



# MALAT1 Up-Regulator Polydatin Protects Brain Microvascular Integrity and Ameliorates Stroke Through C/EBP $\beta$ /MALAT1/CREB/PGC-1 $\alpha$ /PPAR $\gamma$ Pathway

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## Abstract

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a long non-coding RNA contributing to protect the blood–brain barrier (BBB) after stroke. We searched for small molecules that may up-regulate MALAT1 and focused on polydatin (PD), a natural product, as a possible candidate. PD enhanced MALAT1 gene expression in rat brain microvascular endothelial cells, reducing cell toxicity and apoptosis after oxygen and glucose deprivation (OGD). These effects correlated with reduction of inflammatory factors and enhancement of expression of BBB markers. We found opposite changes after MALAT1 silencing. We determined that C/EBP $\beta$  is a key transcription factor for PD-mediated MALAT1 expression. PPAR $\gamma$  activity is involved in MALAT1 protective effects through its coactivator PGC-1 $\alpha$  and the transcription factor CREB. This suggests that PD activates the MALAT1/CREB/PGC-1 $\alpha$ /PPAR $\gamma$  signaling pathway to protect endothelial cells against ischemia. PD administration to rats subjected to brain ischemia by transient middle cerebral artery occlusion (tMCAO) reduced cerebral infarct volume and brain inflammation, protected cerebrovascular endothelial cells and BBB integrity. These effects correlated with increased expression of MALAT1, C/EBP $\beta$ , and PGC-1 $\alpha$ . Our results strongly suggest that the beneficial effects of PD involve the C/EBP $\beta$ /MALAT1/CREB/PGC-1 $\alpha$ /PPAR $\gamma$  pathway, which may provide a novel therapeutic strategy for brain ischemic stroke.

**Keywords** MALAT1 · Ischemic stroke · Cerebrovascular endothelial cells · Polydatin

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Wenchen Ruan and Jingwei Li have contributed equally to this work.

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## Abbreviations

ABCG1	ATP-binding cassette sub-family G member 1
BBB	Blood–brain barrier
CD36	Cluster of differentiation 36
C/EBP $\beta$	CCAAT/enhancer-binding protein $\beta$
COX-2	Cyclooxygenase-2

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CREB	cAMP response element binding
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
HUVEC	Human umbilical vein endothelial cells
IL-6	Interleukin-6
LDH	Lactate dehydrogenase
lncRNA	Long non-coding RNA
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OGD	Oxygen and glucose deprivation
PBS	Phosphate-buffered saline
PGC-1 $\alpha$	Peroxisome proliferator-activated receptor gamma co-activator 1 $\alpha$
PPAR $\gamma$	Peroxisome proliferative activated receptor
rBMVEC	Rat brain microvascular endothelial cell
SD rats	Sprague–Dawley rats
tMCAO	Transient middle cerebral artery occlusion
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
TTC	2,3,5-Triphenyltetrazolium chloride
ZO-1	Zonula occludens-1

## Introduction

Cerebral ischemic stroke is one of the leading causes of death and long-term disability worldwide, and in most cases, it is without effective treatment (Wang et al. 2017; Schellinger et al. 2004). Therefore, it is urgent to search for novel and effective treatments for this disease.

Under normal physiological conditions, cerebrovascular endothelial cells play a leading role in maintaining the integrity of the blood–brain barrier (BBB) and brain homeostasis (Hawkins and Davis 2005; Sandoval and Witt 2008). Cerebral ischemia injures cerebrovascular endothelial cells and damages the BBB, injured vascular endothelial cells express immunoglobulin superfamily adhesion molecules (VCAM-1, ICAM-1, etc.) and selectin family members (E-selectin, P-selectin, etc.), and secrete TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, MCP-1 and other inflammatory mediators, which can cause neuroinflammation and thus neuronal damage (Ruetzler et al. 2001; Huang et al. 2006). The combination of brain endothelial cell injury, endothelial cell inflammation, increased cerebral vascular permeability and blood–brain barrier leakage increases neuronal damage (DEL and Zoppo 2006; Zhang et al. 2012; Yin et al. 2014). For these reasons, the protection of cerebrovascular endothelial cells has become an important therapeutic strategy to prevent cerebrovascular dysfunction against ischemic stroke (Fisher 2008).

Long non-coding RNAs (lncRNA) play an important role in the occurrence and development of many diseases

including stroke (Qureshi and Mehler 2012). Elucidating of the regulatory mechanisms of lncRNA may enhance the understanding of the molecular mechanisms of ischemic stroke and help to establish a more complete treatment strategy. Recent studies reported changes in lncRNA expression profile in cultured rat brain microvascular endothelial cells (rBMVEC) submitted to oxygen and glucose deprivation (OGD) (Zhang et al. 2016). In vivo and in vitro experiments revealed significant differences in the expression of some lncRNAs in cerebral microvascular endothelial cells after cerebral ischemia and that the up-regulation of the lncRNA MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1) was the most significant. Subsequent in vitro and in vivo experiments further confirmed that knockout of MALAT1 exacerbates cerebrovascular endothelial cell damage after cerebral ischemia (Zhang et al. 2017). These studies suggest that MALAT1 may play an important role in the pathological process of ischemic stroke. Other studies have found that lncRNA MALAT1 is also involved in the regulation of endothelial angiogenesis. Michalik et al. found that silencing MALAT1 in vivo reduced vascular endothelial cell proliferation and inhibited neovascularization (Michalik et al. 2014), which indicates that MALAT1 could promote the proliferation of endothelial cells in vivo. In addition, Liu et al. found that MALAT1 silencing can also regulate the proliferation, migration and angiogenesis of retinal vascular endothelial cells (Liu et al. 2014), further indicating the important role of MALAT1 in regulating endothelial cell function. However, the specific molecular mechanisms of MALAT1 in cerebral vascular endothelial cells damaged by ischemia are not fully understood. We propose that finding small molecular probes that increase MALAT1 expression in cerebral microvascular endothelial cells may lead to further developing these probes as therapeutic drugs to ameliorate endothelial injury and brain ischemia.

We constructed human umbilical vein endothelial cells (HUVEC) stably expressing a luciferase reporter gene driven by MALAT1 promoter (MALAT1-pro-luc-HUVEC). These cells can be used as a screening assay to search for small molecular compounds that directly regulate the expression of MALAT1 in endothelial cells. Using these cells, we screened our in-house natural product library. We found a natural product, polydatin (PD), which could directly up-regulate the expression of MALAT1 in our system. PD is a monomeric active ingredient isolated from Chinese traditional medicine *Polygonum cuspidatum* which has beneficial effects on cardiovascular and cerebrovascular system (Jinmo et al. 2010). PD reduces cerebral ischemia, but the mechanisms of this effect have not been clarified. In this study, we found that PD can not only directly regulate the expression of MALAT1 in cerebral vascular endothelial cells, but also protect cerebral vascular endothelial cells from OGD injury in vitro and from cerebral ischemia in vivo. This suggests

that PD may ameliorate endothelial and brain injury after cerebral ischemia by up-regulating MALAT1 expression in cerebral vascular endothelial cells. The discovery that PD up-regulates MALAT1 expression will also provide experimental evidence, indicating that MALAT1 up-regulation could be a new target for the treatment of ischemic stroke.

## Methods

### Reagents

The natural compound PD (polydatin, powder purity over 98%) was obtained from Yunnan Xili Company (China). Pioglitazone (agonist of PPAR $\gamma$ ), GW9662 and T0070907 (antagonists of PPAR $\gamma$ ) were obtained from Selleck (Houston, TX, USA). Compound 666-15 (inhibitor of CREB) and SR-18292 (inhibitor of PGC-1 $\alpha$ ) were purchased from MedChemExpress (MCE). For all the experiments, these compounds were prepared by diluting the stock with culture medium (the final concentration of DMSO was less than 0.1%). Cell culture medium and supplements were purchased from Invitrogen (Carlsbad, CA, USA). All other regular reagents were obtained from Sigma-Aldrich.

### Isolation and Culture of Rat Brain Microvascular Endothelial Cells

Primary rat brain microvascular endothelial cells (rBMVEC) were isolated from 3 to 4 weeks old Sprague–Dawley (SD) rats. The rats were killed by cervical dislocation and sterilized in 75% ethanol for 5 min, then the whole brain was quickly removed and placed in cold Hank's Balanced Salt Solution (HBSS) solution. The cerebral cortex was carefully separated and then shredded followed by digestion by type II collagenase and DNAase (Sigma-Aldrich, St. Louis, USA) for 1.5 h at 37 °C. The digested brain tissue was mixed with 20% BSA solution and centrifuged for 8 min (1000 $\times$ g). The precipitation after centrifugation was digested again by type II collagenase and dispersase (Sigma-Aldrich, St. Louis, USA) for 1 h at 37 °C. After digestion, the precipitation obtained by centrifugation was resuspended in endothelial cell medium (ECM, ScienCell, San Diego, CA, USA) containing puromycin (final concentration 4  $\mu$ g/mL) then transferred to a culture dish pre-coated with type I collagen and cultured in a humidified atmosphere of 5% CO<sub>2</sub>/95% air for 48 h, then replaced with an ECM medium without puromycin. When the cultures reached 80% confluency, the purified endothelial cells were passaged and subjected to the following subsequent experiments.

### Cell Culture

Human embryonic kidney cells (HEK-293T) and human umbilical vein endothelial cells (HUVEC) were obtained from ATCC (Manassas, Virginia, USA) and cultured in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. All cells were maintained in incubator with an atmosphere of 95% air and 5% CO<sub>2</sub>.

### Drug Treatment

In the in vitro studies, HEK-293T cells were pretreated with the CREB inhibitor 666-15 (1  $\mu$ M) (Xie et al. 2015) or the PGC-1 $\alpha$  inhibitor SR18292 (50  $\mu$ M) (Sharabi et al. 2017) for 2 h, and then incubated with PD (20  $\mu$ M) for another 24 h, followed by RT-PCR or luciferase reporter gene assay experiments. In the OGD cellular model, rBMVEC cells pretreated with PPAR $\gamma$  antagonist GW9662 (20  $\mu$ M) or T0070907 (10  $\mu$ M) for 3 h (Xu et al. 2015; Zilleßen et al. 2016), were incubated with PD (20  $\mu$ M) for 24 h, and then placed in an OGD condition for another 12 h, followed by RT-PCR, Western blotting, TUNEL assay or immunostaining experiments.

### Western Blotting Analysis

To determine the levels of protein expression, HUVEC and rBMVEC cells obtained from different treatment groups were washed with cold PBS and collected with Tris-Glycine SDS lysis buffer (Chang et al. 2017), and animal tissues were lysed with RIPA buffer (Vazyme, Jiangsu, China) (Xu et al. 2015). The mixtures were collected as whole cell extracts. Cell extracts were separated by 10% SDS-PAGE, and then transferred onto PVDF membranes. The membranes were blocked for 1.5 h with 3% bovine serum albumin (BSA) in Tris-buffered saline plus Tween 20 (TBST, pH 7.4) and then probed with the specific primary antibodies of rabbit anti-C/EBP $\beta$  (1:1000), rabbit anti-phospho-CREB (1:500), rabbit anti-CREB (1:1000), rabbit anti-PPAR $\gamma$  (1:1000), rabbit anti-PGC-1 $\alpha$  (1:1000), and mouse anti- $\beta$ -actin (1:2000) overnight at 4 °C. C/EBP $\beta$  and  $\beta$ -actin antibodies were purchased from Santa Cruz and other antibodies were obtained from Abclonal. After several washes with TBST, the membranes were incubated with the secondary antibodies of anti-rabbit IgG (1:10,000; SunShineBio, China) or anti-mouse IgG (1:10,000; SunShineBio, China) for 1 h at room temperature, and then blots were visualized using chemiluminescence with a Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). The resulting bands were quantified using densitometric analysis and were normalized to the levels of  $\beta$ -actin protein.

## Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To determine the levels of gene expression, HEK-293T, HUVEC, and rBMVEC cells obtained from different treatment groups were washed with cold PBS and total RNA was extracted using Trizol reagent (Vazyme, Nanjing, China). Reverse transcription was performed using a cDNA synthesis kit (Vazyme, Nanjing, China) following standard techniques. For PCR analysis, 5  $\mu$ L of cDNA was used as a template and amplified using specific primers (Table 1). Each PCR mixture contained 5  $\mu$ L of cDNA, 1  $\mu$ L of each primer (10  $\mu$ mol/L), 3.5  $\mu$ L of ddH<sub>2</sub>O, 10.5  $\mu$ L of SYBR Green Premix (Vazyme, Nanjing, China). The relative quantitative expression was measured in triplicate on a Real-time PCR Applied Biosystems Step One Plus Detection System (USA) where the 18S rRNA levels served as a control. The amplification conditions were 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s. The specificity of amplification was confirmed by a melting curve and the relative gene expression was analyzed by the  $2^{-\Delta\Delta CT}$  method (Kenneth and Thomas 2001). For the specific experimental operation of RT-PCR, we refer to the previous literature (Guo et al. 2017).

## Detection of Toxicity of PD by MTT and LDH Assay

HUVEC and rBMVEC cells were pretreated with different concentrations of PD at a density of 60–70% for 24 h. Then

the culture medium was collected and lactate dehydrogenase (LDH) was measured by the LDH assay kit (Beyotime Biotechnology) according to the manufacturer's instructions, and the remaining cells were incubated with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C. After 4 h, the medium was replaced with DMSO, and absorbance was measured at 570 nm in a BD plate-reader.

## Oxygen and Glucose Deprivation (OGD)

HUVEC and rBMVEC cells were pretreated with PD at a density of 60–70% for 24 h, and then the complete medium was replaced with glucose-free Earle's balanced salt solution (EBSS, pH 7.4) and transferred to a hypoxia chamber containing a mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub> for 12 h. Control cells without OGD were maintained under normal conditions. After OGD, the culture medium (EBSS) was collected for LDH detection by the LDH assay kit, and the remaining cells were collected for Western blotting and RT-PCR to analyze the expressions of related proteins and genes, respectively.

## Luciferase Reporter Gene Assay

All luciferase reporter gene experiments were performed on HEK-293T cells and MALAT1-pro-luc-HUVEC. Cells

**Table 1** Primer sequences

RT-PCR primers	Forward sequence 5'–3'	Reverse sequence 5'–3'
MALAT1 (human)	GTGATGCGAGTTGTTCTCCG	CTGGCTGCCTCAATGCCTAC
PGC-1 $\alpha$ (human)	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTACATCTAGTTCA
C/EBP $\beta$ (human)	CTTCAGCCCCTACCTGGAG	GGAGAGGAAGTCGTGGTGC
PPAR $\gamma$ (human)	GGGATCAGCTCCGTGGATCT	TGCACTTTGGTACTCTGAAGTT
CD36 (human)	GGCTGTGACCGGAAGTGTG	AGGTCTCCAAGTGGCATTAGAA
ABCG1 (human)	ATTCAGGGACCTTCTCTATTCGG	CTCACCATTATTGAAGTCTCCCG
MALAT1 (rat)	GGCTCCGCTGTGCTACATTA	TGTTTTGTTGGCCTTGGGGT
PPAR $\gamma$ (rat)	CTTTGGTACTTTATGGAGCCTAA	CAGCTTCAGCTGGAGTTCCAG
PGC-1 $\alpha$ (rat)	GTGCAGCCAAGACTCTGTATGG	GTCCAGGTCATTACATCAAGTTC
C/EBP $\beta$ (rat)	CTTCAGCCCCTACCTGGAG	GGAGAGGAAGTCGTGGTGC
TNF- $\alpha$ (rat)	GAGAGATTGGCTGCTGGAAC	TGGAGACCATGATGACCGTA
IL-1 $\beta$ (rat)	CCTCTGCCAAGTCAGGTCTC	GAATGTGCCACGGTTTTTCTT
IL-6 (rat)	CACAAGTCCGGAGAGGAGAC	CAGAATTGCCATTGCACAAC
COX-2 (rat)	CGGAGGAGAAGTGGGGTTTAGGAT	TGGGAGGCACTTGCGTTGATGG
ICAM-1 (rat)	CTCTTGCGAAGACGAGAACC	GCCACAGTTCTCAAAGCACA
VCAM-1 (rat)	ACAAAACGCTCGCTCAGATT	GTCCATGGTCAGAACGGACT
MCP-1 (rat)	GAATGGGTCCAGAAGTAC	TTCAGATTTATGGGTCAAG
Occludin (rat)	GGGCATCTTTGGAGGAAGTATGA	CCCAGTCTAATGACGCTGGTAAC
Claudin-5 (rat)	CAGACTCCGAGGCAAGTTAGG	TAAGGCAGCATCCAGCACTCC
ZO-1 (rat)	AGATGTCACACCAAATGCAGTC	CATGCAAATCAAGGTCATCACT
18S rRNA	CTTTGGTCTGCTCGCTCCTC	CTGACCGGGTTGGTTTTGAT

cultured in 96-well plates pre-coated by Poly-L-Lysine (Sigma-Aldrich, St. Louis, USA) at 70% confluence were transfected with 100 ng reporter gene plasmid and 25 ng of  $\beta$ -galactosidase ( $\beta$ -Gal) plasmid as internal control using Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's instructions. After 24 h of transfection, cells were treated with different concentrations of compounds for another 24 h. Cells in each well were lysed with Reporter Lysis Buffer (150  $\mu$ L) from the luciferase reporter assay kit (Promega) for 20 min at 37 °C, and then the cell lysis solution (80  $\mu$ L) was transferred into a 96-well white plate, followed by addition of luciferase assay buffer (40  $\mu$ L) to measure luciferase activity in accordance with the Luciferase Reporter Assay System instructions. The remaining cell lysis solution was tested for  $\beta$ -Gal activity using a  $\beta$ -Gal reporter assay kit purchased from Beyotime Institute of Biotechnology.

### Knockdown and Overexpression of Target Genes

Small interfering RNA (siRNA) was used to knockdown the expression of C/EBP $\beta$ , and the overexpression of C/EBP $\beta$  was mediated by the plasmid pcDNA3.1-C/EBP $\beta$ . The knockdown efficiency of MALAT1 by siRNA in primary rBMVEC cells is very low, so antisense LNA GapmeR of MALAT1 (300635-101, Exiqon), a method to specifically knockdown lncRNA expression in cells was employed to silence the expression of MALAT1 in primary rBMVEC cells, and the method of siRNA was used for knockdown of MALAT1 in HEK-293T cell and HUVEC cell lines. The overexpression of MALAT1 was mediated by pcEGFP-C1-MALAT1. The silencing of PGC-1 $\alpha$  was also achieved by the siRNA method. The transfection of siRNA, GapmeR and plasmids was achieved using Lipofectamine™ 3000 obtained from Invitrogen. GapmeR is a potent antisense oligonucleotide used for highly efficient inhibition of mRNA and lncRNA function. The specific experimental procedures for cell transfection have been described in previous published literature (Ruan et al. 2018). The GapmeR and siRNA sense strands were listed in Table 2.

### Measurement of Apoptotic Cells Level by TUNEL Staining

The culture medium from rBMVEC cells was collected after OGD treatment for the detection of LDH release, and apoptotic cells were measured by Apoptosis Fluorescein Detection kit (TUNEL), which was purchased from Beyotime Institute of Biotechnology. Briefly, the cells fixed on the crawler were infiltrated in 4% of paraformaldehyde for 10 min. The cells were then washed 3 times for 2 min each with PBS (pH 7.4), then cells were infiltrated in the TUNEL reagents for 60 min followed by washing with PBS. After

**Table 2** GapmeR and siRNA sequences

Gene	Sequence 5'–3'
MALAT1 (rat) GapmeR (GapmeR-MALAT1)	Unknown, provided by Exiqon, product number: 300635-101
Control GapmeR (GapmeR-CN)	GCTAGTCGTTAGGATT
PGC-1 $\alpha$ (rat) siRNA	GGCUUGAGCUUACCACCU ATT
MALAT1 (human) siRNA	GUAAGCCUGAACUAUC ATT
C/EBP $\beta$ (human) siRNA	GAAGACCGUGGACAAGCA CTT
Control siRNA	UUCUCCGAACGUGUCACG UTT

washing, an anti-fluorescent quencher was added dropwise to the cells and the total number of TUNEL-positive cells was counted under a fluorescence microscope set at excitation and emission wavelengths of 550 nm and 570 nm, respectively. The apoptosis rate of cells was calculated by morphometric analysis Image-pro plus (Media cybernetics, MD, USA).

### Immunofluorescence Staining

Primary rBMVEC cells exposed to drug treatment were plated on coverslips pre-coated with type I collagen. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin. Then coverslips were incubated with anti-ZO-1 antibody (1:100; Abclonal) overnight at 4 °C. Nuclei were labeled with Hoechst 33,342 (Beyotime, Haimen, China) at room temperature. After washing three times with PBS, the cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Life, USA) for 60 min. The fluorescence was then examined using a confocal microscope (Olympus FV1000, Japan). The specific experimental procedures for immunofluorescence staining refer to the previous literature (Chang et al. 2017). The intensity of fluorescence was calculated by morphometric analysis Image-pro plus (Media cybernetics, MD, USA).

### Transient Middle Cerebral Artery Occlusion (tMCAO) in SD Rats

Transient middle cerebral artery occlusion (tMCAO) was performed as previously described (Wang et al. 2018a, 2018b). The rats were randomly divided into three groups, sham, vehicle, and PD treatment. Total of 112 male SD rats were used in this study. Each group consisted of 8–12 successful treated rats. The right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery

(ECA) of individual rats were visualized. A monofilament nylon suture (diameter of approximately 0.26 mm) with a round tip was inserted into the ICA through the ECA stump and gently advanced to the MCA. After 2 h of MCAO, the filament was withdrawn to restore blood flow (reperfusion). Cerebral blood flow (CBF) was measured using a Laser Doppler flowmetry machine, and following the manufacturer's instruction (MoorFLPI-2, Moor Instruments Inc. Delaware, USA). Briefly, anesthetized rats were placed in the prone position with the skull exposed but unopened. The CBF was measured in both cerebral hemispheres and recorded immediately after the tMCAO surgery. CBF was analyzed by the MoorFLPI software and shown with arbitrary units in a 16-color palette. Body temperature was remained at 37 °C with a temperature control system. In addition, all rats had free access to food and water. Rats in the administration group were injected with a 0.1 mL volume via a single intravenous dose of PD (30 mg/kg, dissolved in saline) at 10 min before surgery, and vehicle group was injected with solvent under the same conditions. The rats were tested for behavioral changes and scored 24 h after the operation, subsequently sacrificed and the entire brain was taken for subsequent experiments. All procedures were performed according to the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals published by the US National Academy of Sciences (<http://oacu.od.nih.gov/regs/index.htm>) and were approved by the Administration Committee of Experimental Animals in Jiangsu Province and the Ethics Committee of China Pharmaceutical University.

### Measurement of Neurological Performance

At 24 h after tMCAO, the surviving rats were tested for behavioral changes and scored as described previously, with minor modifications (Longa et al. 1989). Neurological performance scores were assessed using a 5-point scale: 0, no observable neurologic deficits; (1) failure to extend the left forepaw (a mild focal neurologic deficit); (2) circling to the contralateral side (a moderate focal neurologic deficit); (3) falling to the left (a severe focal deficit); and (4) unable to walk spontaneously.

### Measurement of Infarct Size

After assessing the neurological deficit, the rat brain was collected and placed on ice, then cut into coronal sections of 2 mm. Sections were soaked in 2% TTC (2,3,5-triphenyltetrazolium chloride) (Sigma-Aldrich) phosphate buffer for 20 min at 37 °C in the dark. The infarcted tissue was stained white, while the normal brain tissue was red. The percentage of cerebral infarction volume per rat according

to the following formula: (total infarction area/total area of brain slice) × 100%. The infarct areas and the total brain areas were calculated by morphometric analysis with Image-pro Plus (Media cybernetics, MD, USA) (Wang et al. 2016).

### Evaluation of Evans Blue Dye Extravasation

Evans Blue (2% in saline, 4 mL/kg; Sigma, St. Louis, MO, USA) was intravenously administered 24 h after tMCAO, and then 1 h later, rats were perfused with saline to remove intravascular dyes. The sample was homogenized in 50% trichloroacetic acid solution. The supernatant was obtained by centrifugation and diluted fourfold with ethanol. The fluorescence intensity was measured with a fluorescence detection system (Magellan V6.6; Safire 2, Tecan excitation 620 nm and emission 680 nm).

### Rat Cerebral Microvessel Isolation

Rats in the sham, vehicle, and the PD administration groups were euthanized. The brains were rapidly removed and placed in a cold PBS solution. The right brain was shredded and placed in a tube containing PBS followed by homogenization. The homogenized brain tissue was centrifuged several times at 720×g to remove blood cells, and then the centrifuged homogenate tissue was spread on top of 16% dextran, followed by centrifugation at 4500×g for 20 min to collect the lowest layer (cerebral microvascular tissue). The upper homogenate tissue repeats this procedure to obtain more vascular precipitation. The obtained vascular deposits were stored in a –80 °C refrigerator for subsequent Western blotting and RT-PCR experiments. In this section, we refer to the previously published literature (Wang et al. 2016) for specific experimental operations.

### Statistical Analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 5 software (San Diego, CA) with either unpaired two-tailed *t*-test or one-way ANOVA as appropriate. The statistical test used and *n*-numbers are indicated in each figure legend. The differences were considered significant if the *p* < 0.05.

## Results

### PD Up-Regulates the Expression of MALAT1 in rBMVEC and HUVEC Cells in Dose- and Time-Dependent Manner

Luciferase reporter gene assay is a common method for high throughput drug screening. Based on this method (Fig. 1a),

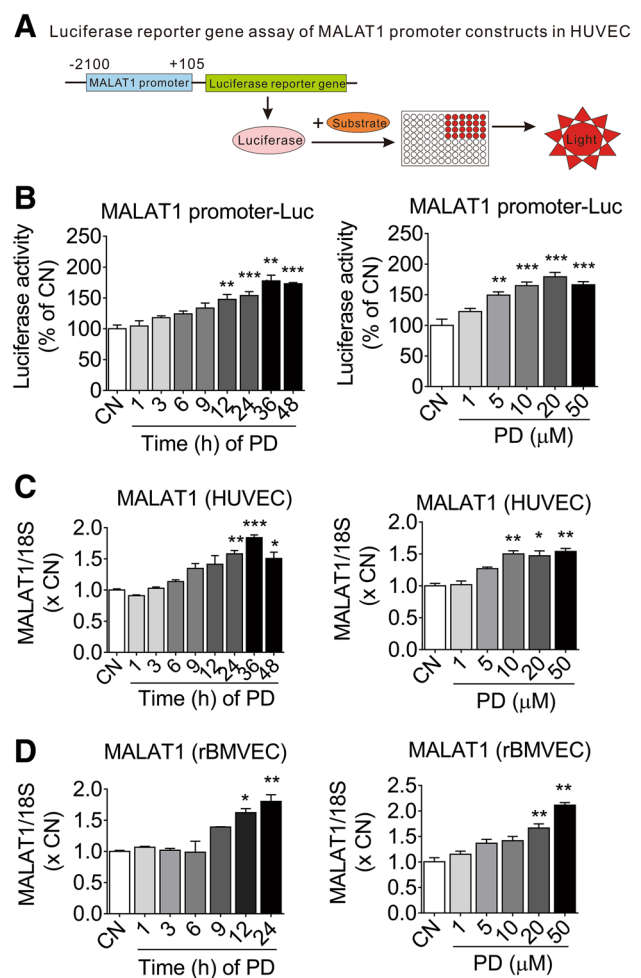
we constructed a HUVEC cell line that stably expressed luciferase reporter gene driven by MALAT1 promoter (−2100~+105) and used it to screen our in-house natural product library to seek for MALAT1 regulators. We found that PD could significantly up-regulate luciferase activity driven by MALAT1 promoter (Fig. 1b). MTT and LDH assays were used to detect the effect of PD on HUVEC and rBMVEC cell viability. The results showed that PD has no cytotoxicity in both cell cultures (Fig. S1A, B). In order to detect the effect of PD on the expression of endogenous MALAT1 in HUVEC and rBMVEC cells, we used the RT-PCR method to detect the changes of MALAT1 expression in both cells. The results showed that PD could up-regulate the expression of endogenous MALAT1 in the time- and dose-dependent manners in both endothelial cell cultures (Fig. 1c, d).

### PD, a New MALAT1 Regulator, Significantly Attenuates OGD-Induced Endothelial Cell Injury

We submitted HUVEC and rBMVEC cultures to OGD, mimicking in vivo ischemia conditions to verify whether PD could prevent endothelial cell injury induced by ischemic stroke (Fig. 2a). PD significantly enhanced the expression of MALAT1 and reduced cellular death in endothelial cells submitted to OGD (Figs. 2b, c, S2A, B). Furthermore, in order to verify whether the endothelial protection by PD is associated with up-regulation of MALAT1, GapmeR-MALAT1 was used to silence the expression of MALAT1 in endothelial cells (Fig. 2d). When MALAT1 was knocked-down by GapmeR-MALAT1 in rBMVEC cells, the death of endothelial cells was increased and the endothelial protection of PD was significantly attenuated (Fig. 2e). Similar results are also shown in HUVEC cells (Fig. S2C, D). These data indicate that MALAT1 plays an important role for endothelial protection in ischemia and is involved in the PD-mediated endothelial protection.

To further study the effects of PD on endothelial inflammation and vascular integrity under OGD conditions, we examined the expression changes of endothelial pro-inflammatory cytokines and BBB markers. PD significantly inhibited the OGD-induced gene expression of endothelial pro-inflammatory factors (TNF- $\alpha$ , IL-6, COX-2) and enhanced the gene expression of BBB markers (Claudin-5, Occludin, ZO-1) in rBMVEC cells (Fig. 2f). PD treatment also enhanced the protein expressions of Occludin and ZO-1 (Fig. 2g). In addition, the knockdown of MALAT1 mediated by GapmeR-MALAT1 reversed these effects of PD in endothelial cells (Fig. 2f, g).

The immunostaining data also showed that PD had a significant inhibitory effect on OGD-induced reduction of the ZO-1 protein expression, whereas knockdown of MALAT1 by GapmeR-MALAT1 reversed this effect of PD (Fig. 2h).



**Fig. 1** Polydatin, a novel MALAT1 up-regulator, time-dependently and dose-dependently increases MALAT1 expression in endothelial cells. **a** A schematic drawing for screening compounds that may regulate MALAT1 expression based on the MALAT1 promoter (−2100~+105) luciferase reporter gene assay in HUVEC cells. **b** Polydatin (PD) time-dependently and dose-dependently increased luciferase activity of MALAT1 promoter-luciferase in HUVEC. Cells were incubated with PD (20  $\mu$ M) for different times or with various concentrations of PD for 36 h. **c** PD time-dependently and dose-dependently increases the expression of MALAT1 in HUVEC Cells. Cells were incubated with various concentrations of PD for 24 h or with PD (20  $\mu$ M) for different times. **d** PD time-dependently and dose-dependently increases the expression of MALAT1 in rBMVEC cells by RT-PCR. Cells were incubated with PD (20  $\mu$ M) for different times or with various concentrations of PD for 24 h. Data are shown as the mean  $\pm$  SEM ( $n=3$ ) after normalization to the control (DMSO). One-way ANOVA with Bonferroni post test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control (CN)

Apoptosis of cerebral vascular endothelial cells induced by ischemia is a common phenomenon in the early stage of ischemic stroke. In order to evaluate whether PD can protect endothelial cells from apoptosis caused by OGD, we detected the apoptosis rate of endothelial cells by TUNEL assay and found that PD reduced the apoptosis rate of

**Fig. 2** Polydatin protects against OGD-induced endothelial cell injury through regulating MALAT1 expression in rBMVEC cells. **a** A schematic diagram of PD inhibition of OGD-induced endothelial cell damage by up-regulating MALAT1 expression. The rBMVEC cells were incubated with PD (10, 20  $\mu$ M) for 24 h, then cultured under OGD conditions for another 12 h. The MALAT1 expression was measured by RT-PCR (**b**), and the cell viability was determined by LDH assay (**c**). **d** The expression of MALAT1 was significantly inhibited by GapmeR-MALAT1 transfected into rBMVEC cells for 24 h. GapmeR-MALAT1-mediated MALAT1 silencing abolished the PD prevention of cell death induced by OGD (**e**). **f, g** MALAT1 knockdown also reversed the PD inhibition of mRNA expressions of pro-inflammatory factors (TNF- $\alpha$ , IL-6, COX-2) and attenuated the PD enhancement of mRNA expressions of BBB markers (Claudin-5, ZO-1, Occludin) and protein expressions of ZO-1 and Occludin in rBMVEC cells. **h** Immunofluorescence method determined that PD protected OGD-induced reduction of ZO-1 expression, but MALAT1 silencing reversed this effect. **i** PD significantly inhibited the apoptosis of rBMVEC cells induced by OGD for 12 h, determined by TUNEL assay, and this effect was reversed by MALAT1 silencing. Data are shown as the mean  $\pm$  SEM ( $n = 3$ ) after normalization to the control (CN). One-way ANOVA with Bonferroni post test. \*\*\* $p < 0.001$  versus CN group; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  versus GapmeR-NC group; \$ $p < 0.05$ , \$\$ $p < 0.01$ , \$\$\$ $p < 0.001$  versus GapmeR-NC + PD group

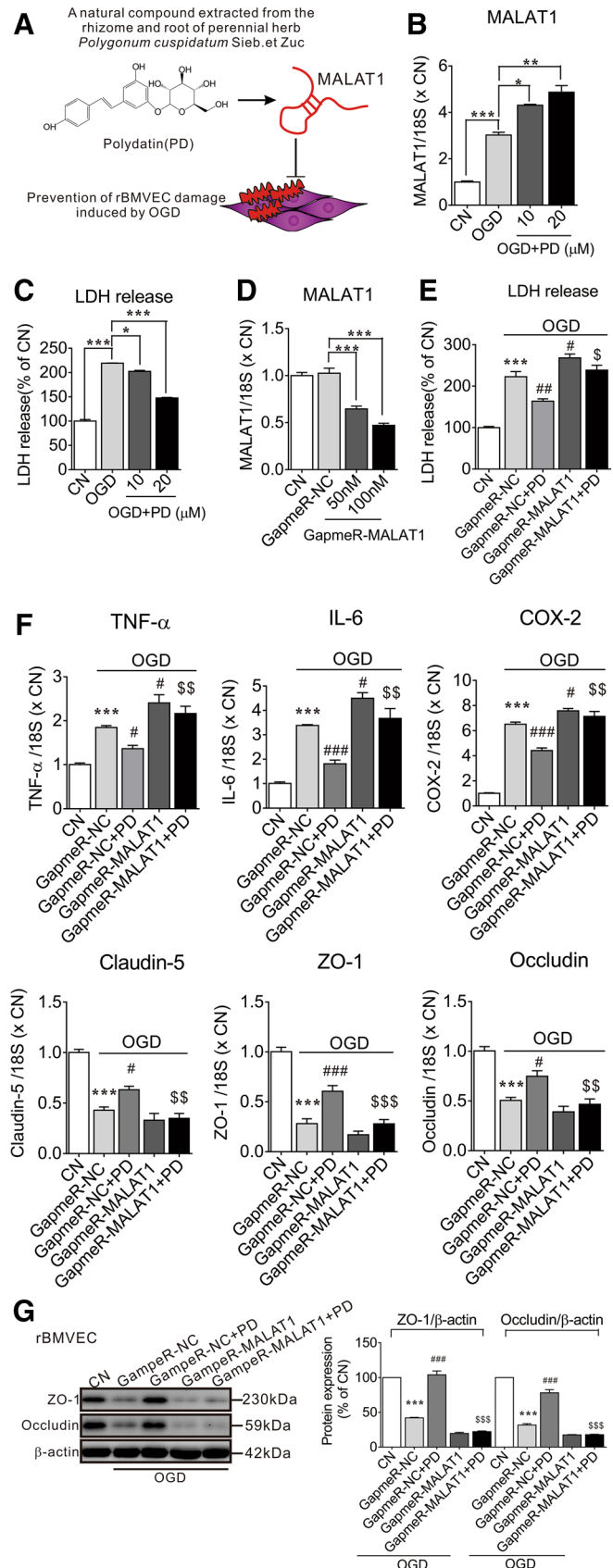
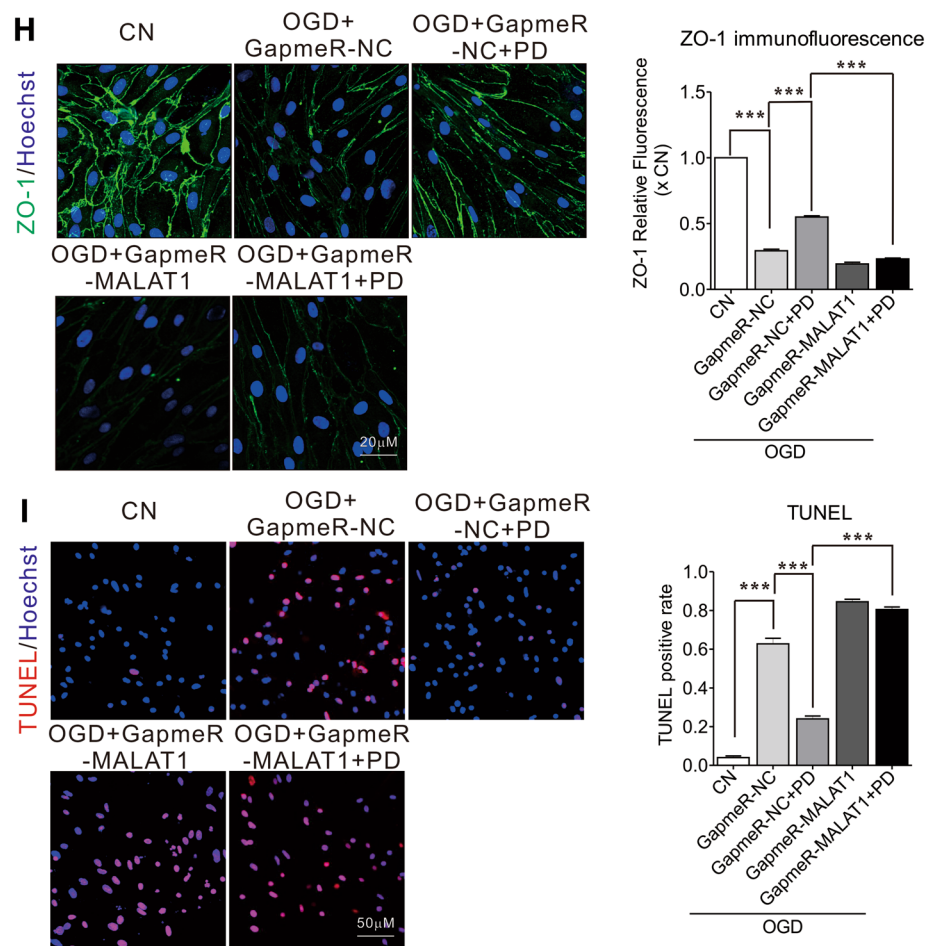




Fig. 2 (continued)



rBMVEC cells induced by OGD from 62.75 to 24%, an effect reversed by GapmeR-MALAT1 with MALAT1 knock-down (Fig. 2i).

The above results indicated that the MALAT1 up-regulator PD could prevent OGD-induced endothelial cell injury by inhibiting the endothelial inflammation and enhancing the expression of BBB markers in rBMVEC cells, which was mediated through enhanced MALAT1 expression.

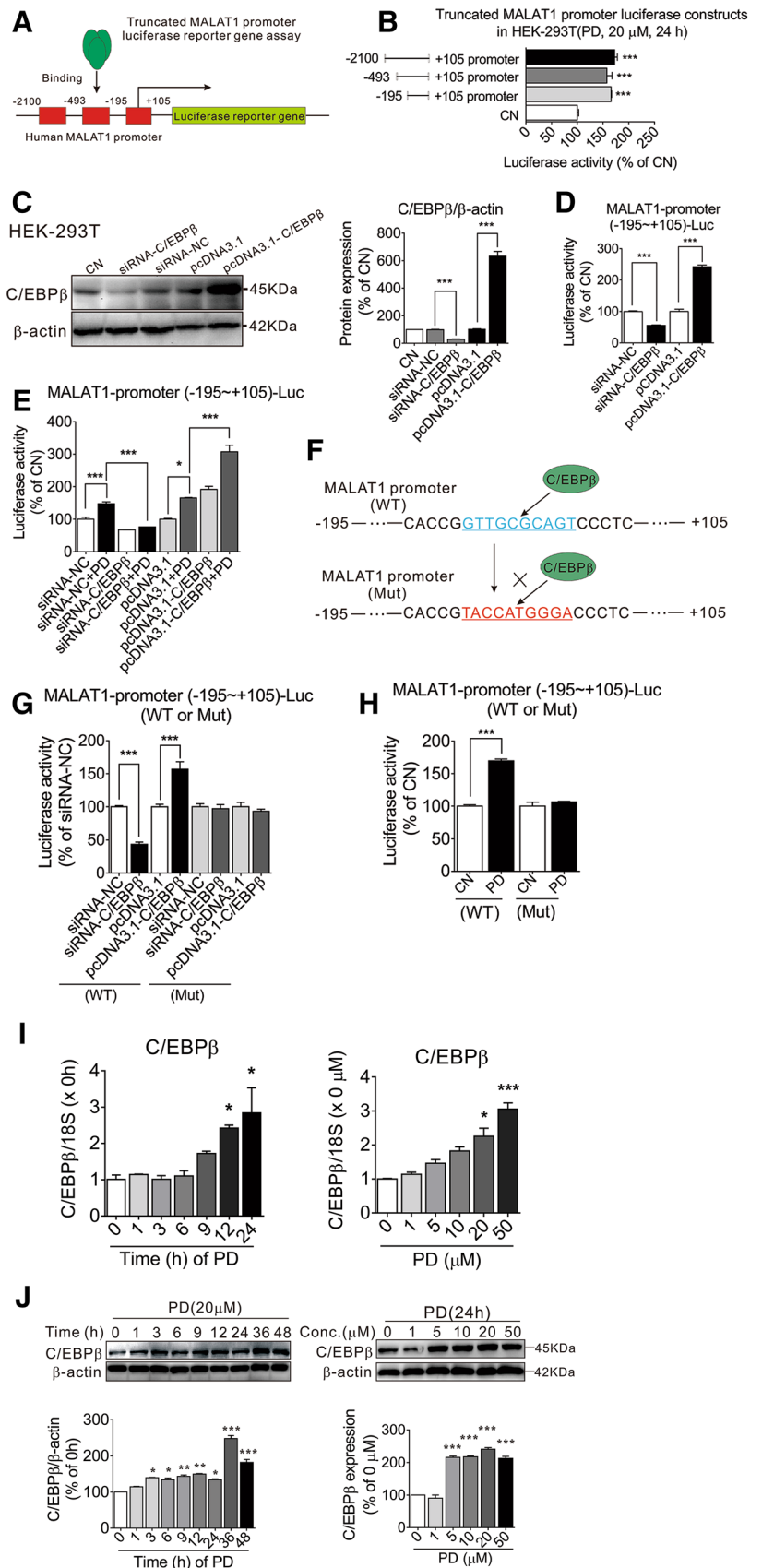
### C/EBP $\beta$ is Responsible for the MALAT1 Up-Regulation by PD

In order to further investigate which transcription factor mediates the up-regulation of MALAT1 expression by PD, we constructed luciferase reporter gene systems with three truncated MALAT1 promoter fragments to estimate the binding location of possible transcription factors acting on MALAT1 promoters (Fig. 3a). The results showed that PD significantly up-regulated the activity of the reporter gene driven by the  $-195 \sim +105$  promoter region of MALAT1 (Fig. 3b), indicating that the transcription factor may act on the  $-195 \sim +105$  promoter region of MALAT1 to regulate MALAT1 expression by PD. PROMO database analysis

shows that one of the transcription factors, C/EBP $\beta$  may bind to the  $-195 \sim +105$  promoter region of MALAT1 with higher possibility. To verify whether PD regulates the expression of MALAT1 by C/EBP $\beta$ , we knockdown and overexpressed C/EBP $\beta$  in the HEK-293T cells (Fig. 3c) to test whether C/EBP $\beta$  has an effect on the reporter gene activity of MALAT1. The results showed that knockdown of C/EBP $\beta$  significantly suppressed the reporter gene activity driven by the  $-195 \sim +105$  promoter of MALAT1, whereas overexpression of C/EBP $\beta$  enhanced this reporter gene activity (Fig. 3d). Furthermore, changes in the expression of C/EBP $\beta$  significantly affected this reporter gene activity driven by PD (Fig. 3e). These results indicate that C/EBP $\beta$  is an important transcription factor for PD-mediated MALAT1 expression.

To further prove that C/EBP $\beta$  may directly regulate the expression of MALAT1, we mutated the possible binding base sequence of C/EBP $\beta$  in the MALAT1 promoter region to confirm the interaction between C/EBP $\beta$  and MALAT1 promoter (Fig. 3f). The results showed that the change of C/EBP $\beta$  expression could not affect the activity of the reporter gene driven by the MALAT1 promoter containing mutant possible binding sequence (Fig. 3g), and PD could not

**Fig. 3** C/EBP $\beta$  is responsible for the MALAT1 up-regulation by polydatin. **a** A schematic diagram of luciferase reporter gene constructs containing different lengths of the MALAT1 promoter fragment. **b** Polydatin (PD) significantly increased the activity of luciferase reporter genes driven by three truncated MALAT1 promoter fragments in HEK-293T cells. **c** The expression of C/EBP $\beta$  protein was knocked down by the C/EBP $\beta$  siRNA transfection (siRNA-C/EBP $\beta$ ) and was overexpressed by the pcDNA3.1-C/EBP $\beta$  plasmid transfection for 24 h in HEK-293T cells. **d** The C/EBP $\beta$  silencing significantly decreased the activity of the reporter gene driven by MALAT1 promoter (-195~+105), while the overexpression of C/EBP $\beta$  remarkably increased its activity. **e** The C/EBP $\beta$  silencing significantly reversed the PD enhancement of the reporter gene driven by the MALAT1 promoter (-195~+105), while the overexpression of C/EBP $\beta$  remarkably increased the PD effect. **f** The wild type and mutant base sequences of C/EBP $\beta$  binding site in MALAT1 promoter (-195~+105) region. **g** C/EBP $\beta$  knockdown or overexpression had no effect on the activity of the reporter gene driven by the MALAT1 promoter (-195~+105) region containing mutant bases of possible C/EBP $\beta$  binding site (Mut). **h** PD had no effect on the activity of the Mut. **i, j** PD time-dependently and dose-dependently increased the mRNA and protein expressions of C/EBP $\beta$  in rBMVEC cells. Data are shown as the mean  $\pm$  SEM ( $n=3$ ) after normalization to the control. One-way ANOVA with Bonferroni post test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control (CN, 0  $\mu$ M or 0 h)



regulate the activity of this mutant reporter gene (Fig. 3h), suggesting that C/EBP $\beta$  may bind to the  $-195 \sim +105$  promoter region of MALAT1 to mediate the PD regulation of MALAT1 expression. In primary rBMVEC and HUVEC cells, PD treatment increased the expression of C/EBP $\beta$  gene and protein in a time- and dose-dependent manner (Figs. 3i, j, S2A–D), indicating that C/EBP $\beta$  is a key transcription factor that regulates the expression of MALAT1 by PD in endothelial cells.

### PD Effects are Mediated by the CREB-PGC-1 $\alpha$ -PPAR $\gamma$ Pathway as Downstream of MALAT1 in Endothelial Cells

We knockdown and overexpressed MALAT1 in HEK-293T cells in order to determine downstream signaling pathways of MALAT1 after PD treatment (Fig. 4a). Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a ligand-activated nuclear receptor, playing an active role in the treatment of ischemic stroke (Liu and Wang 2017). Pioglitazone, the small molecular agonist of PPAR $\gamma$  could significantly reduce the cerebral infarction volume and the release of inflammatory factors in ischemic rats (Medhi et al. 2010), and significantly reduced the risk of ischemic stroke in clinical trials (Kernan et al. 2016). We determined whether MALAT1 influences PPAR $\gamma$  activity in HEK-293T cells, using the PPRE-driven reporter gene assay system to detect PPAR $\gamma$  activity (Fig. 4b). We found that the knockdown of MALAT1 by siRNA significantly inhibited PPAR $\gamma$  activity. Conversely the overexpression of MALAT1 by pEGFP-C1-MALAT1 enhanced PPAR $\gamma$  activity (Fig. 4c).

We determined gene expressions of PPAR $\gamma$  and that of its downstream target genes, CD36 and ABCG1. The results show that MALAT1 knockdown reduced ABCG1 gene expression but the gene expressions of CD36 and ABCG1 were increased by overexpression of MALAT1. Since modulation of MALAT1 did not affect PPAR $\gamma$  gene expression (Fig. 4d), our results suggest that MALAT1 may regulate PPAR $\gamma$  activity through its co-factors.

We examined the effect of MALAT1 on the expression of PGC-1 $\alpha$ , an important coactivator regulating PPAR $\gamma$  activity. We found that MALAT1 knockdown inhibited PGC-1 $\alpha$  gene expression and conversely, the overexpression of MALAT1 enhanced PGC-1 $\alpha$  gene expression (Fig. 4e). In addition, the PPAR $\gamma$  activity increased by overexpression of MALAT1 was significantly reversed by the PGC-1 $\alpha$  inhibitor SR-18292 (Fig. 4f) (Sharabi et al. 2017). This suggests that MALAT1 may enhance the expression of PGC-1 $\alpha$  to increase PPAR $\gamma$  activity.

PD treatment time and dose dependently increased PPAR $\gamma$  activity in endothelial cells (Fig. 4g). This effect was reversed by MALAT1 knockdown and conversely enhanced

by MALAT1 overexpression (Fig. 4h). The role of PD in enhancing PPAR $\gamma$  activity can be blocked by the PGC-1 $\alpha$  inhibitor SR-18292 (Fig. 4i). Taken together, our results suggest that PD may increase PPAR $\gamma$  activity through the stimulation of the MALAT1/PGC-1 $\alpha$  pathway.

In addition, the MALAT1 upstream transcription factor C/EBP $\beta$  also increased PPAR $\gamma$  activity (Fig. 4j) and was also involved in the enhanced PPAR $\gamma$  activity as a result of PD treatment (Fig. 4j).

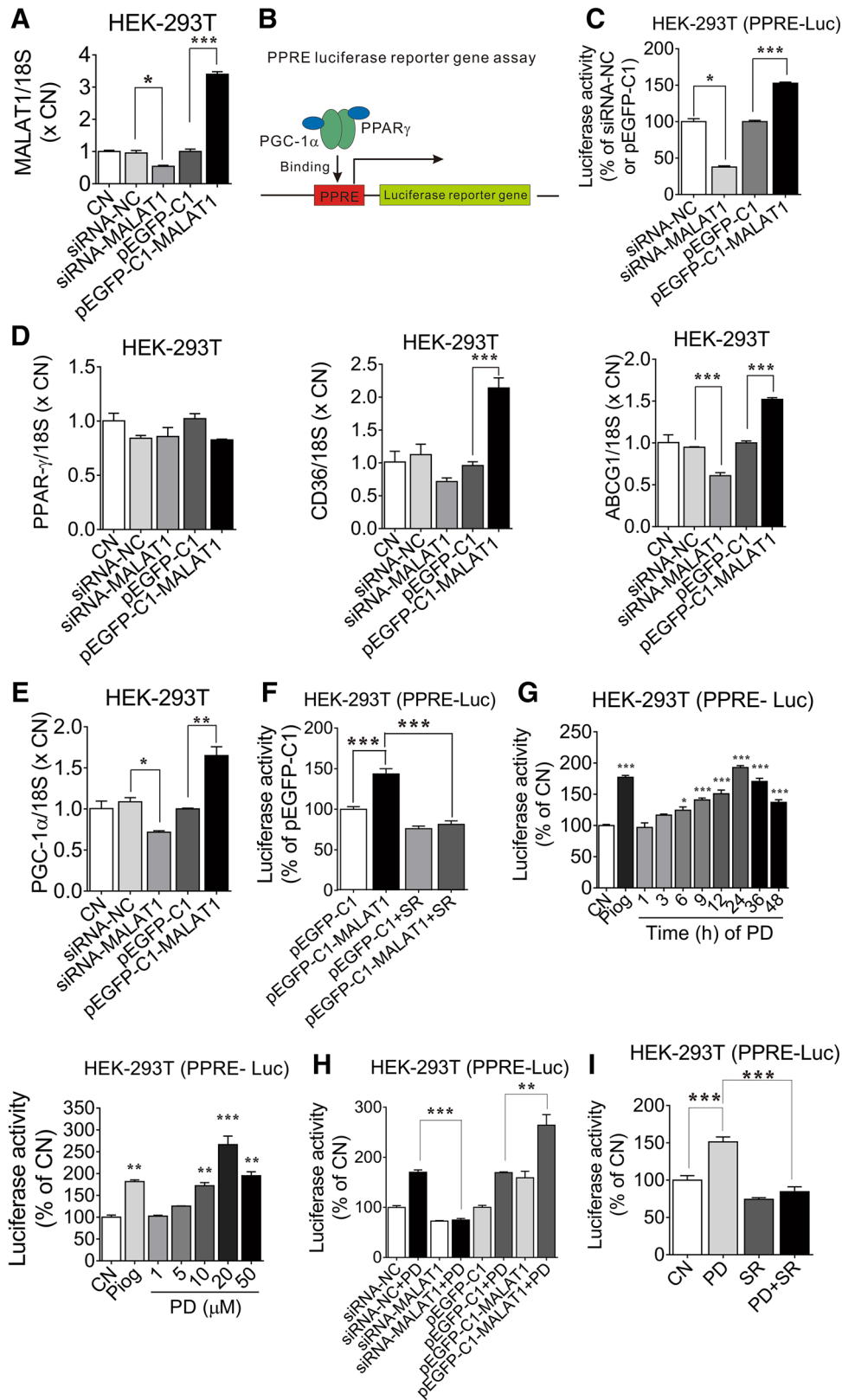
CREB is a transcription factor that has been reported to regulate the expression of PGC-1 $\alpha$  (Singh et al. 2015). MALAT1 could bind to phosphorylated CREB (p-CREB) to maintain the CREB activity (Yao et al. 2016). We proposed that MALAT1 may regulate PGC-1 $\alpha$  expression through CREB activation. To test this hypothesis, we used a reporter gene driven by CRE to detect the transcriptional activity of CREB (Xue et al. 2011) (Fig. 4k). We found that the knockdown of MALAT1 significantly reduced the transcriptional activity of CREB, while the overexpression of MALAT1 enhanced CREB activity (Fig. 4l). In addition, 666-15, an inhibitor of CREB, can significantly reverse the upregulation of PGC-1 $\alpha$  and PPAR $\gamma$  activity induced by overexpression of MALAT1, but not the expression of PPAR $\gamma$  (Fig. 4m, n). This suggests that MALAT1 promotes PGC-1 $\alpha$  expression and PPAR $\gamma$  activity by increasing CREB transcriptional activity.

In HEK-293T cells, PD enhanced CREB transcriptional activity in a dose- and time-dependent manner (Fig. 4o). PD-induced enhancement of PPAR $\gamma$  activity and PGC-1 $\alpha$  expression can be blocked by the CREB inhibitor 666-15 in HEK-293T and rBMVEC cells (Fig. 4p–r), suggesting that PD may increase the PGC-1 $\alpha$  expression and PPAR $\gamma$  activity through modulating CREB transcriptional activity.

C/EBP $\beta$ , as a MALAT1 upstream transcription factor, also enhanced the activity of CREB (Fig. 4s), further supporting that PD effects involved the C/EBP $\beta$ -MALAT1-CREB-PGC-1 $\alpha$ -PPAR $\gamma$  pathway (Fig. 4t).

In rBMVEC cells, PD increased the expressions of phosphorylated CREB and PGC-1 $\alpha$  protein in a dose- and time-dependent manner, but PD had no effect on the protein expression of PPAR $\gamma$  (Fig. 4u, v).

In order to further confirm CREB-PGC-1 $\alpha$  as downstream signaling pathway of MALAT1, we detected the expressions of phosphorylated CREB, PGC-1 $\alpha$ , and PPAR $\gamma$  in rBMVEC cells after the MALAT1 knockdown by GapmeR-MALAT1. MALAT1 knockdown significantly down-regulated the protein expressions of phosphorylated CREB and PGC-1 $\alpha$ , but had no effect on the protein expression of PPAR $\gamma$  (Fig. 4w). When rBMVEC cells pretreated with PD were submitted to OGD, PD significantly enhanced the protein expressions of phosphorylated CREB and PGC-1 $\alpha$ , but had no effect on the PPAR $\gamma$  protein expression, while knockdown of MALAT1 reversed these effects (Fig. 4x).



**Fig. 4** The CREB-PGC-1 $\alpha$ -PPAR $\gamma$  pathway as downstream of MALAT1 is involved in the PD effects. **a** RT-PCR analysis of MALAT1 expression mediated by siRNA-MALAT1 and pEGFP-C1-MALAT1 in HEK-293T cells. **b** A schematic diagram of the system of PPRE-driven luciferase reporter gene expression for PPAR $\gamma$  activity. **c** MALAT1 silencing reduced the PPAR $\gamma$  activity, while MALAT1 overexpression increased the PPAR $\gamma$  activity in HEK-293T cells. **d, e** RT-PCR analysis of the gene expression of PPAR $\gamma$ , CD36, ABCG1, and PGC-1 $\alpha$ . **f** The PGC-1 $\alpha$  inhibitor SR-18292 blocked the MALAT1 overexpression-enhanced PPAR $\gamma$  activity. **g** Polydatin (PD) time-dependently and dose-dependently increased the PPAR $\gamma$  activity. **h** MALAT1 silencing attenuated PD-induced PPAR $\gamma$  activity, while MALAT1 overexpression enhanced the PD effect. **i** The PGC-1 $\alpha$  inhibitor SR-18292 totally inhibited PD-induced PPAR $\gamma$  activity. **j** C/EBP $\beta$  silencing attenuated PD-induced PPAR $\gamma$  activity, while C/EBP $\beta$  overexpression enhanced PD effect. **k** A schematic diagram of the system of CRE-driven luciferase reporter gene expression for CREB activity. **l** MALAT1 silencing reduced the CREB transcriptional activity, while MALAT1 overexpression increased the CREB activity in HEK-293T cells. **m, n** The CREB inhibitor 666-15 (1  $\mu$ M) attenuated the MALAT1 overexpression-induced the PGC-1 $\alpha$  gene expression and the PPAR $\gamma$  activity. **o** PD time-dependently and dose-dependently increased the CREB transcriptional activity in HEK-293T cells. **p, q** The CREB inhibitor 666-15 (1  $\mu$ M) totally blocked the PD-induced PGC-1 $\alpha$  gene expression in HEK-293T and rBMVEC cells. **r** The CREB inhibitor 666-15 (1  $\mu$ M) totally blocked the PD-induced PPAR $\gamma$  activity in HEK-293T cells. **s** C/EBP $\beta$  silencing reduced the CREB transcriptional activity, while C/EBP $\beta$  overexpression increased the CREB activity in HEK-293T cells. **t** A schematic diagram of the upstream signaling pathway of PPAR $\gamma$  activity induced by PD involving C/EBP $\beta$ , MALAT1, CREB and PGC-1 $\alpha$  proteins. **u, v** PD time-dependently and dose-dependently increased the phosphorylated CREB (p-CREB) and PGC-1 $\alpha$  protein levels but not PPAR $\gamma$  in rBMVEC cells. **w** The MALAT1 silencing by GapmeR-MALAT1 in rBMVEC cells reduced the protein expression levels of the phosphorylated CREB and PGC-1 $\alpha$  but not PPAR $\gamma$ . **x** The MALAT1 silencing by GapmeR-MALAT1 attenuated the PD effects on the enhancement of protein expression levels of the phosphorylated CREB and PGC-1 $\alpha$  in rBMVEC cells under OGD condition. Data are shown as the mean  $\pm$  SEM ( $n=3$ ) after normalization to the control (DMSO, CN). One-way ANOVA with Bonferroni post test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus CN or siRNA-NC group; # $p < 0.05$ , ### $p < 0.001$  versus GapmeR-NC group; \$\$\$ $p < 0.001$  versus GapmeR-NC + PD group

### PD Protects Against OGD-Induced Cerebrovascular Endothelial Cell Injury Through the Regulation of the PGC-1 $\alpha$ /PPAR $\gamma$ Pathway

In order to confirm whether PGC-1 $\alpha$ -PPAR $\gamma$  pathway plays an important role for PD protection of OGD-induced endothelial injury, we used two PPAR $\gamma$  antagonists, GW9662 (Lisa et al. 2002) and T0070907 (Lee et al. 2002) in rBMVEC cells. The effect of PD on protection against endothelial cell death induced by OGD was significantly reversed by GW9662 and T0070907 (Fig. 5a). Furthermore, the PD inhibition of OGD-induced endothelial inflammatory cytokines (the TNF- $\alpha$  and IL-6 gene expression) and BBB markers (Claudin-5, Occludin and ZO-1 gene expression, and the Occludin and ZO-1 proteins) was also reversed by GW9662 and T0070907 (Fig. 5b–d). In addition, the

immunostaining data show that GW9662 also reversed the PD enhancement of ZO-1 protein expression in rBMVEC cells submitted to OGD (Fig. 5e). Additionally, the PD inhibition of OGD-induced endothelial cell apoptosis was significantly attenuated by GW9662 (Fig. 5f).

Similarly, PGC-1 $\alpha$  gene silencing by PGC-1 $\alpha$  siRNA (Fig. 5g) reversed the PD protection of OGD-induced endothelial cell injury (Fig. 5h) and the PD inhibition of OGD-induced endothelial inflammatory cytokines (TNF- $\alpha$  and IL-6 gene expression) and BBB markers (Claudin-5, Occludin and ZO-1 gene expression, and the Occludin and ZO-1 proteins) expressions (Fig. 5i–k). These results indicate that PD protected cerebrovascular endothelial cell injury against OGD mainly through the PGC-1 $\alpha$ /PPAR $\gamma$  pathway regulation.

### PD Reduces Cerebral Infarct Volume, Protects Cerebrovascular Endothelial Cells and BBB Integrity in Rats Subject to tMCAO

To confirm the beneficial effects of PD on ischemia in vivo, rats were subjected to tMCAO and treated by PD. The experiment design is shown in Fig. 6a. The results show that PD administration significantly reduced the infarct volume and improved neurological deficit in ischemic rats (Fig. 6b). In order to evaluate the protective effect of PD on BBB integrity, Evans blue staining was used to evaluate the permeability of BBB. The results show that the permeability of BBB was significantly increased in the vehicle group at 24 h post ischemia, while PD administration remarkably improved BBB integrity, indicating that PD could protect the BBB integrity in ischemic rats (Fig. 6c).

In order to study the molecular mechanisms for above PD effects in vivo, we extracted cerebrovascular tissue of rats from the sham, vehicle and PD-treated groups and determined gene and protein expression. The results show that PD significantly enhanced MALAT1 expression (Fig. 6d), and markedly increased the gene and protein expression of C/EBP $\beta$  and PGC-1 $\alpha$ , but not PPAR $\gamma$  (Fig. 6e, f). PD also enhanced the gene and protein expression levels of the BBB markers, Claudin-5, Occludin and ZO-1 (Fig. 6g, h). In addition, PD administration significantly inhibited ischemia-induced the gene expression levels of inflammatory factors, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, COX-2, ICAM-1, VCAM-1 and MCP-1, and also reduced ischemia-induced the protein expression levels of COX-2, ICAM-1 and VCAM-1 (Fig. 6i, j).

## Discussion

lncRNAs have been reported to regulate physiological and pathological responses (Valluri et al. 2017; Heward and Lindsay 2014). The lncRNA MALAT1 contributes to regulate endothelial cell function, and was proposed to be

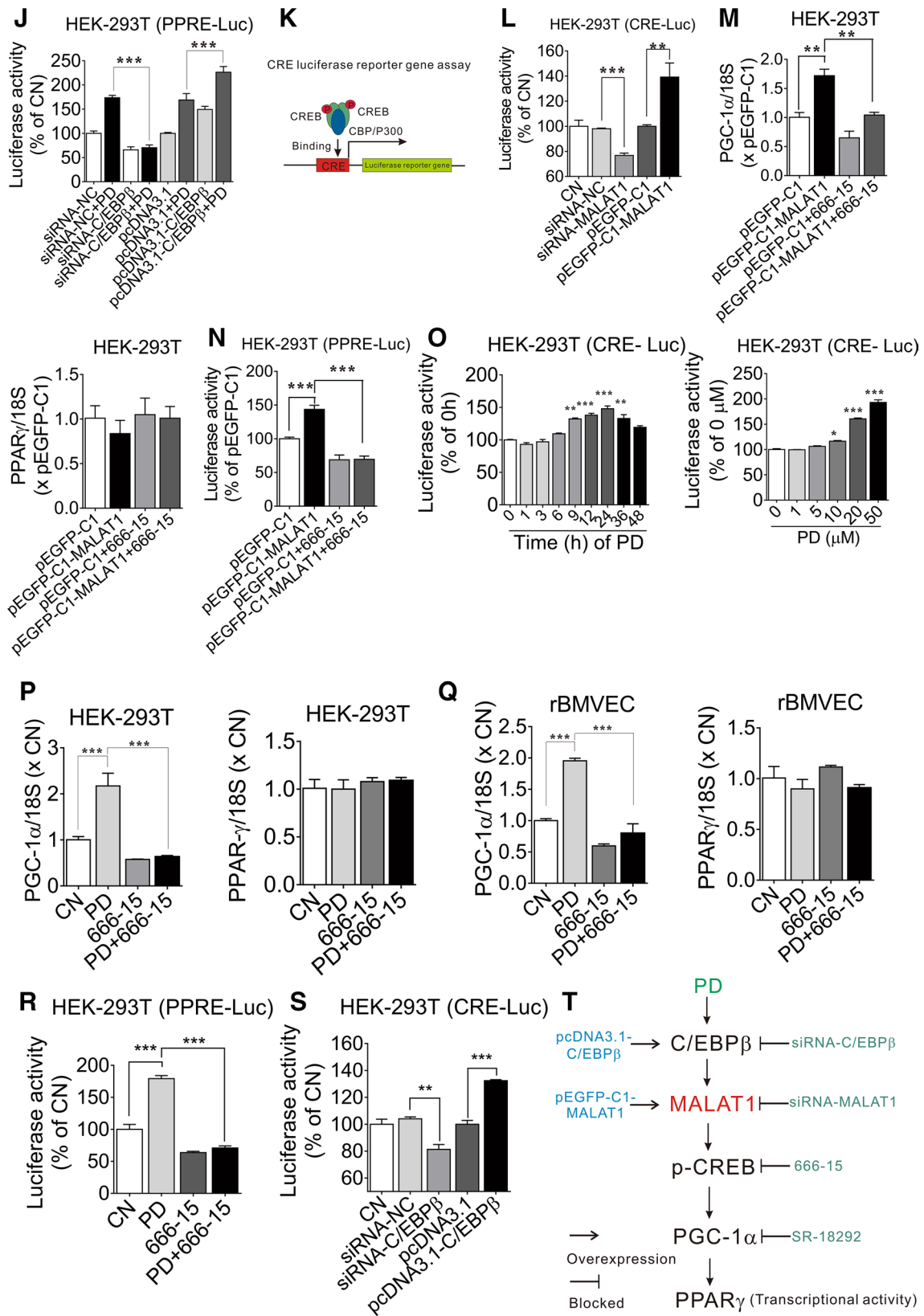


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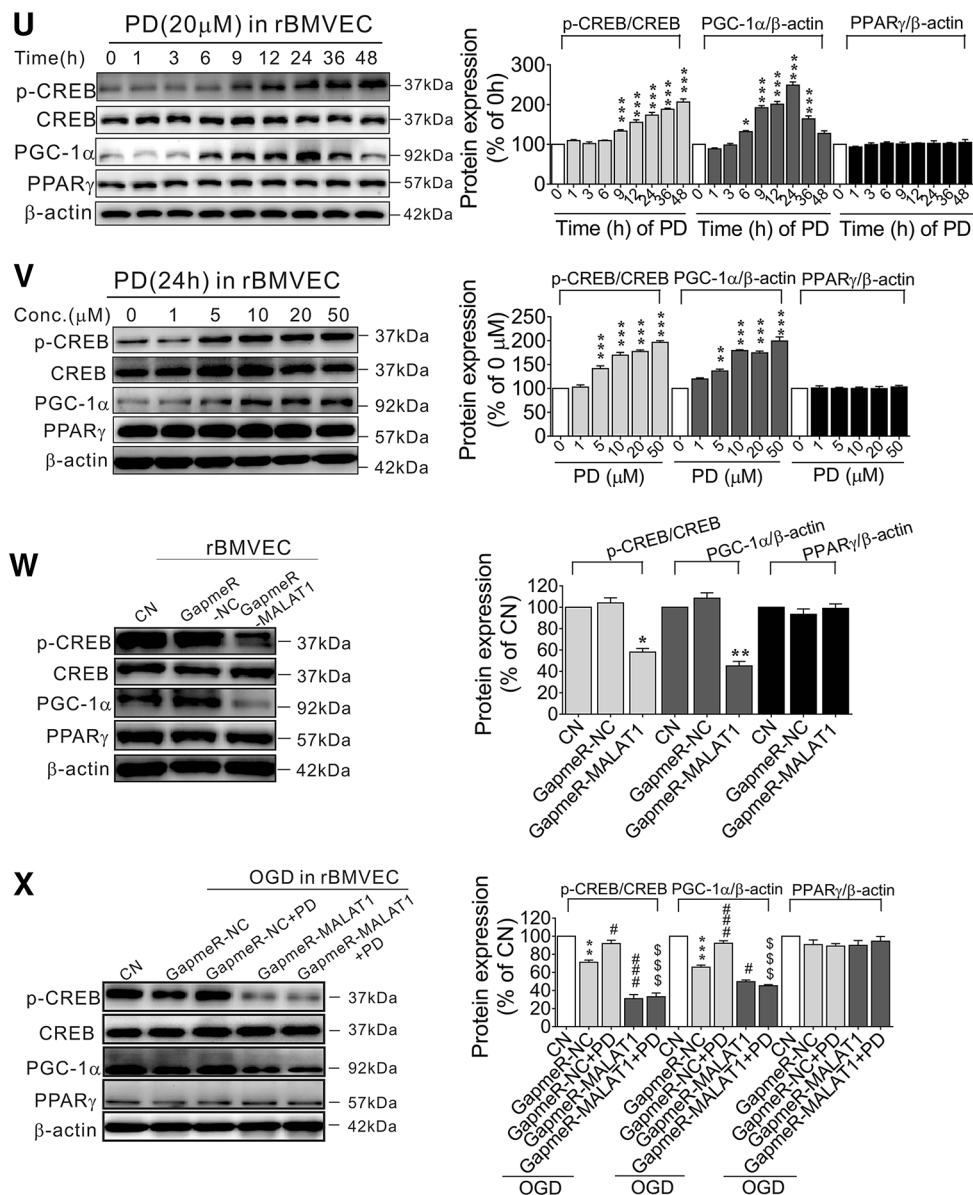


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involved in the mechanisms of vascular endothelial cell damage caused by ischemic stroke (Zhang et al. 2016, 2017). It was proposed that MALAT1 could protect endothelial cells against inflammatory response and apoptosis (Puthanveetil et al. 2015). Thus, promoting the expression of MALAT1 may ameliorate brain damage caused by ischemia, and a small molecular compound that up-regulates the expression of endogenous MALAT1 may be a promising therapeutic strategy for ischemic stroke.

In this study, we established a reporter gene system driven by -2100~+105 promoter region of MALAT1 in HUVEC cells. Our in-house compound library containing 1200 natural products were screened and a component of *Polygonum*

*cuspidatum*, PD, was shown to remarkably up-regulate the expression of MALAT1 in HUVEC and rBMVEC cells in a dose- and time-dependent manner. In addition, our *in vivo* results indicate that PD could enhance MALAT1 expression in cerebrovascular endothelial cells to exhibit beneficial effects in rats with ischemia. We studied the molecular mechanisms involved in the reported PD neuroprotection against ischemic stroke (Gao et al. 2016; Cheng et al. 2006).

In the present study, we demonstrated that PD reduced cerebral infarct volume and improved blood–brain barrier damage in rats subject to tMCAO. Our data indicate that the neuroprotection of PD may be mediated by maintaining the BBB integrity of rats with ischemia. Cerebrovascular

**Fig. 5** Polydatin protects against OGD-induced endothelial cell injury through the PGC-1 $\alpha$ /PPAR $\gamma$  pathway in rBMVEC cells. **a–c** The specific PPAR $\gamma$  inhibitors GW9662 (GW) or T0070907 (T007) reversed the PD reduction of OGD-induced cell death and inflammatory cytokines (TNF- $\alpha$ , IL-6) gene expression, and also reversed the PD enhancement of the gene expression of tight junction protein (Occludin, Claudin-5, ZO-1). **d, e** GW or T007 remarkably attenuated the PD enhancement of the protein expression of Occludin and ZO-1 by Western blotting or immunostaining. **f** TUNEL staining showed that GW reversed the PD inhibition of the OGD-caused endothelial cell apoptosis in rBMVEC cells. **g** The knockdown of PGC-1 $\alpha$  protein expression in rBMVEC cells was mediated by PGC-1 $\alpha$  siRNA (siRNA-PGC-1 $\alpha$ ). LDH assay show that the PGC-1 $\alpha$  knockdown significantly reversed the PD inhibition of OGD-induced cell death (**h**) and the inflammatory cytokines (TNF-1 $\alpha$  and IL-6) gene expression (**i**), and also reversed the PD enhancement of the gene expression of tight junction protein (Occludin, Claudin-5, ZO-1) in rBMVEC cells (**j**). **k** The PGC-1 $\alpha$  silencing totally attenuated the PD enhancement of the protein expression of ZO-1 and Occludin in rBMVEC cells under OGD condition. Data are shown as the mean  $\pm$  SEM ( $n = 3$ ) after normalization to the control (DMSO). One-way ANOVA followed by Bonferroni post-hoc test (**a–f, h–k**) and unpaired two-tailed T test (G). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control (CN); # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  versus OGD or OGD + siRNA-NC; \$ $p < 0.05$ , \$\$ $p < 0.01$ , \$\$\$ $p < 0.001$  versus PD + OGD or siRNA-NC + PD + OGD

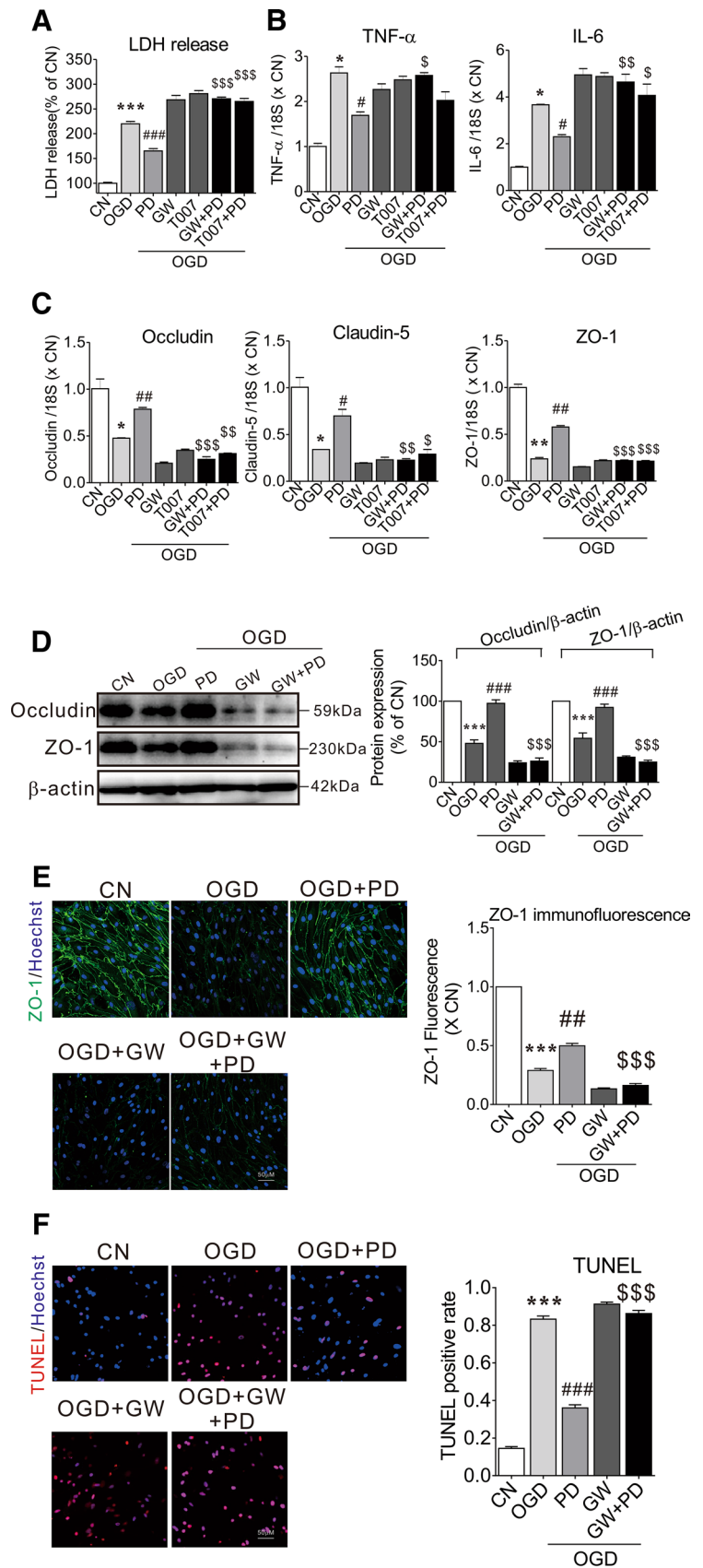
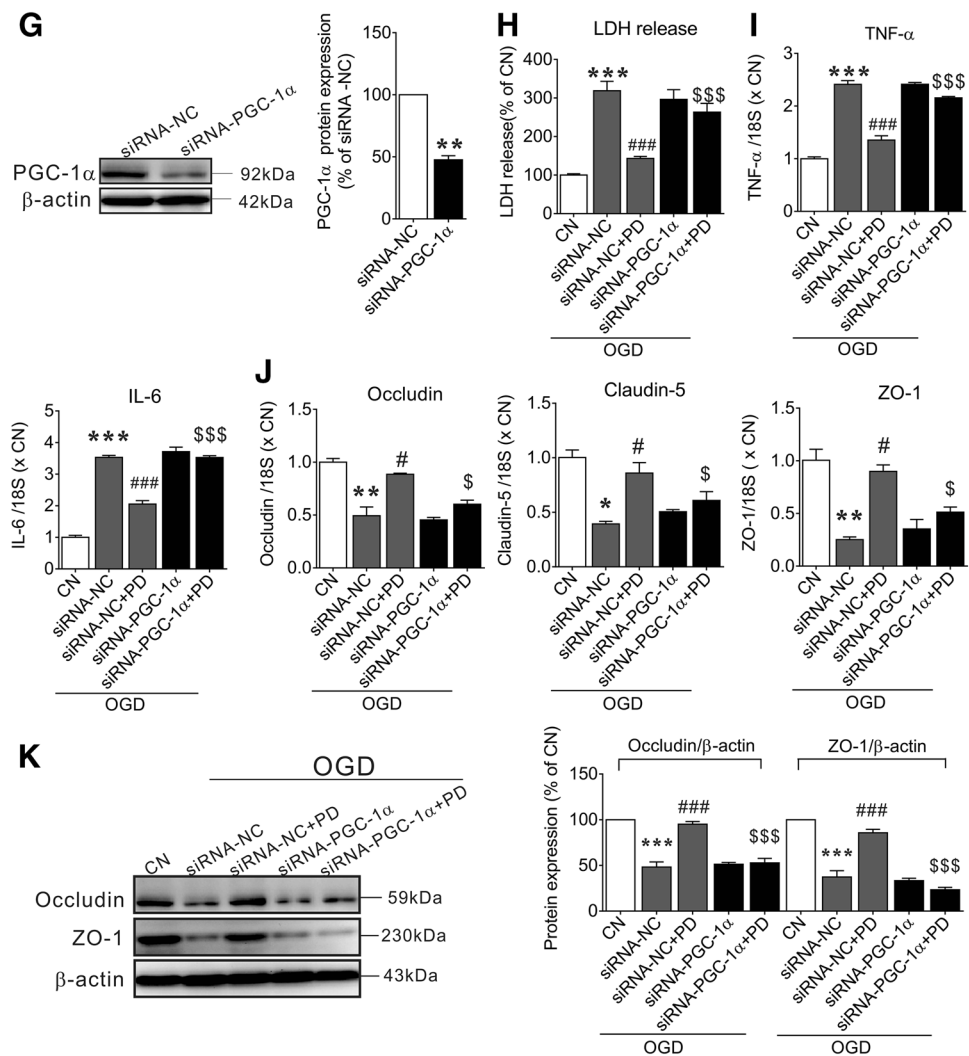




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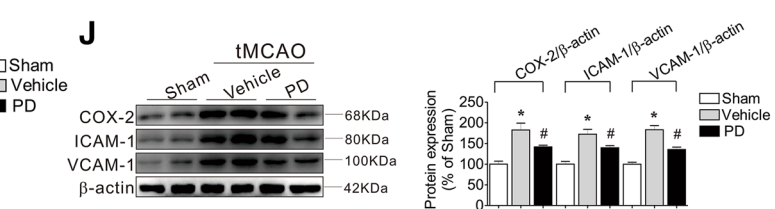
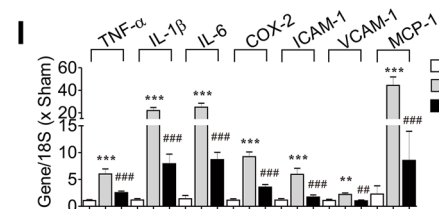
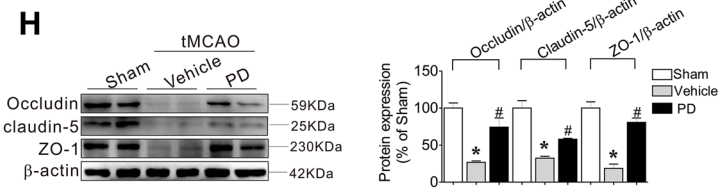
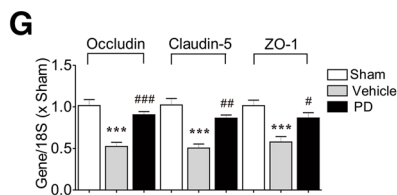
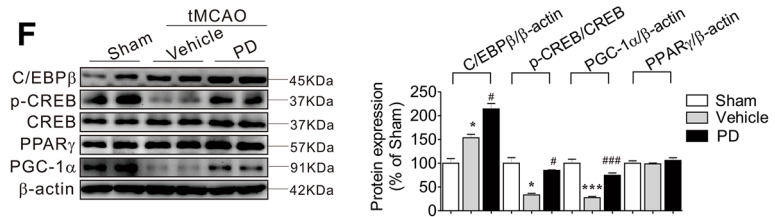
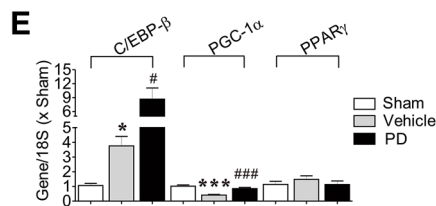
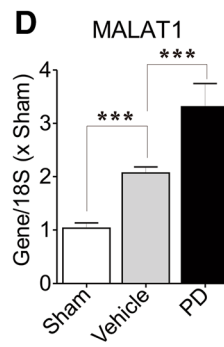
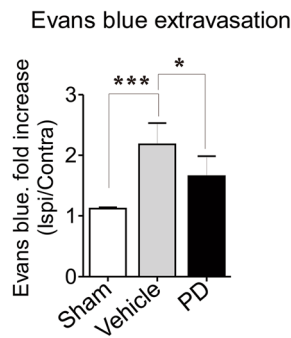
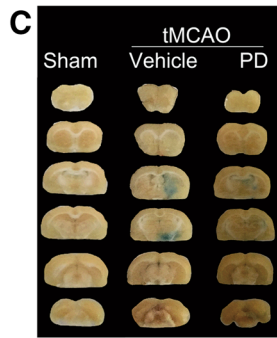
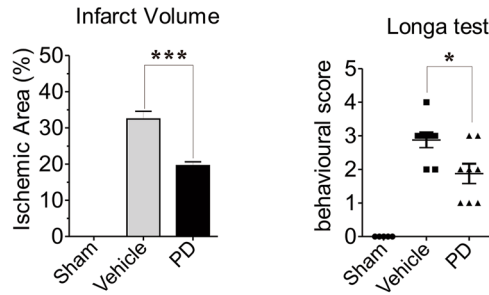
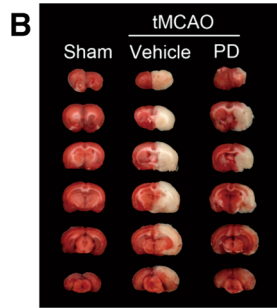
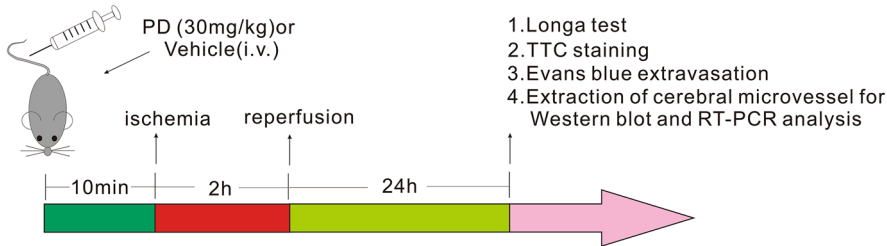
endothelial cells, the main components of BBB structure, are first damaged after ischemia and produce variety of pro-inflammatory factors, which further exacerbate ischemic brain damage. Our results showed that PD could improve cell viability loss and inhibit apoptosis in rBMVEC cells after OGD injury, and significantly inhibited the expression of pro-inflammatory factors (TNF- $\alpha$ , IL-6, COX-2) and the degradation of tight junction proteins (Claudin-5, Occludin, ZO-1). We found that the protective effect of PD against endothelial injury was reversed by MALAT1 knock-down, indicating that PD protection against ischemic stroke involved the regulation of MALAT1 expression.

Although the crucial role of MALAT1 in regulating endothelial cell function has been well recognized, the upstream factors mediating MALAT1 expression are still unclear. C/EBP $\beta$ , a transcription factor, is involved in the control of cellular growth, proliferation and pro-inflammatory process and participates in pathological responses in endothelial cells (Manea et al. 2013; Chuang et al. 2014). We discovered that C/EBP $\beta$  may bind to the -195~+105

promoter region of MALAT1 to regulate MALAT1 expression by the experiment of luciferase reporter gene driven by three truncated human MALAT1 promoter segments. And the C/EBP $\beta$  binding base sequence can be found in the MALAT1 -195~+105 promoter region of human and rat origins, so we think that C/EBP $\beta$  is a key transcription factor regulating the expression of MALAT1. We also found that PD increased C/EBP $\beta$  gene expression and the effect of PD on increase in MALAT1 expression was abolished by C/EBP $\beta$  gene silencing. PD up-regulation of C/EBP $\beta$  gene expression was also observed in cerebrovascular endothelial cells isolated from the brain of rats with ischemia. These results indicate that C/EBP $\beta$  may be a key transcription factor responsible for regulating MALAT1 expression by PD.

Our study further elucidated the downstream mechanisms of PD-induced MALAT1 up-regulation in cerebrovascular endothelial cells. PPAR $\gamma$  has been proved to exert a vital role in the treatment for endothelial dysfunction and ischemic stroke (Xu et al. 2017) and C/EBP $\beta$  could regulate the PPAR $\gamma$  activity (Wu et al. 2018). This indicates

### A Rat tMCAO Model



**Fig. 6** Polydatin ameliorates brain ischemic injury and protects the blood–brain barrier integrity in rats subject to tMCAO. **a** Experimental design of the tMCAO model. Rats were intravenously injected with polydatin (PD, 30 mg/kg) 10 min before ischemia. The rat brains were removed to perform histological analysis 24 h after reperfusion. **b** PD reduced cerebral infarct volume as assessed by TTC staining and improved sensorimotor recovery as evaluated by the Longa test. **c** PD significantly reduced ischemia-induced blood–brain barrier (BBB) disruption, which was tested by Evans blue extravasation assay. **d–f** The brain microvessels from rats with ischemia were isolated to test the expressions of MALAT1, C/EBP $\beta$ , PGC-1 $\alpha$ , and PPAR $\gamma$ . RT-PCR and Western blotting results showed that PD markedly enhanced the MALAT1 expression and the mRNA and protein expression of C/EBP $\beta$  and PGC-1 $\alpha$  but not PPAR $\gamma$ . PD also increased the protein expression of phosphorylated CREB (**f**). **g, h** Ischemia reduced the gene and protein expressions of tight junction proteins (Occludin, Claudin-5, ZO-1), while PD administration significantly enhanced the expressions of tight junction proteins (Occludin, Claudin-5, ZO-1). **i, j** PD administration reduced the gene expression of inflammatory factors (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, COX-2, ICAM-1, VCAM-1, MCP-1) and the protein expression of COX-2, ICAM-1 and VCAM-1 in brain microvessels from rats subjected to tMCAO. Data are shown as the mean  $\pm$  SEM ( $n=8-12$ ) after normalization to the control (Sham). One-way ANOVA with Bonferroni post test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus Sham group; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  versus vehicle group

that MALAT1 may be an important mediator for C/EBP $\beta$ -regulated PPAR $\gamma$  activity. In this study, we uncovered that MALAT1 significantly increased PPAR $\gamma$  activity, without influencing its protein expression. A recent study has shown that MALAT1 binding to CREB maintains CREB phosphorylation by inhibiting PP2A-mediated dephosphorylation, which leads to continuous CREB signaling activation (Yao et al. 2016). Moreover, CREB is an important transcription factor regulating PGC-1 $\alpha$  expression (Singh et al. 2015), a transcriptional coactivator interacting with PPAR $\gamma$ . This suggests that the CREB-PGC-1 $\alpha$ -PPAR $\gamma$  pathway may act downstream of MALAT1.

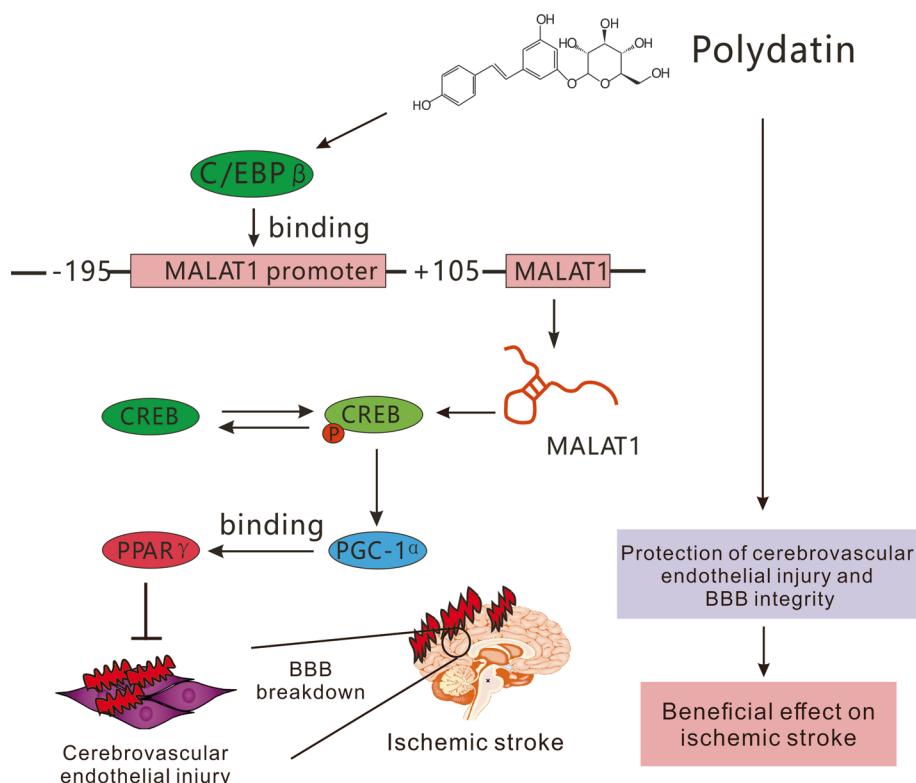
In the present study, we confirmed that MALAT1 regulated CREB phosphorylation to maintain its transcriptional activity. Furthermore, our data showed that the effect of MALAT1 on regulating PGC-1 $\alpha$  was attenuated by the inhibition of CREB activity, indicating that MALAT1 possibly binds with CREB to maintain the phosphorylation of CREB to further activate the CREB signaling pathway, thus promoting PGC-1 $\alpha$  expression. We further confirmed that PD could significantly up-regulate CREB phosphorylation, the expression of PGC-1 $\alpha$  and the activity of PPAR $\gamma$ , suggesting that PD might activate MALAT1/CREB/PGC-1 $\alpha$ /PPAR $\gamma$  signaling pathway to protect endothelial cells against ischemia.

Based on the understanding of mechanism underlying PD-regulated MALAT1 expression, we further proved that the activation of PGC-1 $\alpha$ /PPAR $\gamma$  signaling pathway is essential for the protective effect of PD against ischemia-induced endothelial injury. It has been reported that PPAR $\gamma$  could

directly bind to the subunit P65/P50 of NF- $\kappa$ B, forming a transcriptional repression complex and reducing the binding activity of NF- $\kappa$ B to DNA (Su et al. 2000), thus inhibiting its transcriptional activity and suppressing the production of downstream inflammatory factors. Our data showed that the effects of PD on inhibition of OGD-induced endothelial cell injury and enhancement of the tight junction proteins expressions were reversed by PPAR $\gamma$  inhibition or PGC-1 $\alpha$  gene silencing. Altogether, our results demonstrate that PD plays a protective role in ischemia-induced endothelial injury by activating the PGC-1 $\alpha$ /PPAR $\gamma$  pathway *via* MALAT1.

One limitation of our present study is that the mechanism of PD regulating C/EBP $\beta$  expression in rBMVEC cells has not been clarified. Microscale thermophoresis (MST) or surface plasmon resonance (SPR) technology should be used to further detect whether PD directly binds to C/EBP $\beta$  protein to regulate its transcriptional activity or only regulates its expression. Previous studies showed that PD enhanced Nrf2-ARE pathway by activating Sirt1 to exert anti-oxidative activities. MALAT1 has been particularly shown to activate Nrf2 signaling and regulate the deacetylation activity of Sirt1 (Ye et al. 2017; Huang et al. 2015). Thus, whether Sirt1- or Nrf2-mediated anti-oxidative pathway participates in the protective role of PD in endothelial injury caused by ischemia would be further studied.

Local brain inflammation is one of the pathological markers of ischemic stroke damage. Post-ischemic inflammation is mainly caused by activation of microglia and macrophages and peripheral leukocytes into brain parenchyma. A few minutes after ischemic stroke, microglia are activated to produce a series of inflammatory mediators that migrate from the surrounding tissues of the lesion to the lesion. At the same time, endothelial cells activate and express a variety of adhesion molecules, leading to destruction of the blood–brain barrier and leukocyte adhesion and exudation (Wang et al. 2007; Doyle et al. 2008). Leukocytes exuded in the lesion together with activated multiple glial cells release various harmful molecules such as protease, glutamate, cytokines, chemokines, free radicals, prostaglandins, and nitric oxide. These inflammatory molecules can aggravate the adhesion and exudation of inflammatory cells and cause a vicious cycle of inflammation, and can damage cells, blood vessels and extracellular matrix, resulting in blood–brain barrier destruction, brain edema and cell death (Zoppo 2009). In our study, we only studied that PD improves ischemic stroke which is associated with inhibition of endothelial cell damage. As for whether PD affects the inflammatory response of immune cells, we are not clear. From the published literature, PD can indeed inhibit the inflammatory response of immune cells (Lou et al. 2015; Zhao et al. 2017), but whether this effect is involved in the PD ameliorating ischemic stroke has not been determined. We will address this issue in the subsequent studies.



**Fig. 7** Schematic diagram for the proposed mechanisms of beneficial effects of polydatin on ischemic stroke. Polydatin promotes the expression of MALAT1 by up-regulating C/EBP $\beta$  transcription factor, and then MALAT1 may bind to the phosphorylated CREB to maintain its phosphorylation, resulting in promoting the expression of PGC-1 $\alpha$ . PGC-1 $\alpha$  acts as a transcriptional coactivator to enhance the activity of PPAR $\gamma$ , resulting in expression of PPAR $\gamma$  target genes,

which protect against the cerebrovascular endothelial cells injury and further blood–brain barrier (BBB) damage caused by ischemia. Therefore, PD, as a MALAT1 regulator with beneficial effects on brain ischemic stroke, may inhibit cerebrovascular endothelial cell damage through C/EBP $\beta$ -MALAT1-CREB-PGC-1 $\alpha$ -PPAR $\gamma$  signaling pathway, thereby maintaining the BBB integrity and reducing brain damage caused by ischemia

We selected male oSD rats for our *in vivo* experiments with the tMCAO model to avoid the effect of reproductive hormones during the rat estrous cycle. In future experiments, we will continue to study the effects of PD on brain damage in female tMCAO rats. In addition, we only studied the effects of PD treatment on brain injury in tMCAO rats 10 min before surgery. In the subsequent experiments, we will determine the therapeutic window of PD amelioration of brain damage caused by ischemia. In behavioral tests, we used Longa test to assess the brain protection of PD in the acute phase of ischemic stroke, and the results suggest that PD can improve behavioral deficits. However, in the subsequent recovery of long-term behavior, we do not know the effect of PD. In the acute phase of cerebral ischemia, BBB damage leads to impaired brain parenchyma and thus loss of behavior. Longa test can be used to evaluate the efficacy of the compound from a behavioral point of view (Longa et al. 1989; Wang et al. 2016). But in the late stage of cerebral ischemia, damaged tissues are repaired and behavioral changes are slowly recovered. In order to evaluate the efficacy of compounds comprehensively, it is necessary to

examine their effects on long-term behavioral recovery. In the published literature (Akinrinmade et al. 2017; Shi et al. 2017; Belayev et al. 2018), long-term behavioral recovery has been regarded as an important indicator for evaluating the protective effects of compounds on ischemic stroke. So in the follow-up study, we will determine the effect of PD on long-term behavioral recovery after ischemic stroke.

## Conclusion

In summary, our study, for the first time, discovered that PD, a novel MALAT1 regulator, protected against ischemia-induced endothelial injury *in vitro* and *in vivo* through modulating C/EBP $\beta$ /MALAT1/CREB/PGC-1 $\alpha$ /PPAR $\gamma$  pathway (Fig. 7). These findings may promote the study of MALAT1 up-regulators such as PD to be further developed as therapeutic agents for ischemic stroke.

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**Author Contributions** All authors listed contributed immensely to this study. WR and JL performed the experiments and wrote the paper. YX, YW, FZ, and XY performed the animal experiments and analyzed the data. HJ, LZ, JMS, LS, TP, as experts in molecular pharmacology provided technical supports and designed the research.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that there are no competing interests.

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