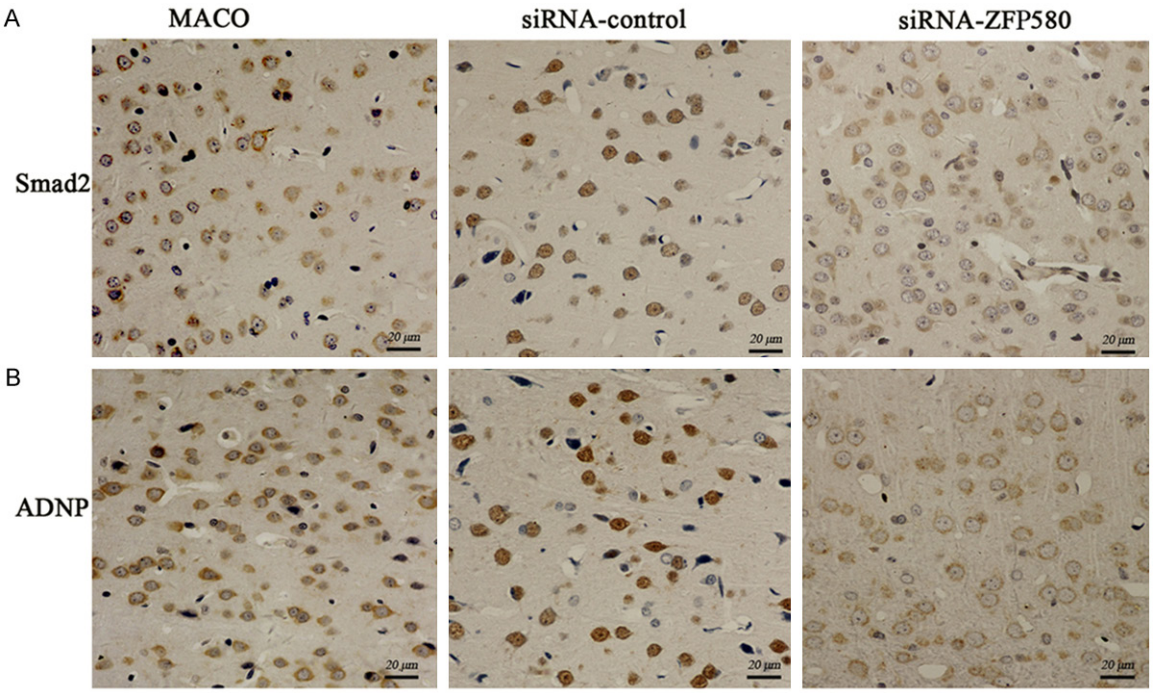

Figure 4. Immunohistochemistry of *Smad2* (A) and ADNP (B). Rats (n=5 each group) were euthanatized 12 h after reperfusion of all groups. Brains were removed out and immunohistochemistry was performed.

sc-109176; β-actin: Boster BM0627; TGF-β: Santa Cruz, CA, USA, sc-31608; *Smad2*: Cell Signaling Technology, Inc. USA #5678; ADNP: Santa Cruz, CA, USA, sc-393377) against each protein was added (1:1000). Then secondary antibody was added for western blot using the ECL (Thermo, America) by the gel imaging system (DNR bio-imaging system, America).

Immunohistochemistry

For histological analysis, animals of operating groups were anesthetized 12 h after treatment. Brains were removed and post-fixed with formalin, and cryoprotected in 30% sucrose in PBS for over 48 h at 4°C. Immunohistochemistry was performed [16] using the same antibodies as above. Tissue was stained with DAB regent in the dark for 2 min after incubated in the secondary antibody and observed under an OLYMPUS BX51 microscope (Janpa).

Statistical analysis

The statistical analyses were performed using the Student's t-test. Values presented as mean ± S.D. Statistical significance was defined as $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$).

Results

Effect of reperfusion on ZNF580 expression

To investigate the effects of reperfusion on ZNF580, the mRNA and protein level in the model were assessed by real-time PCR and western blotting. As shown in **Figure 1A**, the mRNA level of ZNF580 were up-regulated in all treatment group ($P < 0.01$ for all), and the highest peak reached 2-fold at 12 h compared with the control group. As shown in **Figure 1B**, the result of protein level was consistent with that of mRNA level.

Effect of reperfusion on mRNA expression

To investigate whether TGF-β pathway was regulated by ZNF580, in vivo siRNA transfection was conducted. Real-time PCR were performed to detect the expression level of TGF-β at 12 h after reperfusion. As shown in **Figure 2**, The mRNA level of zfp850, tgf-β, *smad2* and adnp was detected by real-time PCR. Animals were euthanatized at 12 h after reperfusion when ZNF580 at the highest level. In ZFP580 mRNA level, the control and siRNA-ZFP580 group were decreased compared with the MACO group

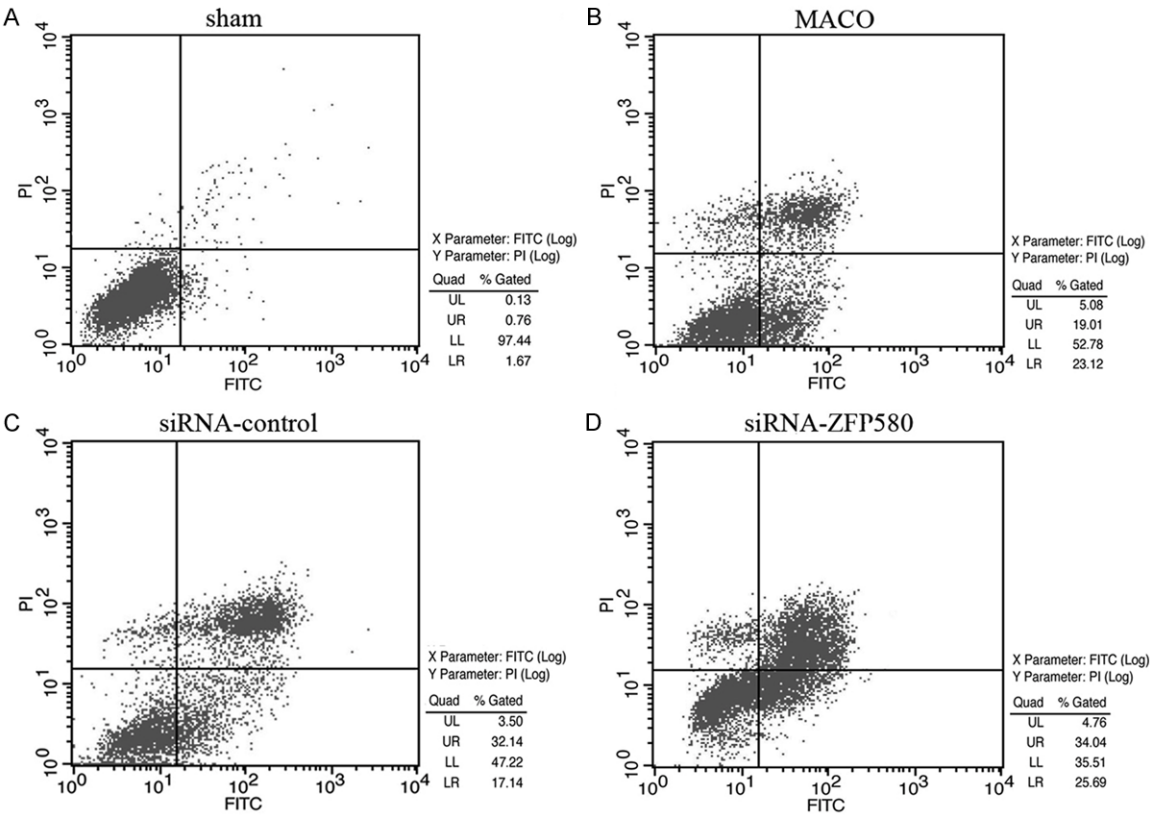

Figure 5. Rats (n=5 each group) were euthanatized 12 h after reperfusion of all groups. Cell death mode was analyzed by dual-parameter flow cytometry utilizing Annexin V-FITC and PI. Representative dot plot of sham cells from three independent experiments (A). Representative dot plot of MACO cells from three independent experiments (B). Representative dot plot of siRNA-control cells from three independent experiments (C). Representative dot plot of siRNA-control cells from three independent experiments (D). The percentages of apoptotic and necroptotic cells were shown graphically.

($P < 0.01$). In TGF-β mRNA level, the MACO and siRNA-ZFP580 group were increased compared with the control group ($P < 0.01$). In Smad-2 mRNA level, the siRNA-ZFP580 group was decreased compared with the MACO group ($P < 0.01$). In ADNP mRNA level, the siRNA-ZFP580 group was decreased compared with the MACO group ($P < 0.01$).

Effect of reperfusion on protein expression

To investigate whether TGF-β pathway was regulated by ZNF580, in vivo siRNA transfection was conducted; western blotting was performed to detect the expression level of TGF-β at 12 h after reperfusion. As shown in **Figure 3**, The protein level of ZFP850, TGF-β, *Smad2* and ADNP was determined. It was shown that ZNF580 was successfully knocked down by the siRNA (**Figure 3A**). The expression of TGF-β was slightly decreased 3.5% compared with the control siRNA group but not statistical significantly (**Figure 3B**). Though siRNA ZNF580 did not influence the TGF-β expression, it reduced the level of *smad2* effector factor. As shown in **Figure 3C**, a significant increase of *Smad2* was observed (3.3-fold compared to sham group) in the ischemic injury groups, which was consistent with that of TGF-β. However, when ZNF580 was silenced (siRNA-ZNF580), the *Smad2* levels exhibit 3 fold decrease compared with the control (siRNA-control). Similar to *Smad2*, the ADNP was also increased after reperfusion at 12 h and decreased when ZNF580 was silenced in ischemic injury (**Figure 3D**).

Effect of Smad2 and ADNP on Immunohistochemistry

To further analyze the expression of *Smad2* and ADNP in the brain of the rats in the operating groups, immunohistochemistry was performed. The number of positive spot was counted and used for statistical analysis. As shown

in **Figure 4**, *Smad2* and ADNP were expressed in all groups, and the numbers of positive spots in the siRNA ZNF580 group were less than the other two groups, which suggested the least level expression of them.

Effect of ZNF580 on cell necrosis

To determine the cell death in the model at 12 h after reperfusion, the percentages of apoptotic, necroptotic and necrotic cells were determined by FACS analysis of Annexin V/PI double-staining. The cells negative for both PI and Annexin V staining are viable cells (in the lower left quadrant, LL), the PI-negative Annexin V-positive staining cells are apoptotic cells (in the lower right quadrant, LR), the PI and Annexin V-positive staining cells are primarily necroptotic cells (in the upper right quadrant, UR), and the PI- positive and Annexin V-negative staining cells are necrotic cells (in the upper left quadrant, UL). As shown in **Figure 5**, compared with the sham group, the significant increases in the percentages of necrosis cells were observed in MACO group. The results indicated that the reperfusion could induce the necrosis cell in the model.

Discussion

Previous study indicated that the activation of TGF-β signaling pathway play an important role in the development of ischemic diseases [17, 18]. It indirectly mediated anti-inflammatory response through suppression of endothelial cell activation [18]. ZNF580 has been identified to be a transcription factor recently and participate in the process of vascular inflammatory and homeostasis of endothelial cells [19, 20]. Other studies also demonstrate that ZNF580 can regulate the expression of MMP-2 and VEGF, which involved in the migration and proliferation of vascular endothelial cells [21]. TGF-β signaling pathway is activated via two different serine/threonine kinase receptors, types I and II. As an important downstream factor in the TGF-β signaling pathway, *Smad2* is specifically activated by type I receptors [22]. The phosphorylated *Smad2* translocated into the nuclei and then interact with transcription factors thus regulate the transcription of target genes [23]. In our study, the data indicate that the transcriptional and translational level of ZNF580, TGF-β, *Smad2* and ADNP were significantly increased in the cerebral artery occlusion-reperfusion model (MACO), *Smad2* together with ADNP decreased by varying degrees after silencing ZNF580 while TGF-β seems to be little affected in the model. The expression of ZNF580 was significantly increased after reperfusion, which implicated that it may accelerate the proliferation of vascular endothelial cells and be involved in the recovering from cerebral ischemia. We also found that TGF-β increased as that of ZNF580, but not under the siRNA interference in ischemic injury. However, the protein of *Smad2* was decreased under the siRNA interference in ischemic injury. It indicated that ZNF580 might regulate *Smad2* but not TGF-β as well as preventing cell from the injury of inflammation by interacting with *Smad2*.

Activity-dependent neuroprotective protein (ADNP) belongs to a homeodomain of the zinc finger-containing protein family. Previous study indicated ADNP is essential for mouse embryonic brain formation and performance a high expression in the cerebral cortex and cerebellum brain [24]. It can participate in the development of the embryonic brain and protect adult brain from the damage caused by oxidative stress [25]. ADNP could reduce the release of cell toxin which harmful to brain and edema surrounding the damage tissue. High expression of ADNP may inhibit the activation of p53 signaling pathway, resulting in reduction of inflammatory cytokines and Caspase3-dependent cell apoptosis. In our study, the mRNA and protein level of ADNP were upregulated in the MACO model and decreased by silencing ZNF580, which revealed ADNP can be regulated by ZNF580. In addition, research has reported that the expression of ADNP is upregulated in astrocytes by VIP, it reveals that the regulation to ADNP is complicate process consist of multiple factors [26]. However, the underlying molecular mechanisms between ZNF580 and ANDP were still remaining elusive.

Based on our results of ZNF580, TGF-β, *Smad2* and ADNP significantly increased in the cerebral artery occlusion-reperfusion model (MACO), as well as *Smad2* together with ADNP decreased by varying degrees after silencing ZNF580 while TGF-β seems to be little affected in the model. we purposed that ZNF580 could affect ADNP and TGF-β signaling pathway via regulating *Smad2* level. Furthermore, ZNF580 would influence the ADNP levels. It suggests that ZNF580 plays a role in danmaging repair in

ischemic injury through regulating the *smad2* and ADNP levels. which provides the clues for further research in the cerebrovascular diseases.

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Disclosure of conflict of interest

None.

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