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RESEARCH PAPER

ZEB2, interacting with MDM2, contributes to the dysfuntion of brain microvascular endothelial cells and brain injury after intracerebral hemorrhage

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ABSTRACT

ZEB2 has been shown to be upregulated in the brain tissues of rats with intracerebral hemorrhage (ICH), but its role in ICH-caused brain injury remains unclear. Here, an ICH rat model was established via intracerebral injection of autologous blood, and the lentivirus-mediated ZEB2 short hairpin RNA (sh-ZEB2) or negative control (scramble) were administered 0.5 hours after ICH. Silencing ZEB2 alleviated ICH-induced neurologic deficits and the increase of BBB permeability, brain water content and ZEB2 expression. Next, OGD (oxygen glucose deprivation) plus hemin was used to treat primary brain microvascular endothelial cells (BMECs) to simulate the ICH condition *in vitro*. OGD plus hemin upregulated ZEB2 expression and apoptosis, but reduced cell viability, migration, TEER (transendothelial electric resistance) and the expression of vascular-endothelial (VE-) cadherin, occludin and claudin-5, which was reversed by inhibiting ZEB2. Mechanism researches showed that ZEB2 interacted with MDM2 to up-regulate MDM2 protein expression, and then increased E2F1 protein level by suppressing its ubiquitination, which in turn promoted the transcription of ZEB2 to induce its protein expression, so as to enhance the interaction between ZEB2 and MDM2, thereby contributing to OGD plus hemin-induced endothelial dysfunction. Additionally, the joint interference of ZEB2 and MDM2 *in vivo* had better mitigative effects on ICH-induced brain injury compared with silencing ZEB2 alone. In summary, ZEB2 interacted with MDM2 to promote BMEC dysfunction and brain damage after ICH.

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Introduction

Intracerebral hemorrhage (ICH) accounts for 10%-15% of all strokes, with high morbidity and mortality [1]. The formation of hematoma and its expansion in the brain parenchyma can cause direct damage to the brain tissues. Brain edema, blood-brain barrier (BBB) destruction and cell death caused by secondary brain injury are the main causes of death and disability in patients [2–4]. Conventional treatments for ICH include removing hematoma, reducing edema and reducing intracranial pressure [5]. However, there is no effective treatment to improve the prognosis of ICH patients [2]. Therefore, it is necessary to find new therapeutic targets for ICH.

Zinc finger E-box binding homeobox 2 (ZEB2) is composed of two zinc finger clusters and a central inhibition zone, and is a member of the ZEB family [6]. It plays an important role in regulating cell adhesion, migration and cytoskeleton reorganization in cancer and neural development [7,8]. The functions of ZEB2 in endothelial cells are seldom studied. In rat renal artery endothelial cells, the knockdown of ZEB2 significantly inhibited the angiotensin II–induced cell proliferation and migration [9]. In human umbilical vein endothelial cells, overexpression of ZEB2 promoted cell proliferation and migration activity, and suppressed apoptosis induced by high glucose [10]. In addition, ZEB2 has been reported to be upregulated in the brain tissues of an ICH rat model [11], but its specific mechanism in the progression of ICH remains to be explored.

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The MDM2 protein is a multifunctional protein, and its overexpression in many tumors is associated with poor prognosis of cancer patients [12]. Growing evidence indicates that MDM2 can participate in many cellular processes, including cell differentiation, apoptosis, cycle control, and genome stability [13]. It is reported that MDM2 is up-regulated in a cell model of cerebral ischemia induced by oxygen glucose deprivation/reoxygenation, and interference with MDM2 promotes the proliferation of human brain microvascular endothelial cells (BMECs) and reduces apoptosis [14]. Moreover, a previous report showed that MDM2 expression reaches a peak 24 hours after ICH, and the MDM2/P53 pathway is involved in the process of ICH-induced neuronal apoptosis [15]. We were committed to studying the role of MDM2 in ICH-induced brain injury and endothelial dysfunction.

In this study, we demonstrated that silencing ZEB2 alleviated ICH-induced brain injury in an ICH rat model, and hindered OGD plus hemin-induced BMEC dysfunction in an ICH cell model. Mechanism studies showed that the interaction between ZEB2 and MDM2 might contribute to the promoting effect of ZEB2 on brain injury and BMEC dysfunction.

Materials and methods

ICH model

Male Sprague-Dawley rats (weighing 280 to 300 g) were exposed to a temperature and humidity controlled environment and provided with adequate food and water. Rats were randomly assigned to different research groups, and an ICH model was established by autologous blood injection (n = 48) as previously reported [16]. Briefly, animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (3.5 mL/Kg). Next, a craniotomy was performed and a 27-gauge needle was inserted into the left striatum (from the Bregma: 0.2 mm anterior, 3.5 mm lateral, and 5.5 mm indepth). Autologous blood (100 μL) collected from the femoral artery was injected at a rate of 10 μL/min. The craniotomy was then sealed with bone wax. The rats in the sham group (n = 8) only underwent needle insertion.

Adenovirus-mediated (2×10^{11} pfu) ZEB2 short hairpin RNA (ZEB2 shRNA, sh-ZEB2), MDM2 shRNA (sh-MDM2), and negative control (scramble) were obtained from Gene Pharma (Shanghai, China). 30 min after ICH, 40 modeled rats were randomly selected, and 4 μL of scramble (n = 16), 4 μL of sh-ZEB2 (n = 16), or 3 μL of sh-ZEB2 plus 3 μL of sh-MDM2 (n = 8) were injected into the left ventricle (from the Bregma: 0.8 mm posterior, 1.5 mm lateral, and 3.5 mm deep) at a rate of 1 μL/min with a Hamilton micro syringe. Then, the rats administrated with scramble/sh-ZEB2 were randomly divided into two groups of 8 rats.

Isolation of brain microvascular endothelial cells (BMECs) and cell culture

The brains of neonatal rats were isolated, and the cerebellum, midbrain, brainstem, white matter, leptomeninges, and surface blood vessels were removed. The cortices were collected and washed with PBS for three times. Next, the cortices were digested with 0.1% collagenase II (Gibco, Carlsbad, CA, USA) for 30 min at 37°C, filtered through a mesh filter, and centrifuged for 15 min at 4°C. The precipitate was collected and resuspended with 20% BSA. After further centrifugation, the precipitate was digested with 0.1% collagenase II/dispersive enzyme (Solarbio) for 1 h. Then, the suspension was added into 50% Percoll (Solarbio), and centrifuged at 1000 × g for 20 min. BMECs were obtained and cultured in DMEM (Gibco) containing 10% fetal bovine serum (Gibco) in 37°C with 5% CO_2.

Cell transfection and treatment

Adenovirus-mediated ZEB2, MDM2 and E2F1 overexpression vectors were constructed using full-length sequences of ZEB2, MDM2 and E2F1. The tagged vector plasmids pCMV-C-Flag, pCMV-C-HA, and pCMV-C-Myc were purchased from Beyotime Biotechnology (Shanghai, China). Cells were infected with Adenovirus-mediated vectors with a multiplicity of infection (MOI) of 100. Cell transfections were performed with Lipofectamine ® 3000 reagent (Thermo, Waltham, MA, USA).

To simulate the ICH condition *in vitro*, BMECs were cultured in a glucose-free and serum-free

DMEM for 10 min in 37°C with 5% CO_2. Next, hemin (10 μM, Sigma, St. Louis, MO, USA) was added into the culture medium, and the cells were transferred to an anaerobic chamber filled with 5% CO_2 and 95% N_2 for 2 h. The cells were then moved back to normal culture conditions to end hemin and oxygen glucose deprivation (OGD) treatment.

Neurologic deficit

We scored the neurological function of rats at 24 h and 72 h after ICH.

For neurologic deficit score, rats were scored from six aspects, including body symmetry, gait, climbing, circle behavior, forelimb symmetry, and compulsory circling. The maximum deficit score was 24, with each test scoring from 0 to 4. Rats with a neurological deficit score higher than 20 at 24 h were excluded from the analysis.

To perform the corner turn test, rats were placed in a 30-degree corner and allowed to rotate freely to the left or right to exit the corner. We recorded the choice of direction during the 10 repetitions and calculated the percentage of left turns.

To carry out the wire hanging test, a metal wire (1 mm × 55 cm) was suspended horizontally between two columns 50 cm above the ground. To prevent rats from using all the paws, we taped the rats' hind limbs and placed a pillow under the metal wire to prevent injury from falling. The rats were placed on the wire and the latency of the fall was recorded. Rats with a latency time less than 10 seconds were excluded from the analysis.

Measurement of brain water content

The rats were euthanized after the behavioral tests performed at 24 hours and 72 hours after ICH. The brains of rats in each group were excised and weighed (wet weight). Next, the brains were immediately dried at 100°C for 24 h and weighed again (dry weight). Brain water content was calculated as [(wet weight − dry weight)]/(wet weight) × 100%.

Measurement of transendothelial electrical resistance (TEER)

BMECs were plated on the top of Transwell chambers (0.4-μm pore size; Greiner, Frickenhausen, Germany). TEER was measured using chopsticks electrodes (Millipore) as previously described. Before calculating the final values, the TEER values of the blank filters were subtracted.

Evans blue extravasation assay

Evans Blue (EB, Sigma-Aldrich) dye extravasation was performed to evaluate the brain-blood barrier (BBB) permeability. 24 h after ICH, 2% EB dye (2 mL/Kg) was injected through the tail vein. Two hours later, the rats were euthanized, and saline was infused through the left ventricle to flush out the intravascular dye. Next, the brains were excised, weighed, and incubated with formamide (1 ml/100 mg tissue) at 37°C for 24 h. Then, the samples were centrifuged at 2,000 × g for 10 min, the supernatant was collected, and the optical density value at 632 nm was measured. The content of EB was determined based on the standard curve and quantified as μg/g brain tissue.

Cell viability

Cell viability of BMECs was assessed by MTT assay. BMECs were seeded in a 96-well plate at a density of 3×10^3 cells per well. After treatment, 5 mg/mL MTT (20 μL; Sigma-Aldrich, St. Louis, MO) was added into each well at 72 h for 4-h incubation at 37°C. Next, the supernatant was carefully discarded and 150 μL of DMSO (Sigma-Aldrich) was added to each well. After the crystals were dissolved by shaking vigorously, the absorbance at 570 nm was measured using a microplate reader.

Cell migration

A Transwell assay was conducted to measure cell migration by using a 24-well Transwell chamber. 2×10^4 BMECs suspended in the serum-free DMEM medium were placed into the upper chamber, and DMEM with 10% FBS was added to the lower chamber. Cells were allowed to incubate for

48 h in a 37°C humidified atmosphere of 5% CO_2. Then, the migratory cells on the lower surface of the membrane were fixed in 90% alcohol and stained with 0.1% crystal violet for 10 min. Five areas were randomly selected under an optical microscope to calculate the average number of cells.

Cell apoptosis

Annexin V-FITC apoptosis detection kit (Beyotime, Shanghai, China) was used to assess apoptosis of BMECs. The treated cells were washed with cold PBS and resuspended in the binding buffer. 5 μL of FITC labeled Annexin-V (20 μg/mL) and 5 μL of PI (50 μg/mL) were sequentially added to each sample in the dark. Then, the cells continued to incubate in the dark for 30 min at room temperature. Apoptosis was examined by using a flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blotting

The brain was removed after lavage with PBS, and brain tissues surrounding the hematoma (except for the hematoma area and 3 mm beyond) were collected. Total protein isolated from brain samples and BMECs was lysed with RIPA lysis buffer. Protein concentrations were quantified using a BCA Protein Assay Kit (Beyotime, Shanghai, China). 50 μg of protein were loaded onto 10% polyacrylamide gels for electrophoresis and electrophoretically transferred onto PVDF membranes. After blocking with 10% skim milk for 2 h, the membranes were incubated with anti-ZEB2 (ab223688, Abcam, 1:1000), anti-MDM2 (ab259265, Abcam, 1:1000), anti-E2F1 (ab179445, Abcam, 1:4000), anti-vascular-endothelial- (VE-) cadherin (ab231227, Abcam, 1:1000), anti-occludin (ab167161, Abcam, 1:1000), anti-claudin -5 (ab131259, Abcam, 1:5000), anti-Flag (ab1170, Abcam, 1:6000), anti-HA (ab182009, Abcam, 1:6000), and anti-Myc (ab206486, Abcam, 1:3000) at 4°C overnight. The membranes were then treated with corresponding secondary antibodies conjugated with HRP for 1 h at room temperature. β-actin was used as an internal control. Blots were visualized by using the ECL detection kit (Amersham Pharmacia Biotech, ON, Canada). The Leica Image Processing and Analysis System was used to quantify the intensity of blots.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from BMECs with Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA) according to the kit instructions. Prime Script RT reagent kit with gDNA Eraser (TaKaRa Biotechnology Co., Ltd., China) was used for cDNA synthesis. QPCR was carried out with SYBR Fast qPCR Mix (TaKaRa Biotechnology Co.). The ZEB2 mRNA expression was calculated by using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. The primer sequences were as follows: GAPDH-Forward: TCG ACA GTC AGC CGC ATC TTC TTT, GAPDH-Reverse: ACC AAA TCC GTT GAC TCC GAC CTT; ZEB2-Forward: GGC GCA AAC AAG CCA ATC CCA, ZEB2-Reverse: TTC ACT GGA CCA TCT ACA GAG GCT T.

Co-immunoprecipitation (Co-IP) and ubiquitination assay

The cell lysate was centrifuged at 12000 × g for 20 min at 4°C, and the supernatant was collected. 1 μg of the corresponding antibodies (including IgG (sc-2027, Santa Cruz Biotechnology), anti-Flag (ab205606, Abcam), anti-Myc (ab18185, Abcam), and anti-HA (ab236632, Abcam)) were added to the sample, and incubated at 4°C overnight. Next, the protein A/G-agarose beads (Santa Cruz Biotechnology, sc-2003) washed with cold lysis buffer were added to the mixture, and continued to be incubated for 4 h. Then, the beads were centrifuged at 2000 × g for 5 min at 4°C, and the supernatant was discarded. The beads were washed with lysis buffer for 3 times, and the precipitates were resolved by SDS-PAGE. Anti-ubiquitin (Ub) antibody (ab7780, Abcam) was used to assess the ubiquitination of E2F1.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed with the EZ ChIP™ Chromatin Immunoprecipitation Kit (Millipore, Burlington, MA) following the manufacturer's

description. Briefly, formaldehyde was added into cells and immobilized the cells for 10 min. Next, cells were lysed, sonicated into 200–1000 bp chromatin fragments, and incubated with anti-ZEB2 or normal serum IgG at 4 °C overnight. The bead-bearing immunoprecipitation complex was eluted and semi-quantitatively analyzed.

Statistical analysis

All data were presented as mean ± SEM and analyzed with SPSS 22.0 software (SPSS Inc, Chicago, IL, USA). The Shapiro-Wilk test was used to analyze whether the data was normally distributed. Levene's test was used to analyze the homogeneity of variances. According to the data normal distribution and homogeneity of variances, student's t-test was used for comparison between two groups, and ANOVA was used for comparison among multiple groups. Differences were considered significant at $P< 0.05$.

Results

Silencing ZEB2 mitigated ICH-induced neurobehavioral deficits, brain edema, and blood-brain barrier (BBB) dysfunction

It is reported that the mRNA level of ZEB2 in the brain tissues of ICH rats is higher than that in the sham group [11]. In order to explore the specific role of ZEB2 in ICH, we used autologous blood injection to establish an ICH rat model, and the expression of ZEB2 was inhibited by administration with ZEB2 shRNA. The results showed that 24 and 72 hours after ICH, rats in the ICH group had higher neurologic deficit scores than that in the sham group (Figure 1(a)). At the both time points after ICH, the performance in the corner turn test in rats was reduced and the falling latency in the wire hanging test was also decreased (Figure 1(b,c)). While the knockdown of ZEB2 alleviated ICH-induced neurobehavioral deficits (Figure 1(a–c)). Besides, interference with ZEB2 significantly reversed the increase of brain water content and BBB permeability caused by ICH (Figure 1(d,e)). Moreover, ZEB2 protein expression was significantly increased at 24 and 72 hours after ICH induction, which was observably suppressed by silencing ZEB2 (Figure 1(f)).

Silencing ZEB2 alleviated BMEC dysfunction induced by OGD plus hemin

To explore the role of ZEB2 in ICH-induced BMEC dysfunction, OGD plus hemin was used to treat BMECs to simulate ICH conditions *in vitro*, and ZEB2 shRNA was transfected into this cell model. ZEB2 protein level was observed to elevate significantly after OGD plus hemin treatment, while it was suppressed by interference with ZEB2 (Figure 2(a)). Silencing ZEB2 markedly rescued the decrease in cell viability, cell migration and TEER induced by OGD plus hemin (Figure 2 (b–d)). Besides, as representatives of tight junctions (TJs) and adhesion junctions (AJs), VE-cadherin, Occludin, and Claudin-5 expression was notably declined in response to OGD plus hemin treatment, while knocking down ZEB2 partially restored their expression (Figure 2(e)). In addition, the increased apoptosis caused by OGD plus hemin was restrained by inhibiting ZEB2 (Figure 2(f)).

ZEB2 interacted with MDM2

A previous study has shown that MDM2 expression reaches a peak 24 hours after ICH [15], indicating that it may be involved in the ICH process. The prediction results of protein interaction showed that ZEB2 might interact with MDM2 (Figure 3(a)). The results of co-IP assay showed that ZEB2 and MDM2 physically interacted (Figure 3(b)). And we also found that OGD plus hemin treatment could enhance the strength of the interaction between ZEB2 and MDM2 (Figure 3 (c)). Next, we explored the regulatory relationship between ZEB2 and MDM2 expression and found that overexpression of ZEB2 observably up-regulated MDM2 protein level, while silencing ZEB2 markedly reduced MDM2 expression (Figure 3(d)). In turn, MDM2 could also positively regulate the protein expression of ZEB2 (Figure 3(e)).

Overexpression of MDM2 reversed the effect of silenceing ZEB2 on BMEC function

To explore the effect of MDM2 on the function of BMECs, ZEB2 shRNA was transfected alone or co-

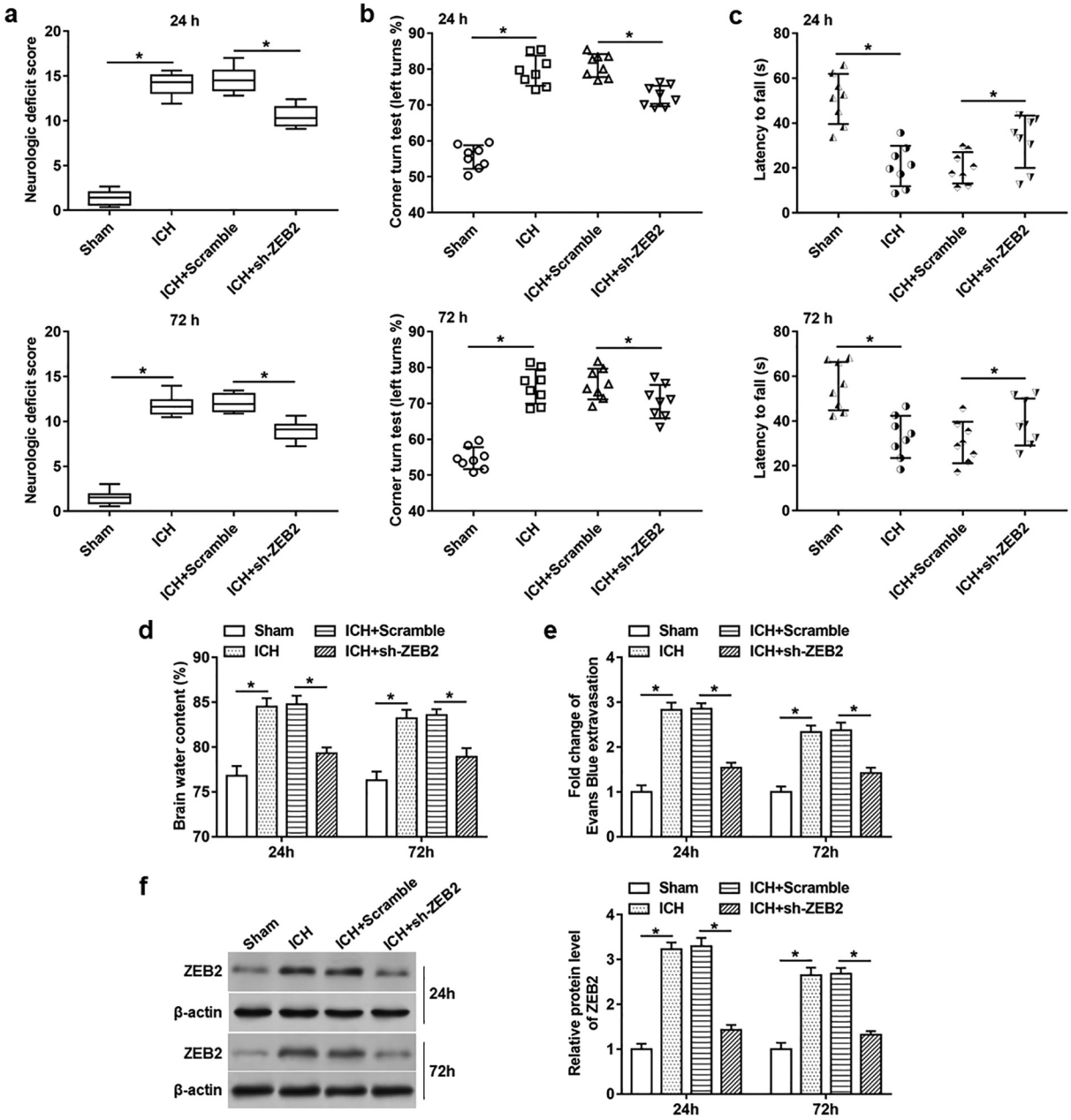

Figure 1. Silencing ZEB2 mitigated ICH-induced neurobehavioral deficits, brain edema, and BBB dysfunction. An ICH model was established by autologous blood injection. 30 minutes after ICH, ZEB2 shRNA (sh-ZEB2) or control (scramble) were injected into the ipsilateral ventricle. 24 and 72 hours after ICH: (a–c) Neurologic deficits of rats were evaluated by a 24-point scoring test, the corner turn test, and the wire hanging test. (d) Brain water content was determined by using wet and dry weight method. (e) The BBB permeability was assessed by EB extravasation assay. (f) ZEB2 protein level was detected by Western blotting. N = 8 in each group, and each test was independently repeated at least three times. *$P< 0.05$.

transfected with Ad-MDM2 in OGD plus hemin-treated cells. The results displayed that the expression of ZEB2 and MDM2 was notably upregulated after treatment with OGD plus hemin, and was decreased after transfection with ZEB2 shRNA (Figure 4(a)). The subsequent overexpression of MDM2 up-regulated the expression of MDM2 and ZEB2 (Figure 4(a)). Besides, the knockdown of ZEB2 restrained the decrease of cell viability, cell migration, TEER, and the expression of VE-cadherin, Occludin and Claudin-5 caused by OGD plus hemin (Figure 4 (b–e)). While overexpression of MDM2 hindered the rescue effect of ZEB2 on cell viability, migration, TEER, and VE-cadherin, Occludin and Claudin-5

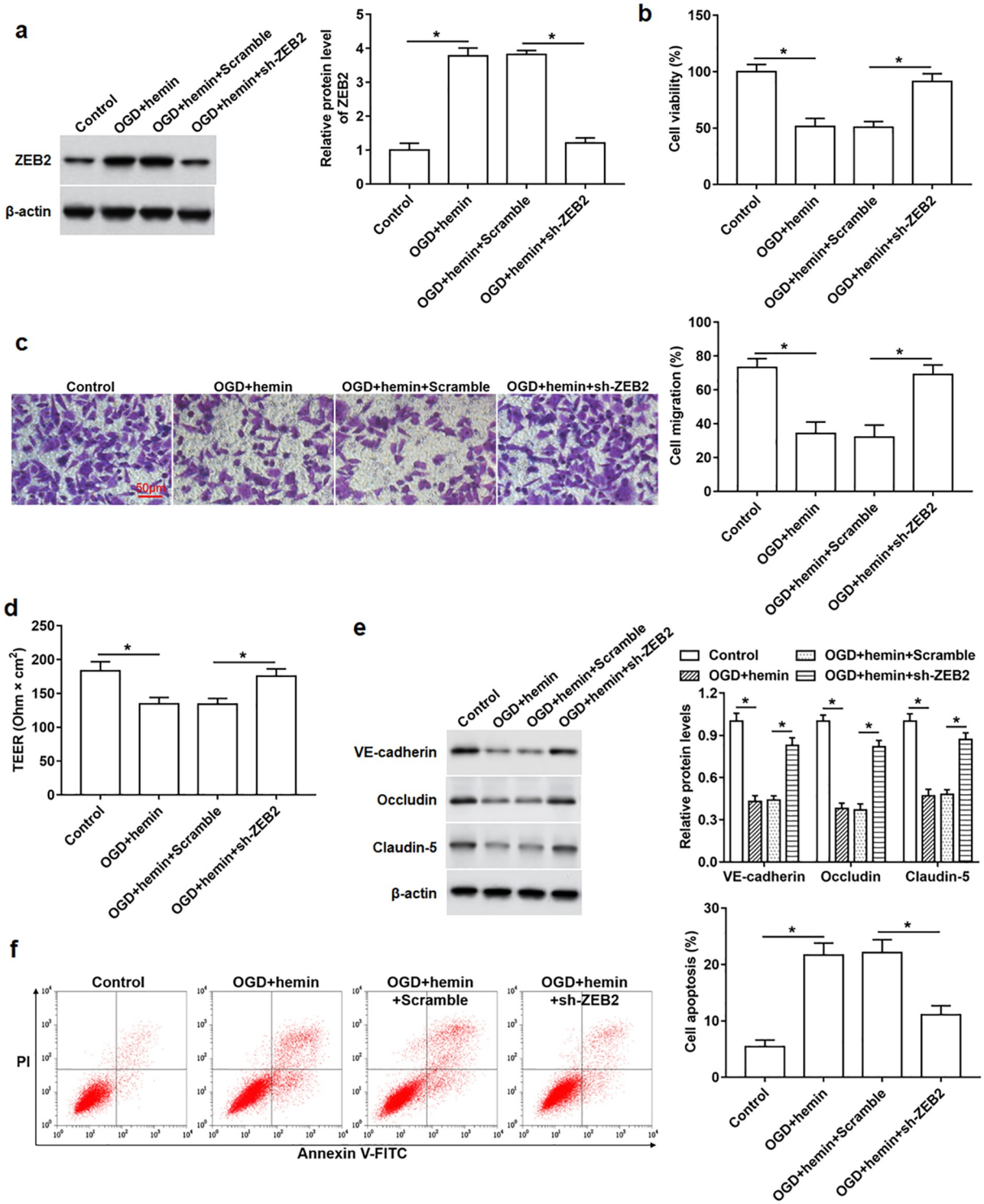

Figure 2. Silencing ZEB2 alleviated BMEC dysfunction induced by OGD plus hemin. ZEB2 shRNA was transfected into BMECs for 24 h, and then cells were treated by OGD plus hemin. (a) ZEB2 expression was detected by Western blotting. (b) Cell viability was measured by MTT assay. (c) Transwell assay was used to analyze cell migration. (d) TEER was measured by using chopsticks electrodes. (e) The protein levels of VE-cadherin, Occludin and Claudin-5 were detected by Western blotting. (f) Apoptosis was determined by flow cytometry. N = 5 in each group, and each test was independently repeated at least three times. *P< 0.05.

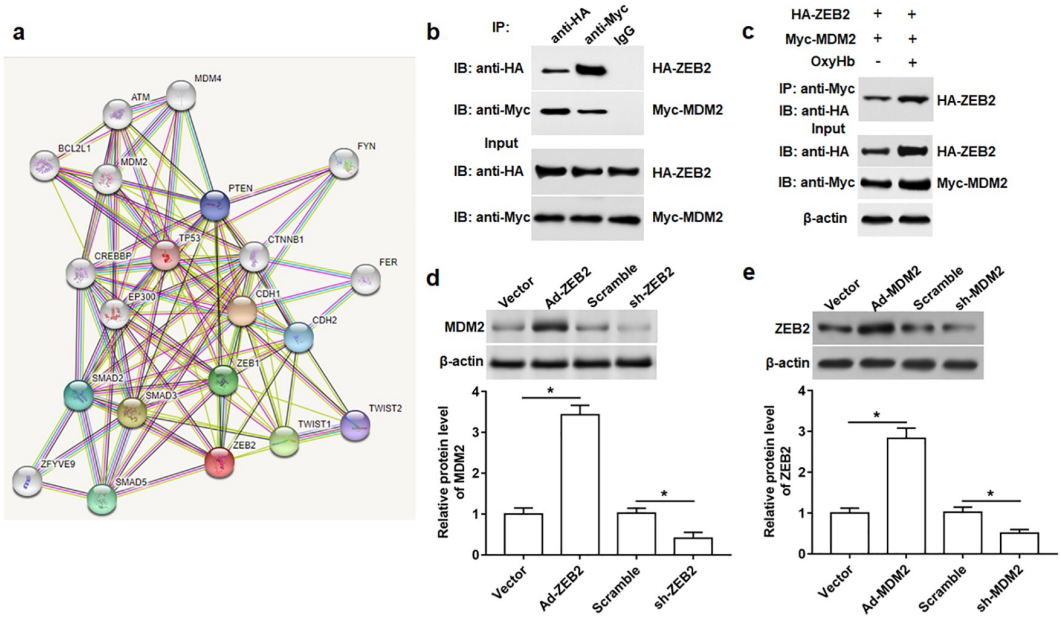

Figure 3. ZEB2 interacted with MDM2 in BMECs. (a) STRING database was used to predict proteins interacting with ZEB2. (b) HA-ZEB2 and Myc-MDM2 were transfected into BMECs, and the cell lysate was immunoprecipitated (IP) using IgG, anti-HA or anti-Myc. The immunoblot (IB) analysis was performed by using anti-Myc or anti-HA. (c) After HA-ZEB2 and Myc-MDM2 were transfected into BMECs, the cells were treated with or without OGD plus hemin. Cell lysate was immunoprecipitated with anti-HA, followed by immunoblot analysis with anti-HA or anti-Myc. (d) MDM2 protein level was detected after overexpressing or inhibiting ZEB2. (e) ZEB2 protein level was measured after overexpressing or suppressing MDM2. N = 5 in each group, and each test was independently repeated at least three times. *P< 0.05.

protein expression (Figure 4(b–e)). Moreover, the inhibitory effect of sh-ZEB2 on apoptosis in OGD plus hemin-treated cells was also abolished by the high expression of MDM2 (Figure 4(f)).

MDM2 induced E2F1 protein expression through the ubiquitination pathway

It is reported that E2F1 expression was increased in the brain tissues after ICH [17], indicating that E2F1 may play a role in the progression of ICH. Zhang et al. demonstrated that MDM2 stabilizes E2F1 protein by inhibiting E2F1 ubiquitination. We first used co-IP assay to verify the binding of MDM2 and E2F1 in BMECs, and the results indicated that MDM2 and E2F1 had obvious interaction (Figure 5(a)). Next, we explored the effect of MDM2 on the stability of E2F1 protein. The data displayed that polyubiquitinated forms of E2F1 was observed in Flag-E2F1 and HA-Ub co-transfection group, while the subsequent overexpression of MDM2 inhibited the ubiquitination of E2F1 (Figure 5(b)). Moreover, we also found that MDM2 overexpression induced E2F1 protein expression in a dose-dependent manner (Figure 5(c)). And in response to MDM2 inhibition, the E2F1 protein level was reduced dose dependently (Figure 5(d)). In addition, we found that ZEB2 promoter had an obvious enrichment in the E2F1 antibody-precipitated complex compared to the IgG (Figure 5(e)). And the mRNA and protein expression of ZEB2 was up-regulated with the increase of E2F1 and down-regulated with the reduction of E2F1 (Figure 5(f,g)). As shown in Figure 5(h), a regulatory loop was formed among ZEB2, MDM2 and E2F1. ZEB2 interacted with MDM2 to up-regulate its protein level, and MDM2 increased E2F1 protein expression by restraining its ubiquitination. As a transcription factor, E2F1 promoted the transcription of ZEB2 to upregulate its protein expression, so as to

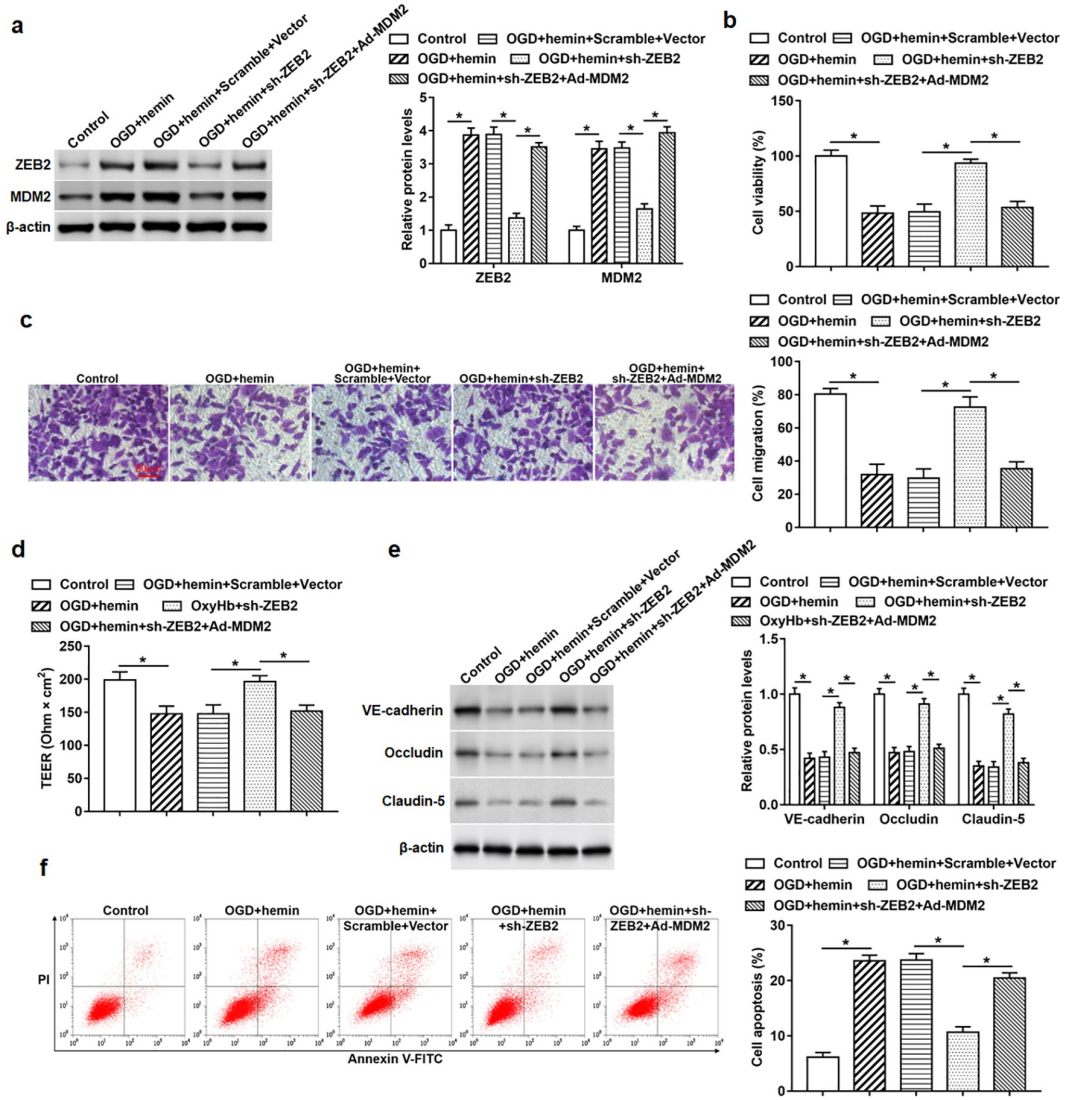

Figure 4. Overexpression of MDM2 reversed the effect of silenceing ZEB2 on BMEC function. BMECs were transfected with ZEB2 shRNA alone or together with Ad-MDM2 for 24 h, and then cells were treated by OGD plus hemin. (a) ZEB2 and MDM2 protein levels were measured by Western blotting. (b) Cell viability was assessed by MTT assay. (c) Cell migration was analyzed by Transwell assay. (d) TEER was evaluated by using chopsticks electrodes. (e) The expression of VE-cadherin, Occludin and Claudin-5 was detected by Western blotting. (f) Apoptosis was determined by flow cytometry. N = 5 in each group, and each test was independently repeated at least three times. *P< 0.05.

enhance the interaction between ZEB2 and MDM2.

Overexpression of E2F1 reversed the effect of silenceing MDM2 on OGD plus hemin-induced BMEC dysfunction

To investigate whether MDM2 participated in the regulation of BMEC function by inducing E2F1 expression, MDM2 shRNA was transfected alone or co-transfected with Ad-E2F1 in OGD plus hemin-treated cells. The data revealed that the protein levels of MDM2 and E2F1 were markedly increased after OGD plus hemin treatment, and was reduced after silencing MDM2 (Figure 6(a)). With the overexpression of E2F1, the expression of MDM2 and E2F1 were both increased (Figure 6(a)). Moreover, interference with MDM2 reversed the reduction of cell viability, cell migration, TEER, and the expression of VE-cadherin, Occludin and Claudin-5 induced by OGD plus hemin (Figure 6(b–e)). While overexpressing E2F1 abolished these roles played by sh-MDM2 (Figure 6(b–e)). In addition, silencing

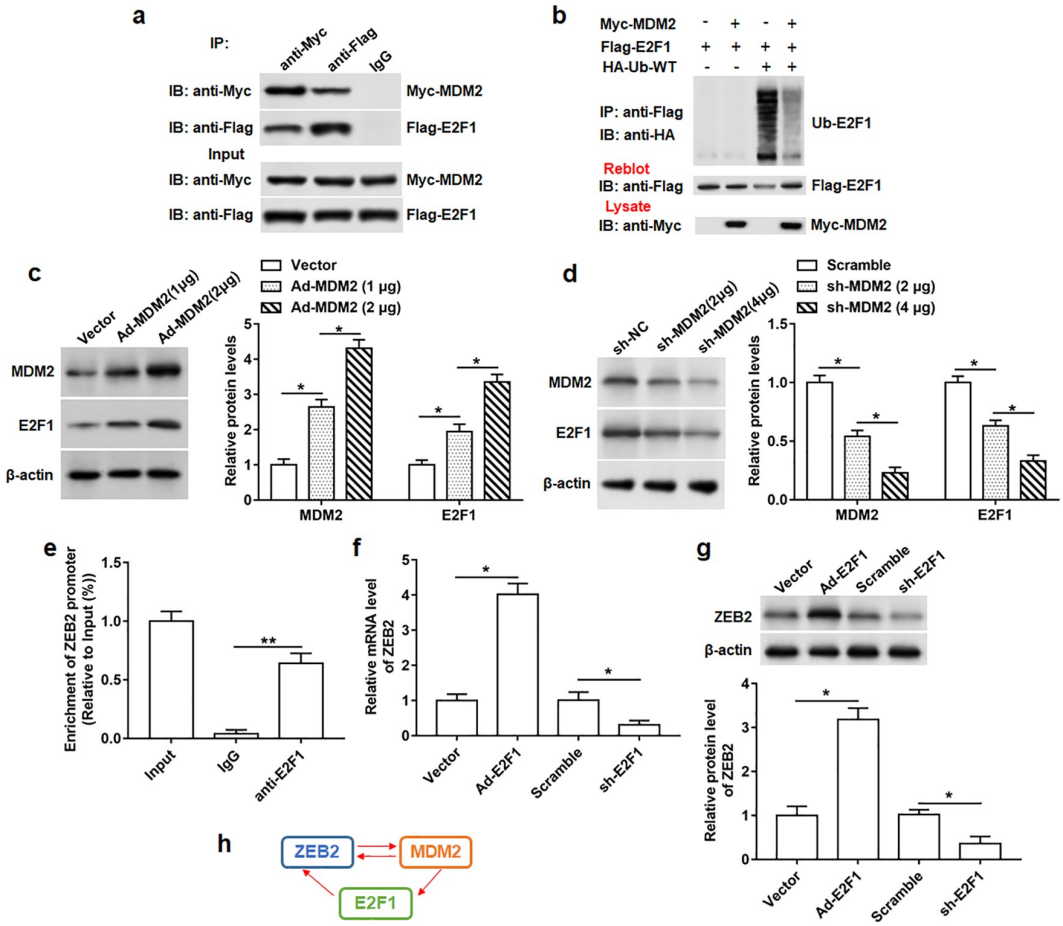

Figure 5. MDM2 induced E2F1 protein expression through the ubiquitination pathway. (a) Myc-MDM2 and Flag-E2F1 were transfected into BMECs, and cell lysate was immunoprecipitated with IgG, anti-Flag or anti-Myc, followed by immunoblot analysis with anti-Myc or anti-Flag. (b) BMECs were transfected with Myc-MDM2, Flag-E2F1, and HA-Ub-WT as indicated. Cell lysate was immunoprecipitated with anti-Flag, followed by immunoblot analysis with anti-HA. (c) 1 μg or 2 μg of Ad-MDM2 was transfected into BMECs, and MDM2 and E2F1 protein expression was detected. (d) 2 μg or 4 μg of MDM2 shRNA was transfected into BMECs, and MDM2 and E2F1 protein levels were measured. (e) ChIP assay was performed by using anti-ZEB2 and IgG. (f, g) BMECs were transfected with Ad-E2F1, E2F1 shRNA or respective controls, and the ZEB2 mRNA and protein expression was analyzed. (h) ZEB2 interacted with MDM2 to upregulate MDM2 protein level, and then increased E2F1 protein expression, which in turn promoted the expression of ZEB2 to enhance the interaction between ZEB2 and MDM2. N = 5 in each group, and each test was independently repeated at least three times. *P< 0.05.

MDM2 restrained the increased apoptosis caused by OGD plus hemin, which was also reversed by E2F1 overexpression (Figure 6(f)).

Silencing ZEB2 mitigated ICH-induced neurobehavioral deficits, brain edema, and BBB dysfunction by interacting with MDM2

To explore the regulatory mechanism of ZEB2 and MDM2 *in vivo*, ZEB2 shRNA was administered alone or co-administered with MDM2 shRNA in the cerebral ventricle of the ICH rats. Our results revealed that, silencing ZEB2 reduced the hematoma area and neurologic deficit scores, and elevated the performance in the corner turn test and the falling latency in the wire hanging test (Figure 7(a–e)). And the joint interference of ZEB2 and MDM2 had a better alleviating effect on ICH-induced cerebral hematoma and neurobehavioral deficits (Figure 7(a–e)). Moreover, compared with the ZEB2 shRNA administration group, the co-administration of ZEB2 shRNA and MDM2 shRNA had a higher inhibitory effect on the increase in brain water content and BBB permeability caused by ICH (Figure 7(f,g)). Furthermore, knockdown of

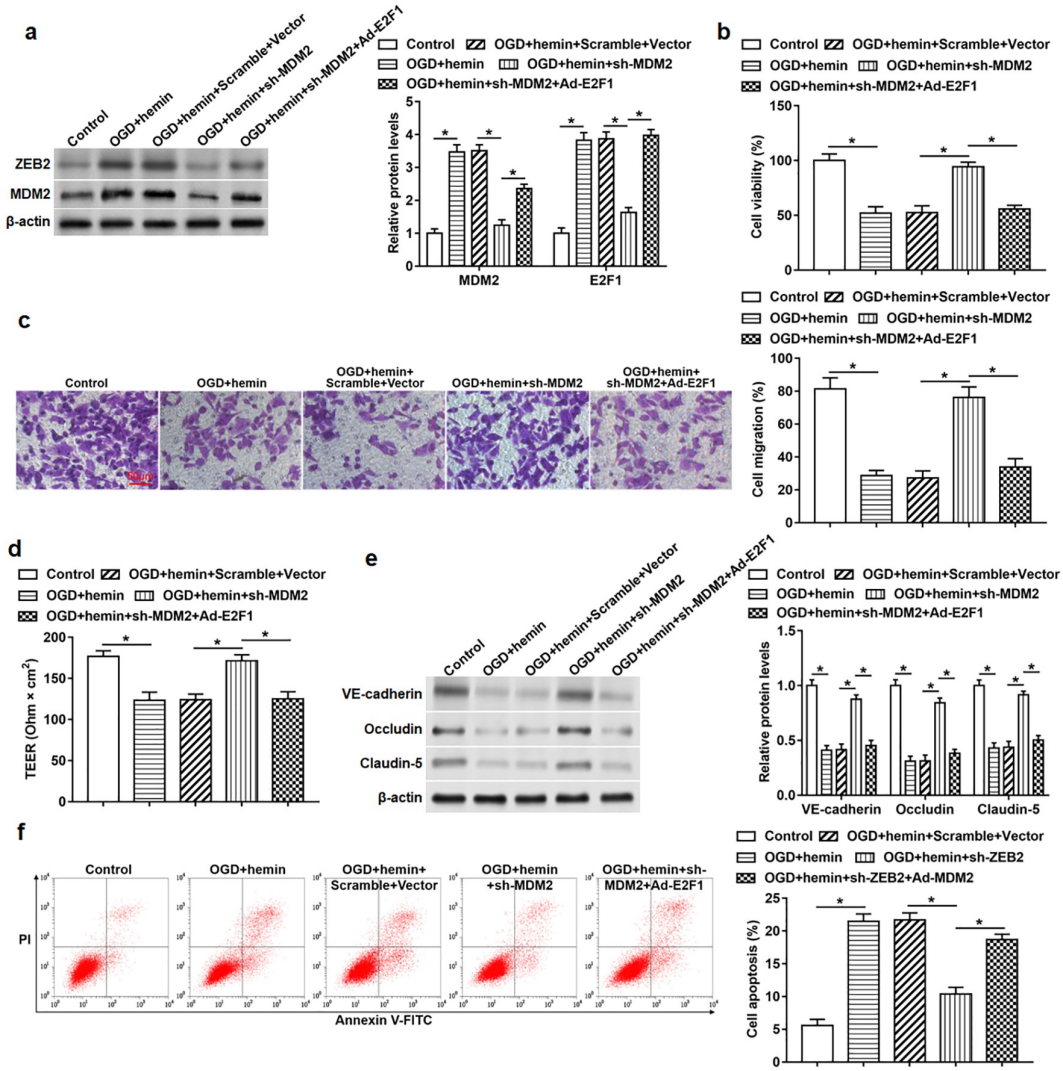

Figure 6. Overexpression of E2F1 reversed the effect of silenceing MDM2 on BMEC function. BMECs were transfected with MDM2 shRNA alone or together with Ad-E2F1 for 24 h, and then cells were treated by OGD plus hemin. (a) MDM2 and E2F1 expression was determined. (b) Cell viability was evaluated by MTT assay. (c) Cell migration was measured by Transwell assay. (d) TEER was assessed by using chopsticks electrodes. (e) VE-cadherin, Occludin and Claudin-5 expressin was detected by Western blotting. (f) Apoptosis was analyzed by flow cytometry. N = 5 in each group, and each test was independently repeated at least three times. *P< 0.05.

ZEB2 significantly inhibited the expression of ZEB2, MDM2 and E2F1 in the brain tissues of ICH rats, which was further suppressed by silencing MDM2 (Figure 7(h)). In addition, we also determined the protein expression of ZEB2, MDM2 and E2F1 in modeled rat BMECs. The results displayed that compared with the sham group, the expression of ZEB2, MDM2 and E2F1 was significantly increased after ICH induction, while they were significantly decreased after the knockdown of ZEB2. After silencing ZEB2 and MDM2 together, the three protein expression was further reduced compared with suppressing ZEB2 alone (Supplementary Figure 1).

Discussion

As shown in Figure 8, OGD plus hemin treatment could increase the protein expression of ZEB2. And ZEB2 interacted with MDM2 to increase MDM2 protein level, and then upregulated E2F1 protein expression by inhibiting its ubiquitination,

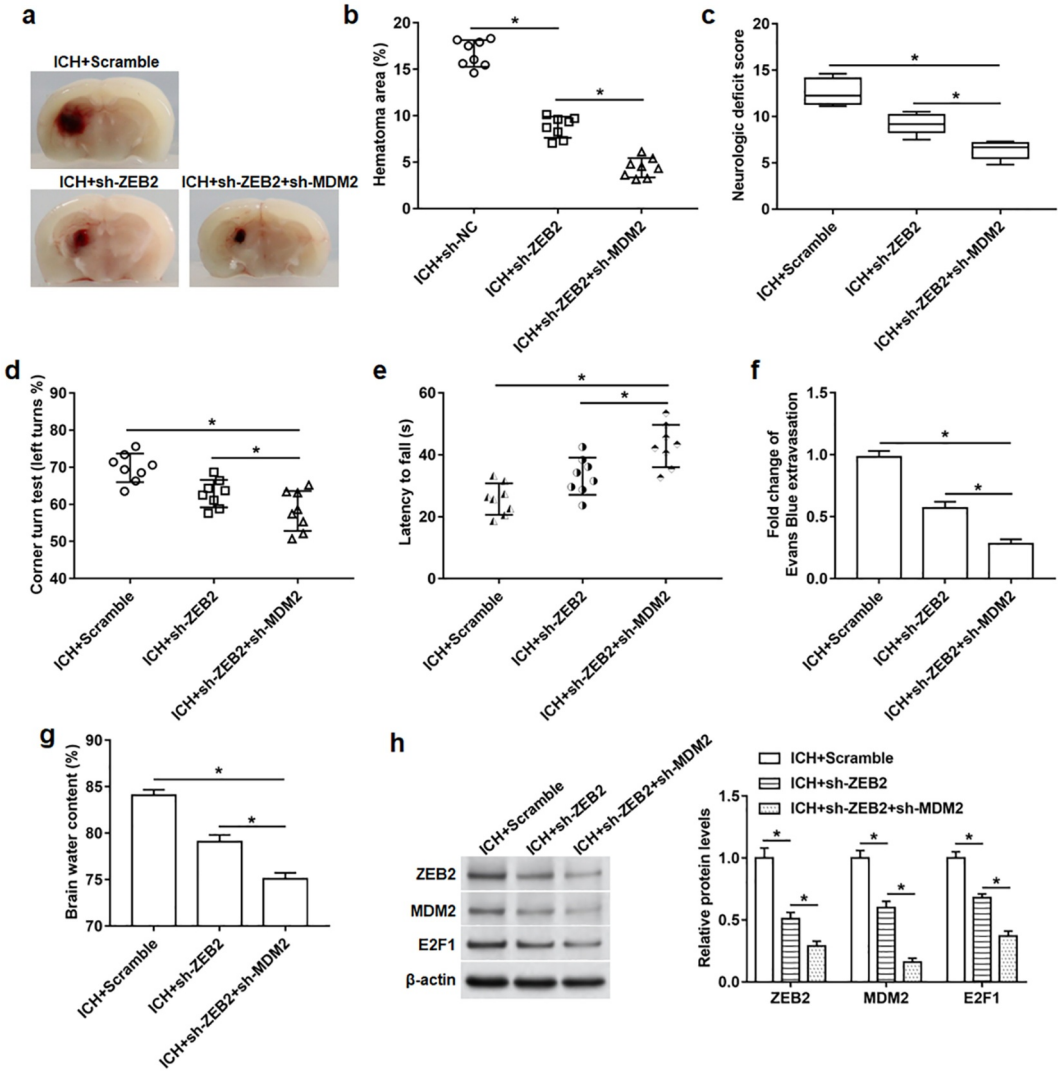

Figure 7. Silencing ZEB2 mitigated ICH-induced neurobehavioral deficits, brain edema, and BBB dysfunction by interacting with MDM2. An ICH model was established by autologous blood injection. 30 minutes after ICH, ZEB2 shRNA was administered alone or co-administered with MDM2 shRNA. 24 hours after ICH: (a) Representative images of brain tissue slice; (b) Image J software was used to qualify the hematoma size; (c–e) Neurologic deficits of rats were evaluated by a 24-point scoring test, the corner turn test, and the wire hanging test; (f) The BBB permeability was evaluated by EB extravasation assay; (g) Brain water content was determined by using wet and dry weight method; (h) ZEB2, MDM2 and E2F1 protein levels were detected by Western blotting. N = 8 in each group, and each test was independently repeated at least three times. *P< 0.05.

which in turn promoted the transcription of ZEB2 to induce its protein level, so as to enhance the interaction between ZEB2 and MDM2, thereby contributing to OGD plus hemin-induced endothelial dysfunction.

As the main component of brain microvasculature, vascular endothelium plays a vital role in maintaining normal physiological functions. An important function of vascular endothelium is to form the BBB, and has a leading function in maintaining the integrity of the BBB and brain homeostasis [18]. Changes in junctions (including TJs and AJs) connecting endothelial cells, endothelial cell death and increased endothelial cell transcytosis can lead to the destruction of the endothelial barrier [19]. The TJs protein claudin-5 and

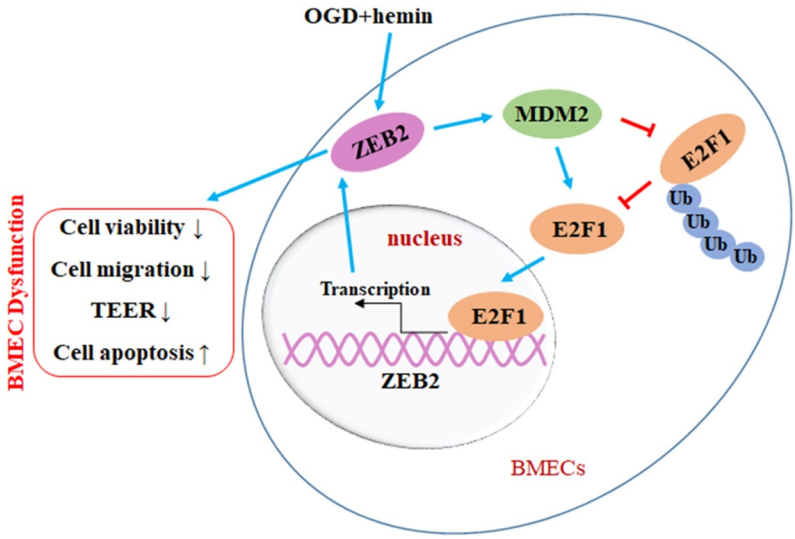

Figure 8. A schematic diagram of the regulatory effects among ZEB2, MDM2, and E2F1. The treatment of OGD plus hemin induced the protein expression of ZEB2. ZEB2 interacted with MDM2 to increase MDM2 protein level, and then upregulated E2F1 protein expression by inhibiting its ubiquitination, which in turn promoted the transcription of ZEB2 to induce its protein level, so as to enhance the interaction between ZEB2 and MDM2, thereby contributing to OGD plus hemin-induced endothelial dysfunction.

occludin play a key role in ensuring the low permeability of the BBB endothelium [20]. The AJs protein VE-cadherin helps maintain the morphology of endothelial cells and control cell motility by binding to the actin-based cytoskeleton [21]. In this study, we used OGD plus hemin to treat BMECs to simulate the condition of ICH *in vitro* to explore endothelial functions.

ZEB2 was originally defined as a transcription factor and a regulator of epithelial-mesenchymal transition in embryonic development and cancers [22]. It is up-regulated in various human tumor tissues [23]. ZEB2 can regulate many aspects of neurodevelopment, including alternative cell fate determination, neuroectodermal formation, proliferation and differentiation [24]. It is reported that ZEB2 regulates the proliferation of glial cells in experimental models of spinal cord injury and ischemic stroke to promote the recovery of nerve functions [25]. In addition, ZEB2 mRNA has been shown to be up-regulated in the brain tissues of the ICH rat model [11]. We found that ZEB2 protein level was upregulated in the brain tissues after ICH, and silencing ZEB2 alleviated ICH-induced neurologic deficits and the increase of BBB permeability and brain water content. In addition, previous studies have shown that silencing ZEB2 suppressed the proliferation and migration of rat renal artery endothelial cells induced by angiotensin II [9]. Overexpression of ZEB2 inhibits human umbilical vein endothelial cell apoptosis induced by high glucose, and promotes cell proliferation and migration [10]. Our data revealed that OGD plus hemin upregulated the ZEB2 expression in BMECs. And the knockdown of ZEB2 reversed the increase in apoptosis, and the reduction in cell viability, migration, TEER and VE-cadherin, occludin and claudin-5 expression induced by OGD plus hemin.

Previous researches didsplayed that MDM2 expression is associated with increased levels of vascular endothelial growth factor, which may contribute to tumor cell formation and promote tumor progression [26]. Besides, Inhibition of MDM2 can promote the proliferation of human BMECs and reduce apoptosis, and also can reduce the area of cerebral infarction in cerebral ischemia mice [14]. In addition, MDM2 expression in brain tissues reached a peak 24 hours after ICH, and the MDM2/p53 pathway was involved in the process of ICH-induced neuronal apoptosis [15]. We mainly study the role of MDM2 in the progression of ICH and the dysfunction of BMECs

induced by ICH. Our results suggested that MDM2 protein level was increased both in *in vitro* and *in vivo* ICH models. Interference with MDM2 rescued increased apoptosis and decreased cell viability, migration, TEER and VE-cadherin, occludin and claudin-5 expression caused by OGD plus hemin. Overexpressing MDM2 partially abolished the protective effect of sh-ZEB2 on BMEC functions. Moreover, the joint interference of ZEB2 and MDM2 *in vivo* had better mitigative effects on ICH-induced neurological dysfunction, brain edema and BBB injury compared with silencing ZEB2 alone. The MDM2 protein contains an N-terminal domain, the central acidic domain adjacent to the zinc finger domain and the C-terminal contains a RING finger domain with E3 ligase activity [27]. It is reported that MDM2, as an E3 ubiquitin ligase, can prolong the half-life of E2F1 by inhibiting the ubiquitination of E2F1 protein [28]. The data in this study displayed that MDM2 induced E2F1 protein expression by blocking the ubiquitination of E2F1.

E2F1 is a member of the E2Fs transcription factor family, and its transcription activity is mainly regulated by the retinoblastoma (Rb) protein [29]. It is frequently up-regulated in many human malignancies and is a key regulator of tumor angiogenesis in the body [30]. Besides, E2F1 plays an important role in regulating ischemia-induced neuronal death [31]. After induction of cerebral ischemia, E2F1 knockout rats show reduced neuronal damage and improved motor function compared with normal rats [32]. E2F1 promotes neurological deficits in mice with ischemic stroke by inhibiting miR-122 transcription [33]. Moreover, a previous report showed that E2F1 expression is up-regulated in the rat ICH model, and PTEN inhibitors restrain heme-induced neuronal damage by down-regulating E2F1 expression in cortical neurons [17]. We found that overexpression of E2F1 hindered sh-MDM2-induced increase in cell viability, migration, TEER and VE-cadherin, occludin and claudin-5 expression, and decrease in apoptosis in the ICH cell model. In addition, it is reported that E2F1 regulates ZEB2 gene expression in small cell lung cancer cells [34]. Similar to the reports, our data demonstrated that E2F1 promoted the transcription of ZEB2 to induce its protein expression, so as to enhance the interaction between ZEB2 and MDM2.

Conclusion

In conclusion, this study showed that silencing ZEB2 alleviated ICH-induced neurological dysfunction, brain edema and BBB injury in an ICH rat model. And the joint interference of ZEB2 and MDM2 had a better mitigative effect on ICH-induced brain injury. Furthermore, ZEB2 interaced with MDM2 to increase MDM2 expression, and then upregulated E2F1 protein level by restraining its ubiquitination, which in turn promoted ZEB2 transcription to induce its protein expression, so as to enhance the interaction between ZEB2 and MDM2, thereby contributing to OGD plus hemin-induced endothelial dysfunction. These findings revealed that genetic targeting of ZEB2 and MDM2 might be a promising strategy for the treatment of ICH.

Availability of data and materials

All data used during the current study are available from the corresponding author on reasonable request.

Credit authorship contribution statement

Qingbao Guo: Conceptualization, Investigation, Methodology, Writing - original draft. Manli Xie: Conceptualization, Investigation, Methodology, Writing - original draft. Miao Guo: Methodology, Software, Visualization. Feiping Yan: Resources, Validation, Data curation, Visualization. Lihong Li: Resources, Validation, Data curation, Software. Rui Liu: Conceptualization, Supervision, Writing - Review & Editing.

Disclosure statement

The authors declare that they have no competing interests.

Ethical approval

This research was approved by the Ethics Committee of Xing Yuan Hospital of Yulin (YLXY-2019-031). All animal experiments were in accordance with the guide for the care and use of laboratory animals established by United States National Institutes of Health (Bethesda, MD, USA).

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