We used the ARRIVE checklist when writing our report.[1]

Experimental design

The objective of the study was to evaluate the role of LRRC8A in cerebral vascular endothelial cells in the blood-brain barrier (BBB) modulation. A transient middle cerebral artery occlusion model was established with C57Bl/6 mice and endothelial cell-specific knockout of LRRC8A was used to test its effect on the outcomes of ischemic stroke. Neurological benefits and BBB integrity post-ischemic stroke were analyzed through behavioral tests and immunostaining studies. For mechanistic exploration, immunocytochemistry, patch clamp, and permeability assays were used to identify the underlying mechanism of LRRC8A-mediated BBB injury. To evaluate the effects of Eupatorin on ischemic stroke, mice were randomized into different groups. Drugs or vehicles were configured by a fixed person. The investigators who performed surgery or testing were blinded to group allocation. Sample sizes were chosen empirically to ensure adequate statistical power and were in line with field standards for the techniques used in the study. Sample sizes for each experiment were shown in the figure legends. Endpoints were determined according to each experiment.

Transient middle cerebral artery occlusion (MCAO)

C57Bl/6 mice were anesthetized with isoflurane (induction dosage 3%; maintenance dosage 1.5%) in an air mixture delivered by a face mask combined with a scavenger system to lower the surgeon's exposure. Following anesthesia, a midline ventral neck incision was made to expose and isolate the right common carotid artery as well as the

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external carotid artery. Both arteries were subsequently ligated. Ischemic conditions were induced by gently inserting a silicone-coated monofilament (0.19 mm, 7–0; Doccol, USA) through the external carotid artery into the internal carotid artery. The monofilament was advanced until it occluded the middle cerebral artery (MCA) for 90 min, after which the monofilament was removed to allow for reperfusion. Laser speckle contrast imaging was used to test the cerebral blood flow (CBF). Surgery was considered successful if CBF was <30% of baseline during occlusion and >50% of baseline after reperfusion.[2] Throughout the surgical procedure, the body temperature of the mice was maintained at 37.0 ± 0.5 °C through a thermostat-controlled heating pad. Following surgery, the mice were transferred to a recovery room set at 23 ± 2 °C until they regained consciousness. Mice were excluded from subsequent analysis if they met any of the following criteria: death during or immediately after surgery, development of subarachnoid hemorrhage, or failure to achieve a reduction in CBF to <30% of baseline during occlusion and an increase to >50% of baseline after reperfusion (approximately 5%).

In the case of sham-operated mice, only the neck skin was incised and blood vessels were exposed, without any manipulation of the vasculature. 24 h after reperfusion, a blinded observer evaluated the neurological score using a 0–5 scale:

0 = No apparent neurological deficit

1 = Weakness in the left forelimb

2 = circling to the left side

3 = Body imbalance and trunk inclination to the left

4 = No spontaneous motor activity

5 = Death.[3]

Considering a neurological score of 5 points was typically regarded as indicative of a modeling injury, mice with a score of 5 at 24 h after reperfusion were excluded from analysis according to previous study.[4] The 24-hour mortality rates post-reperfusion in each group were as follows: 18% in Tie2-cre mice, 0% in LRRC8A$^{fl/fl}$; Tie-2Cre mice, 14% in LRRC8A$^{fl/fl}$; Tie-2Cre mice administered WNK463, 15% in wild-type mice, and 0% in wild-type mice treated with Eupatorin.

After neurological evaluation, brain infarct volume was quantified using three representative MAP2 or Nissl-staining sections encompassing the major MCA territory. The areas of MAP2-negative or Nissl-negative regions and the ipsilateral hemisphere were measured with ImageJ. The percent infarct area per slice was calculated as: (area of infarct / area of ipsilateral hemisphere). The percent volume of infarct was calculated as: (the sum area of infarct / the sum area of the ipsilateral hemisphere).[3-5]

Nissl staining

According to a previous study,[6] the brain sections were stained with toluidine blue in distilled water for 10 min at room temperature. After staining, the sections were sequentially rinsed in 75%, 90%, and 100% ethanol, each step being performed twice for 5 min. Subsequently, the sections were subjected to dehydration in xylene three times for 5 min each. The sections were then sealed with neutral gum and observed using Pannoramic Slide Scanners (Panoramic Scan, Hungary).

Laser Speckle Contrast Imaging

The mice were anesthetized with tribromoethanol. Subsequently, their head skin was opened and secured within a stereotactic frame for precise positioning. CBF was monitored using Laser Speckle Contrast Analysis (PeriCam PSI, PERIMED, Sweden). CBF images were obtained at 10 min before MCAO, 10 min after the onset of MCAO, and 10 min post-reperfusion. Equivalent regions of interest (ROIs) in the ipsilateral brain were established based on a decrease in blood flow to below 30% following occlusion. Mean CBF values were quantified within 10 min at different time points, with final data expressed as a percentage of baseline.

Assessment of BBB function

Evans Blue (EB) dye was used as a tracer to measure BBB permeability. Briefly, mice were injected with a dose of 2.5 mg/kg EB via the lateral tail vein 24 h after MCAO. This allowed the dye to circulate for a period of 2 hours. Afterward, the mice were humanely euthanized, and transcardial perfusion was performed. Subsequently, the brains were surgically extracted. For evaluation of the cortical fluorescence intensity, which serves as an indicator of EB leakage, the brains were subjected to imaging using IVISSpectrum CT Imaging System (SpectrumCT, PerkinElmer.Inc.).

Alternatively, each brain hemisphere was weighed and homogenized, and EB was extracted by incubating the tissue homogenate with 50% trichloroacetic acid (TCA), followed by centrifugation at 12,000 g for 30 min. The absorbance of EB was measured at 610 nm using the spectrometer.

In addition, an assessment of BBB leakage was conducted through IgG

immunostaining (as described below). The region displaying IgG positivity was designated as the leakage area, thereby enabling the quantification of BBB permeability.

Immunohistochemistry and image analysis

Mice were subjected to transcardial perfusion with ice-cold PBS and 4% PFA. The brain was then harvested and post-fixed in 4% PFA at 4 °C for 24 h. After sucrose dehydration, the tissue was embedded in O.C.T. Compound (Tissue-Tek®, CAT#4583). Frozen serial coronal sections (25 μm per section) were then cut with a freezing microtome (Leica CM1950, Germany) and were subsequently mounted onto slides. The sections were incubated with 0.1% Triton X-100 in PBS for 15 min and blocked in 3% bovine serum albumin for 1 h at room temperature. The following primary antibodies were applied to the mounted sections overnight at 4 ℃: rabbit anti-MAP2 antibody (1:200, Abcam, Cat# ab183830), rabbit anti-IBA1 antibody (1:200, FUJIFILM Wako Pure Chemical Corporation, Cat# 019-19741), mouse anti-LRRC8A antibody (1:100, Santa Cruz, Cat# sc-517113), rabbit anti-CD86 antibody (1:200, proteintech, Cat# 13395-1-AP), mouse anti-pdgfrβ antibody (1:100, Santa Cruz, Cat# sc-374573), rabbit anti-Arg1 antibody (1:200, Abcam, Cat# ab96183), rabbit anti-CD31 antibody (1:100, Abcam, Cat# ab222783), rabbit anti-ZO-1 antibody (1:200, Abcam, Cat# ab221547), rabbit anti-VE-cadherin antibody (1:200, Abcam, Cat# ab205336). After that, the sections were washed with PBS, and subsequently incubated with secondary Alexa FluorTM 568 donkey anti-rabbit IgG (H+L) (1:500, Invitrogen, Cat# A10042) or Alexa FluorTM 488

donkey anti-mouse IgG (H+L) (1:500, Invitrogen, Cat# A21202) for 1 h at room temperature in a dark room. The sections were washed with PBS and then cover-slipped with Vectashield mounting medium with DAPI Fluoromount-G (SouthernBiotech, Cat#0100-20). For IgG immunostaining, brain slices were incubated solely with Alexa FluorTM 488 donkey anti-mouse IgG (H+L) (1:500, Invitrogen, Cat# A21202), and the other protocols remained the same.

Immunofluorescence was visualized and images were acquired using a Zeiss confocal-LSM-900 microscope with ZEN2 software. Fluorescence intensity was analyzed using the ZEN2 software. Microglia analysis was based on the cells within the thalamus in the peri-infarct areas.

MAP2, Nissl, and IgG images were digitally captured using the Pannoramic Slide Scanners and analyzed with ImageJ software (v1.52a, NIH). For each animal, three representative sections (25 μm) encompassing the major MCA territory including the striatum, hippocampus, and substantia nigra were used to obtain the average value of the ischemic area, ipsilateral brain area, and the leakage area.

Isolation of mouse brain microvascular endothelial cells

Based on a previous study,[7] 8-10 adult mice (8-12 weeks) were euthanized and brain tissue excluding brainstem, cerebellum, and thalamus were carefully rolled on sterile blotting paper to separate the meninges. The brain tissue was transferred to a centrifuge tube pre-filled with DMEM medium and digested with collagenase (10 mg/ml) and DNase (1 mg/ml) for 1 h on a 37 °C, 180 r/min shaker. After that, the digestion was terminated and the pellet was re-suspended with BSA-DMEM (20%

w/v). Centrifugation was performed to remove the myelin and BSA layer. The pellet was re-suspended again and digested with collagenase/dispase (10 mg/ml) and DNase (1 mg/ml) for 1 h. The digested cells were then diluted with 10 ml of DMEM and centrifuged again. The pellet was re-suspended in 2 ml of DMEM and carefully layered onto a Percoll solution, followed by centrifugation at 700 g for 10 min at 4 °C. Approximately 12 ml of the interface cells were collected and centrifuged at 1000 g for 10 min at 4 °C. The final pellet was resuspended in an endothelial cell complete medium containing puromycin and cultured at 37 °C with 5% CO_2 for the first two days only. The culture medium was subsequently refreshed every 2-3 days without the addition of puromycin.

For the immunocytochemistry experiments, cells were cultured for 4-5 days until they formed the tight junctions. Cells were then incubated with normal (330 moSm, mM: NaCl 95, mannitol 100, KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 6, $MgCl_2$ 1, $CaCl_2$ 2, pH 7.35 adjusted with NaOH) or hypotonic (165 moSm, mM: NaCl 95, KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 6, $MgCl_2$ 1, $CaCl_2$ 2, pH 7.35 adjusted with NaOH) solution for 6 h. The osmolarity was adjusted with sucrose.

Cell culture

Human brain microvascular endothelial cells (hBMVECs) were purchased from ATCC and 2 to 3 generations of cells were used for the test. When density reached 80%-90%, cells were digested with 0.05% trypsin and cultured with F12 full medium plus 10% fetal bovine serum(FBS), 1% penicillin/streptomycin and 1% ECGS in a 37 °C, 5% CO_2 incubator.

Oxygen-glucose deprivation（OGD）

The cell culture medium was removed and replaced with the glucose-free Dulbecco's Modified Eagle's Medium (DMEM) (pre-hypoxia exposure for 2-4 h). Then cell plate was placed in a hypoxic incubator (94% N_2, 1% O_2, 5% CO_2) and subjected to OGD for 12 h. For the negative control, cells were cultured with complete endothelial cell medium in a 95% O_2, 5% CO_2 incubator. After OGD exposure, cells were placed back into the complete medium and cultured in a 95% O_2, 5% CO_2 incubator for re-oxygenation.

Cell viability assay

In brief, cells were seeded in 96-well plates and incubated with 20 μl of methylthiazolyldiphenyl-tetrazolium bromide (MTT, 5 mg/mL, Sigma, Cat# M5655) for 4 h at 37 °C. Subsequently, the solution in each well was carefully discarded, and 150 μl of dimethyl sulfoxide (DMSO) was added for 20 min at room temperature. The absorbance of each well was measured at 570 nm. The results of cell viability were expressed as a percentage of the control group.

siRNA transfection

HBMVECs were grown for 72 h (80-90% confluence) and transfected with either a silencer select siRNA with siLRRC8A (Cat#R312111793/1794, sense: AGACCATCATCAAGGTGAT, Hippbio) or a nontargeting control silencer select siRNA (Hippbio). Briefly, the full medium was changed to a blank DMEM medium and starved for 30 min. Each siRNA (2 μl) was combined with NanoTrans 20TM (4 μl, Cat#B1001, Hippbio) and diluted with DMEM medium in a final volume of 2000 μl.

HBMVECs were transfected by the transfection complex at 37 °C for 4-6 h. Then the cells were returned to F12 media containing 10% FBS, 1% penicillin/streptomycin, and 1% ECGS. Cell lysates were collected on day 2.

Immunocytochemistry

Mouse brain microvascular endothelial cells (mBMVECs) were washed with PBS and fixed with 4% PFA for 20 min. Then PFA was washed and cells were permeabilized with 3% BSA and 0.3% triton for 30 min. 10% donkey serum was used to block cells for 1 h, followed by primary antibody incubation of ZO-1 (1:250; Abcam, Cat# ab221547) and VE-cadherin (1:250; Abcam, Cat# ab205336) overnight at 4 °C. After PBS washing, cells were incubated with secondary antibodies coupled to DyLight 488 (1:500) at room temperature for 2 h and later labeled with DAPI for visualizing nucleus. Images were acquired using the A1Rsi (Nikon) confocal microscope or ImageXpress Micro Confocal system. The fluorescence intensity was quantified using NIS-Elements AR Analysis software (version 5.01.00) and ImageJ software (version 1.52a). Measurements were taken from both the plasma membrane and cytoplasm, and the results were expressed as fluorescence intensity per unit area.

Cell permeability

Tanswell chamber (24-well Plates with 3 μm Pore Size Polycarbonate Membrane Tissue Culture Treated, Corning, USA) was used to test the cell permeability.[8] Briefly, 100 μl of cell suspension containing approximately 1.5×10^5 cells was added to the upper chamber and 600 μl of complete medium was added to the lower chamber. Then the cells were maintained in a 37 °C, 5% CO_2 incubator until the cells reached

full density and no obvious leakage could be detected. Following this, cells designated for analysis at different time points were subjected to a simultaneous OGD exposure for 12 h. Subsequently, they were transferred to a 37 °C, 5% CO_2 incubator for re-oxygenation. After re-oxygenation periods of 1, 3, 5, and 7 h, respectively, FITC-Dextran (1 mg/ml) with different molecular weights (4, 40, and 70 kDa) were added to the cells, and 100 μl of the sample was withdrawn from the lower chamber 1 h later for absorbance measurement.[9] It is important to note that cells from a single well were not employed for analysis at multiple time points. Rather, the duration of re-oxygenation was varied among distinct wells, followed by the addition of FITC-Dextran for permeability assessment.

Flow cytometry and real-time PCR

The brain tissues obtained 24 h post-surgery were broken up and incubated with 1 mg/ml DNase, and then the tissue was pipetted into a single-cell suspension, followed by filter and centrifugation. 1 ml of staining buffer was added to wash the cells and the final concentration of cell suspension was 10^6 ~ 10^7 cells/ml. According to the manufacturer's instructions, fluorescence-labeled specific antibodies were used to incubate the cells on ice in the dark for 30 min, and then the cells were loaded for sorting by CytoFLEX SRT (Beckman Coulter). Data analysis was performed with FlowJo software (FlowJo, LLC).

After cell collection, total RNA was extracted with TRIzol, and RNA was reverse-transcribed to cDNA following the manufacturer’s instructions (Thermo Scientific). The reaction conditions were as follows: 95 °C pre-denaturation 30 s;

95 °C ×5 s, 60 °C×30 s, 72 °C×30 s (40 cycles). For each target gene, mRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (ΔCt=target gene Ct - β-actin Ct value). The primers were designed by Primer 6.0 software and synthesized by Shanghai Shenggong Biological Co., Ltd. The primers (5′-3′) were shown in Table 1.

Electrophysiology

All recordings were performed using the whole-cell patch technique. Based on our previous study,[10] patch electrodes were pulled from borosilicate glass and fire-polished to give resistance of 2–3MΩ when filled with a hypertonic internal solution of the following composition (mM): CsCl 140, $MgCl_2$ 2.4, $CaCl_2$ 0.5, EGTA 5, HEPES 10, Mg-ATP 5, Na-GTP 0.5, pH 7.35 adjusted with CsOH and osmolarity was adjusted to 420 mOsm with sucrose. The isotonic external solution contained the following (mM): NaCl 145, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, glucose 10, HEPES 10, pH 7.4 adjusted with NaOH, and osmolarity was adjusted to 320 mOsm with sucrose. A low-profile perfusion chamber fed by a gravity perfusion system was used for solution exchange (2 ml/min, bath exchange time of ~15 s). A MultiClamp 700B amplifier in combination with pCLAMP 10.0 software (Molecular Devices, CA, USA) was used for experiments. All recordings were performed at room temperature. The current amplitude was measured at its maximum at −60 mV and was filtered at 2 kHz.

Data and statistical analysis

Shapiro-Wilk normality test was conducted before each statistical test to determine if the data followed a normal distribution. For data with normal distribution, a two-sample *t*-test was used to compare two groups. One-way ANOVA with Tukey's

post hoc test and two-way ANOVA with Bonferroni's post hoc test were performed for multiple comparisons. A significant difference was considered for $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. All results were presented as mean ± Standard Error of Means (SEM) and error bars in the figures represent SEMs.

Data availability statement

All data are available in the main text or the supplementary materials.

Table1: Primers used for different genes

Gene	Primers (5′-3′)
Occludin	Forward: TTGAAAGTCCACCTCCTTACAGA
	Reverse: CCGGATAAAAAGAGTACGCTGG
Claudin 5	Forward: GCAAGGTGTATGAATCTGTGCT
	Reverse: GTCAAGGTAACAAAGAGTGCCA
β-actin	Forward: GGCTGTATTCCCCTCCATCG
	Reverse: CCAGTTGGTAACAATGCCATGT
ZO-1	Forward: GCCGCTAAGAGCACAGCAA
	Reverse: TCCCCACTCTGAAAATGAGGA
VE-cadherin	Forward: GCTCACGGACAAGATCAGCTC
	Reverse: ATAGTGGGGCAGCGATTCAT
LRRC8A	Forward: TCTGCCTGCCTTGTAAGTGG
	Reverse: ACGTAGTTGTACTGGTGCCG

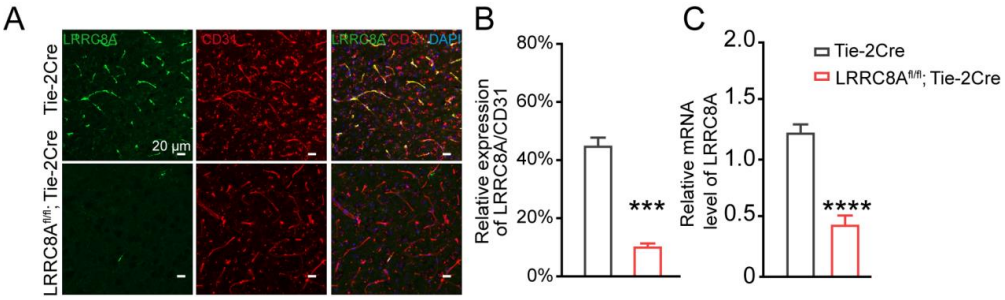

Figure S1 Validation of endothelial cell-specific LRRC8A knockout efficiency. (A) Representative images of LRRC8A (green) and CD31 (red) co-localization in LRRC8A$^{fl/fl}$; Tie-2Cre group and Tie-2Cre group. (B) Quantification of the relative expression of LRRC8A/CD31. N = 3 mice in each group, ***P < 0.001, two-sample t-test. (C) Quantification of the LRRC8A mRNA levels. Brain endothelial cell sorting was used and real-time PCR was performed to quantify the LRRC8A mRNA levels. N = 3 mice in each group, ****P < 0.0001, two-sample t-test.

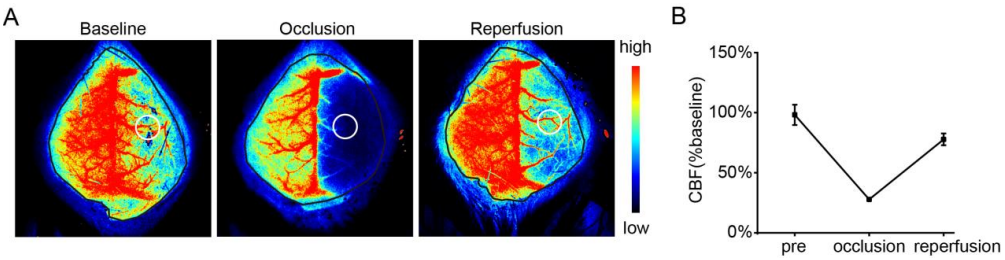

Figure S2 Laser speckle contrast imaging and analysis. (A) Representative CBF images before, during, and 24 h after surgery. Equivalent regions of interest (ROIs) (white circles) in the ipsilateral brain were established based on a decrease in blood flow to below 30% following occlusion. (B) Quantitative analysis of the mean CBF of ROIs within 10 min at different time points. N=6 mice.

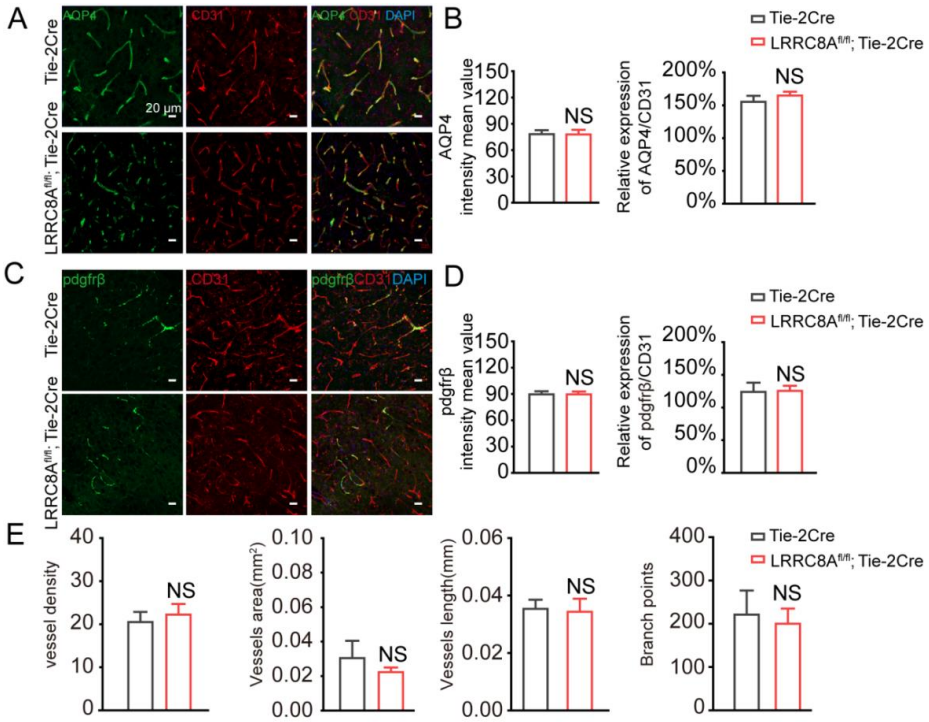

Figure S3 Endothelial cell-specific knockout of LRRC8A has no effect on the brain vasculature and the coverage of astrocytes and pericytes. (A, C) Representative images of CD31, AQP4, pdgfrβ and co-staining in LRRC8A$^{fl/fl}$; Tie-2Cre group and Tie-2Cre group. (B, D) Quantification of the relative expression. N = 4 mice in each group, two-sample *t*-test. (E) Quantification of the vascular morphology in LRRC8A$^{fl/fl}$; Tie-2Cre group and Tie-2Cre group. N = 3 mice in each group, two-sample *t*-test.

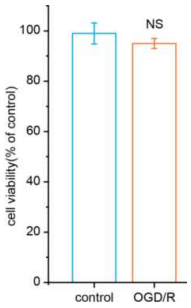

Figure S4 Quantification of cell viability. Data represent five independent

experiments, two-sample *t*-test.

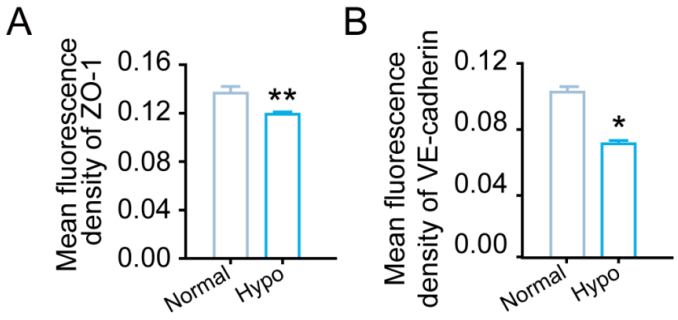

Figure S5 Effect of hypotonic stress on the expression of ZO-1(A) and VE-cadherin (B) in the mBMVECs. Data represent three independent experiments. **P* < 0.05, ***P* < 0.01, two-sample *t*-test.

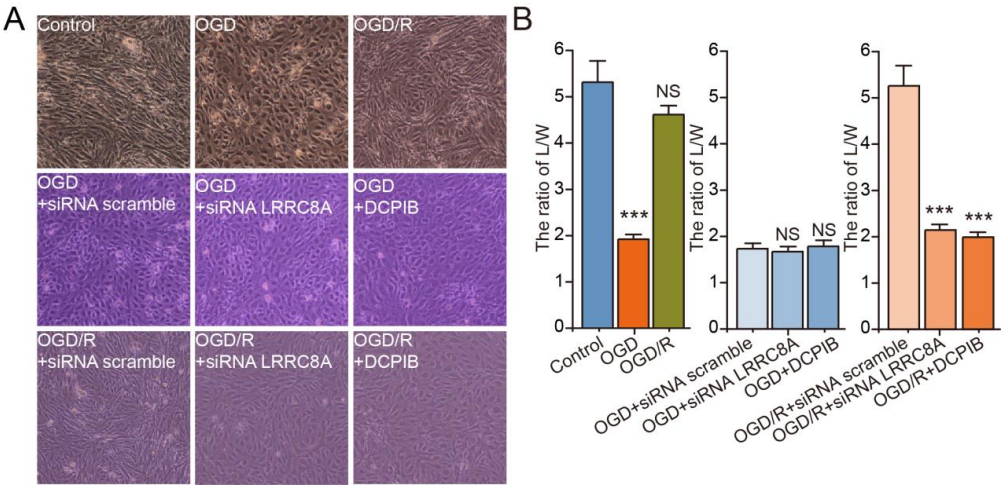

Figure S6 Effect of LRRC8A modulation on the morphological changes of mBMVECs. (A) Representative images displayed the morphologies of mBMVECs with different preconditioning. (B) Quantification of the ratio of length to width (L/W) of mBMVECs in different groups. Data represent three independent experiments. ****P* < 0.001, compared with the first column, one-way ANOVA.

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