The TDP43 CFTS Affect Brain Endothelial Cell Functions by Regulating YAP and Tight Junction Proteins in Cerebral Ischemic Injury.

Xiaotian Xu

University of Houston College of Pharmacy

Changwen Zhang

Cincinnati Children's Hospital Medical Center

Jianxiong Jiang

University of Tennessee College of Medicine: The University of Tennessee Health Science Center College of Medicine

Mei Xin

Cincinnati Children's Hospital Medical Center Center for Health Care Quality: Cincinnati Children's Hospital Medical Center James M Anderson Center for Health Systems Excellence

Jiukuan Hao (✉ jhao6@central.uh.edu)

University of Houston College of Pharmacy <https://orcid.org/0000-0002-4483-183X>

Research

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Abstract

Background: As important components of the blood-brain barrier (BBB), brain endothelial cells (ECs) interact with pericytes, astrocytes, neurons, microglia and *extracellular* matrix in the neurovascular unit to maintain central nerve system (CNS) homeostasis and regulate neurological functions. Pathological changes in brain endothelium plays an important role in progression of ischemic stroke. The compromised BBB under ischemic stroke condition causes neuronal damage. However, the pathophysiological mechanisms of BBB in normal and under ischemic stroke condition has not been fully elucidated.

Methods: C57bl/6 mice were subjected to 1-hour transient middle cerebral occlusion (tMCAO) model, and collected the brain samples after reperfusion for 24hours, 72hours and 1week. Lentivirus (YAP/TAZ shRNA), adenovirus (YAPS112A), TDP43 siRNA, TDP43-CTFS35 overexpression plasmid, Oxygen-glucose deprivation (OGD), and lipopolysaccharides (LPS) were applied to brain endothelial cells *in vitro* experiments. Brain endothelial cells (ECs) functions were tested by cell proliferation, migration and cell viability. Hippo signaling pathway was examined by immunofluorescence and western blotting.

Results: The present study demonstrates that TDP43 is an essential gene to regulate brain ECs normal function, and knockdown of TDP43 reduces tight junction protein expression and inhibits brain ECs migration. Furthermore, ischemic injury and inflammation induce cytoplasmic TDP43-CTFS35 aggregation brain ECs, which weaken TDP43 full-length's function leading to impairing tight junction (TJ) protein expression and cell migration. The expression of cytoplasmic TDP43-CTFS35 in brain ECs increased at 24 hours and 72 hours after MCAO, but disappeared at 1 week after MCAO. The expressions of TJ proteins, ZO-1 and claudin-5, and expression of P-YAP are associated with the dynamic changes of TDP43-CTFS35 expression in brain ECs after MCAO. The underlying mechanism of TDP43-CTFS35's effects on brain ECs is that TDP43-CTFS35 turns off the Hippo signaling pathway by inhibition of PMST1/2 phosphorylation leading to de-phosphorylate YAP, and subsequently causes brain ECs functional changes.

Conclusions: The present study provides new insight knowledge regarding the mechanisms of brain vascular ECs regulation and pathological change in the BBB after cerebral ischemic injury.

Introduction

The BBB formed by vascular ECs with tight-junctions and other types of cells controls movement of molecules and cells across the BBB. Pathological changes at the level of brain endothelium plays an important role in progression of brain diseases. The compromised BBB under ischemic stroke condition causes neuronal damage. However, the approaches to modulate the BBB function for therapy are still very limited. Therefore, understanding the pathophysiology of the BBB in ischemic condition is important for finding a novel effective target to treat neurological diseases such as ischemic stroke, Parkinson disease and Alzheimer disease involved in the BBB dysfunction.

Hippo signaling pathway plays important roles in regulating tissue homeostasis, growth and repair by regulating activity of the YES-associated protein (YAP) and its paralog TAZ (transcriptional co-activator with PDZ-binding motif), the transcriptional coactivators of the Hippo signaling pathway [1–4]. YAP/TAZ can drive the expression of genes that regulate cell proliferation, cell survival, and cell-cell interaction [5, 6]. Recently, studies have shown that YAP promotes endothelial cell proliferation, migration and angiogenesis[7–9]. YAP also plays a role in regulating formation and integrity of tight-junction [10, 11], which indicates its possible role in tight-junction of the BBB. Furthermore, our previous study showed that cytoplasmic form of YAP and nucleus YAP play an independent role in the proliferation and migration of vascular ECs during retinal angiogenesis [12]. Notably, the role of Hippo signaling in brain vascular ECs and BBB disruption after acute ischemia stroke has not been defined.

TAR DNA-binding protein 43 (TDP-43), a 414-amino-acid protein, participates in the regulation of RNA splicing, stability, transcriptional repression, and other cellular functions[13]. The role of TDP43 in neurodegenerative diseases has drawn increasing interest and continued to expand in recent years. Growing evidence show that TDP43 plays roles in amyloid deposition, tau hyperphosphorylation, mitochondrial dysfunction, and neuroinflammation[14, 15]. The majority studies of TDP43 are focused on its aberrant phosphorylation, ubiquitination, cleavage and/or nucleus depletion, cytoplasmic aggregation and neurotoxicity in neurons of ALS, AD, FTL, HD[15, 16]. There is no study on the role of TDP43 in regulation of brain ECs and the BBB functions.

In the present study, we employed primary brain vascular ECs, mouse brain-derived brain endothelial cell and middle cerebral artery occlusion (MCAO) mice model to investigate the role of TDP43 and TDP43-CTFS35 in regulation of brain EC functions, and elucidate underlying mechanisms involved in brain vascular EC proliferation, migration, tight-junctions (TJs) protein expression, and its interactions with Hippo signaling pathway and YAP in normal condition and ischemic condition.

Materials And Methods

2.1 Materials

2.1.1 Chemicals

Lipopolysaccharides (LPS; L2880) and X-tremeGENE™ siRNA transfection reagent (4476093001) were purchased from sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin–streptomycin, 4',6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies (Carlsbad, CA). Anti-Tubulin antibodies (2148S), anti-GAPDH(2118S), anti-YAP(14074T), anti-β-Actin antibody(4967S), anti-TBP antibody(8515S), anti-TAZ(83669S), anti-p-YAP (Ser127) (13008T), anti-YAP/TAZ(93622), anti-MST1(14946S), anti-p-(Thr183)/MST2 (Thr180)(49332S), anti-VEGFR2(9698S), Anti-rat IgG (Alexa Fluor® 488 Conjugate), Anti-rabbit IgG (Alexa Fluor® 555 Conjugate) were purchased from Cell Signaling Technology (Beverly, MA). anti-CD31 antibody(ab7388), anti-claudin-5(ab131259), were purchased from Abcam (San Francisco, CA). Anti-ZO-1 antibody (40-2200), anti-TDP43 (c-terminal) antibody(12892-1-AP), anti-

TDP43 antibody (PA5-17011), BrdU™ Assay Kit(Alexa Fluor™ 488)(A23210), EdU Cell Proliferation Kit for Imaging(Alexa Fluor™ 488)(C10337), Ki-67 antibody (69-5698-82), and MTT Cell Viability Assay (V13154) were purchased from ThermoFisher Scientific (Waltham, MA). Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies (Rockville, MD).

2.2 Methods

2.2.1 Primary brain endothelial cell isolation, treatment, transfection

The method of Primary brain ECs isolation from C57BL/6 mice brains was described previously[17]. Primary brain ECs were infected with a lentivirus containing shRNA targeting YAP and TAZ or adenovirus (YAPS112A) (gifts from Mei Xin, Cincinnati Children's Hospital, Cincinnati, OH, USA). The transfection details have been described previously[12, 18, 19].

2.2.2 The bEnd3 cells line culture, treatment, transfection

In the present study, the mouse brain-derived vascular endothelial cells line (bEnd3 cells) were cultured in DMEM medium with 10% FBS and penicillin-streptomycin at 37°C with 5% CO₂. Then, the bEnd3 cells were pretreated with DMEM serum-free medium containing LPS (0.1, 0.3, 0.5, 0.7, 1ug/mL) for 24 hours. The bEnd3 cells (4×10⁵ cells per well) were seed onto 6-well plates overnight to reach 70% confluence for transfection. The pCMV6-AC-Myc vector (PS100003, OriGene Technologies, Rockville, MD, USA) encoding mutant mouse TAR DNA binding protein (Tardbp) (NM_145556) C-terminal fragments (CTF35, 978bp) was transfected into cells for 4.5 hours using Lipofectamine 3000 (Thermo Fisher Scientific), then replaced with complete medium to culture another 48 hours at 37°C, which was collected for use in subsequent experiments. The mouse Tardbp43 siRNA ON-TARGETplus SMARTPool (L-040078-01-0005) and ON-TARGETplus non-targeting pool (D-001810-10) (Horizon Discovery, USA) were transfected into cells for 4.5 hours using X-tremeGENE™ siRNA Transfection Reagent (4476093001, sigma, USA) for 5 hours, then replaced with complete medium to culture another 48 hours at 37°C, which was collected for use in subsequent experiments.

2.2.3 Oxygen-glucose deprivation (OGD)

Hypoxia was induced by placing cells in the incubator (Thermo Scientific™ Heracell 150i) at 37°C, and introducing a flush with 95% N₂/5% CO₂ gas until the complete removal of O₂. The oxygen level was maintained at 1%. Aglycemia was performed by using Gibco cell culture medium without D-glucose. For experiments using OGD and reperfusion (OGD/R) conditions, the bEnd3 cells were kept in OGD for 4 h followed by normal growth condition and normoxic atmosphere for an additional 24 h.

2.2.4 Cell viability assays

The viability of primary brain ECs and the bEnd3 cells were evaluated by MTT and CCK-8 assays following the manufacturers' instructions. The data are presented as a percentage of the value obtained from cells incubated in fresh medium only.

2.2.5 Immunocytochemistry

For immunocytochemistry, cells were seeded at 0.8×10^6 on 1.5-mm² coverslips for 24 hours. After treatments or transfection, the cells were fixed with 4% ice cold paraformaldehyde for 20 minutes at 4°C. The cells were then air dried, followed by blocking and permeabilization with 1% BSA in PBS with 0.1% Triton for 30min. After that, primary brain ECs were stained with CD31 antibody (1:100) and YAP antibody (1:200), or the bEnd3 cells were stained with CD31 antibody (1:100) and TDP43 antibody (1:100), TDP-43 (C-terminal) antibody (1:100), claudin-5 antibody, or ZO-1 antibody for 24 hours in a humidified chamber at 4°C. After three washes with 0.1% Triton in PBS, secondary antibody (1:500 anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor® 488 Conjugate), catalog number 4412s, cell signaling technology; 1:500 anti-mouse (Alexa Fluor® 555), catalog number 19581S, cell signaling technology) were used for 1 hr. After DAPI staining for 10min, the coverslips were transferred onto glass slides. Images were obtained using a Digital Microscope Camera Systems (Laica DM6000B).

2.2.6 Cell proliferation (Edu and Ki67) assays

To assess the cell proliferation, primary brain ECs were seeded in a 6-well plate with complete culture medium for 24 hours. After treatments, primary brain ECs proliferation was determined using Ki-67 Proliferation Assay[20] according to the manufacturer's instructions. The bEnd3 cells proliferation was determined using Edu Proliferation Assay[21] according to the manufacturer's instructions.

2.2.7 Scratch assay

Primary brain ECs were scratched with a P10 pipette tip in serum-free medium after transfected with YAP/TAZ shRNA lentivirus. Cells were photographed at 0, 16, and 24 h after the scratch. The cells were treated with hydroxyurea, an inhibitor of cell proliferation, for 4 hours before the migration assay. The bEnd3 cells were scratched with a P10 pipette tip in serum-free medium after transfected with TDP43 siRNA or TDP43-CTFS overexpression plasmid. Cells were photographed at 0 and 24 h after the scratch. Invaded area was measured by digital imaging using ImageJ software.

2.2.8 Animals

All animal procedures were approved by the Institutional Animal Care and Use Committees at University of Houston and they complied with pertinent NIH guidelines for care and use of animals. Both male and female mice were used in our study. Transient MCAO (tMCAO) in eighteen C57bl/6 mice: C57bl/6 mice from Charles river laboratories (Wilmington, MA, USA), kept under standardized light/dark (12 hours) and temperature (25°C) conditions, were anesthetized with isoflurane, and subjected to transient MCAO (tMCAO) for 1 hour as described previously [22]. Briefly, the skull was exposed for monitoring of local cerebral blood flow by Laser-Doppler flowmetry (Moor Instruments, Wilmington, DE, USA) with a probe at 1 mm posterior and 3 mm lateral to bregma. An incision was made in the midline of neck to expose the left carotid bifurcation and the external carotid artery (ECA), the occipital and superior thyroid arteries, and the internal carotid artery and pterygopalatine artery. Then a small incision was made in the ECA, and

a 15 mm monofilament nylon suture with a heat-rounded at the tip (head diameter: 0.2–0.3 mm) (6-0, Harvard Apparatus) was inserted into ECA and was advanced towards the origin of the MCA (about 9mm from the carotid bifurcation) until a reduction in cortical cerebral blood flow (CBF) of the left MCA territory less than 25% of baseline. After one hour of occlusion, the suture was withdrawn to allow reperfusion. Animals were excluded from the experiment if the cerebral blood flow did not recover to at least 70% of baseline by 10 min post reperfusion. The brain was then removed 24 hours, 72 hours, and 1 week after induction of ischemia and coronal brain sections were prepared.

2.2.9 Histology

The mice were perfused with 50 ml of ice-cold 0.9% saline followed by 50 ml of 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Their brains were removed and incubated overnight in 4% paraformaldehyde and then dehydrated in 20%–30% sucrose in PBS. Coronal brain slices (14 mm thick) from the right parietal cortex were sectioned with a frozen microtome (Leica) to produce consecutive frozen sections. For immunofluorescence staining, the sections were boiled in citric acid buffer (pH 6.0) for 5 min in a microwave oven. After the sections were cooled, they were treated with 0.3% Triton X-100 and 10% goat serum for 1 h at room temperature. The sections were then incubated overnight at 4°C with a primary antibody (1:200 anti-CD31 antibody, 1:300 anti-TDP43 antibody, 1:100 anti-TDP-43 (C-terminal) Polyclonal antibody, 1:100 anti-ZO-1 antibody, 1:100 anti-Claudin 5 antibody, 1:200 anti-Phospho-YAP antibody, 1:200 anti-YAP/TAZ antibody), and then incubated for 1 h at room temperature with a secondary antibody (1:500 anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor® 488 Conjugate), catalog number 4412s, cell signaling technology; 1:500 anti-mouse (Alexa Fluor® 555), catalog number 19581S, cell signaling technology) in PBS containing 10% blocking solution. The sections were mounted onto slides, stained with DAPI Solution with Antifade (Sigma-Aldrich), and covered with a coverslip.

2.2.10 Western blot analysis

Cells were scraped and lysed in RIPA lysis buffer on ice after treatment of 1 hours at 4°C for protein Extraction. In addition, NE-PER Nucleus and Cytoplasmic Extraction Reagents (78833, Thermo Scientific, Waltham, MA) to proceed cytoplasmic and nucleus protein extraction according to the manufacturer's instructions. Protein was quantified using a BCA assay kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. The cell lysates were solubilized in sodium dodecyl sulfate (SDS) sample buffer (40µg/lane) and separated by 10% SDS-polyacrylamide gel electrophoresis (110 V for 75 min). After electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 3% bovine serum albumin (BSA) and incubated with primary antibodies overnight at 4°C, followed by a horseradish peroxidase–conjugated secondary antibody for 1 hours at room temperature, and detected with the enhanced chemiluminescence plus detection system (Millipore, Billerica, MA). The density of each band was quantified using Quantity One image-analysis software (Hercules, CA).

3. Data and Statistical Analyses

ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the immunofluorescence results. The significance of differences in data sets was analyzed by two-tailed Student's *t* test or one-way analysis of variance tests. The data are expressed as the mean $\pm$ SD and were analyzed using GraphPad Prism 6 software (GraphPad, La Jolla, CA, USA). The *p* value <0.05 was considered statistically significant.

Results

YAP/TAZ are required for primary brain ECs proliferation and migration

To investigate the effects of YAP/TAZ on primary brain ECs proliferation, migration, we isolated primary brain ECs. As shown in Fig. 1A, the primary brain ECs were cultured in 12-well plate with 2 different seeding densities to achieve low (60–70%) and high (100%) confluence respectively. YAP mainly expression in the nucleus of primary brain ECs, once the cell confluence, YAP nucleus sublocation obviously decreased. Meanwhile, western blot confirmed that the expression of P-YAP, P-TAZ and YAP, TAZ were reduced in confluence cells, compared to sparse status ($p = 0.0023$, YAP; $p < 0.001$, TAZ; $p = 0.002$, P-YAP; $p = 0.001$, TAZ) (Fig. 1B). The result is consistent with the previous study, which showed that cell-cell contact affect YAP expression and subcellular localization in HUVECs[23]. We further silenced the YAP/TAZ expression in primary brain ECs. The results of MTT assay showed that YAP/TAZ silencing significantly reduced cell viability of primary brain ECs compared to negative control ($p < 0.001$) (Fig. 2B). Knockdown of YAP/TAZ in primary brain ECs reduced cell proliferation tested by Ki67 assays ($p < 0.001$) (Fig. 2A) and cell migration by scratch assay of primary brain ECs (Fig. 2D). Alternatively, we construct constitutively nucleus YAP expression plasmid (YAPS112A) further confirmed that overexpression of nucleus YAP promoted primary brain ECs proliferation ($p = 0.03$) (Fig. 3). These data indicate that YAP/TAZ plays an essential role in primary brain ECs proliferation, migration.

Silence of TDP43 inhibits migration of bEnd3 cells and expressions of tight-junction proteins and phosphorylation YAP/TAZ in bEnd3 cells.

To investigate whether TDP43 is an essential gene that regulates tight-junction proteins (claudin-5 and ZO-1) and YAP/TAZ transcriptions in brain ECs, we employed TDP43 siRNA to silence the expression of TDP43 in bEnd3 cells ($p < 0.0001$). The results showed that silence of TDP43 significantly decreased the expressions of tight-junction proteins (claudin-5 and zo-1) ($P < 0.001$, claudin-5; $p = 0.047$, ZO-1) and phosphorylation of YAP (P-YAP) in bEnd3 cells, but no effect of overall YAP/TAZ expression ($p = 0.0014$) (Fig. 4A-E). The wound healing assay indicated that silencing TDP43 remarkable inhibited the wound closed (Fig. 4F-G), compared to control group ($p = 0.0025$). This result indicates that loss function of TDP43 inhibits endothelial cell migration and TJs proteins expression.

Expression of TDP43-CTFS is increased in bEnd3 cells induced by LPS, but it did not change in OGD condition.

We employed LPS or OGD conditions to stimulate bEnd3 cells *in vitro*. The results show that the full-length of TDP43 protein expression has no changes both in OGD ($p = 0.3741$) and LPS stimulation ($p = 0.1691$) (Fig. 5A-C). However, the immunofluorescent staining showed that some of TDP43 was located in cytoplasm of bEnd3 cells after LPS stimulation ($p < 0.0001$), but didn't show the similar phenomenon after OGD treatment ($p = 0.9417$) (Fig. 5D-F). Considering cytoplasmic TDP43 aggregation are C-Terminal TDP-43 Fragments (CTFS)[24, 25], we then detected TDP43-CTFS (TDP43-CTFS) expression in bEnd3 after LPS and OGD, and the western blots indicated that LPS markedly increased expression of TDP43-CTFS protein in bEnd3 ($p < 0.0001$) (Fig. 6A, C), but it had no response to OGD condition ($p = 0.4353$) (Fig. 6A, B). Parallel immunofluorescent staining was performed to detect sub-cellular localization of TDP43-CTFS in bEnd3. As shown in Fig. 6D-F, LPS promoted TDP43-CTFS located in cytoplasm of bEnd3, but not OGD stimulation ($p = 0.9352$, OGD; $p = 0.0001$, LPS).

The changes of full-length of TDP43, TDP43-CTFS, tight junction proteins and P-YAP expression in MCAO mice model.

We adopted MCAO mouse model to further investigate the role of TDP43 in brain ECs. As shown in Fig. 7B, TDP43 co-stained with ECs marker CD31 was obviously upregulated in peri-infarct region (24h, 72h, 1w), but there was no detection of TDP43 co-stained with CD31 located in the corresponding region of the contralateral hemisphere. Moreover, TDP43-CTFS showed a time-dependent dynamic location changes from cytoplasm to nucleus in ECs of MCAO. TDP43-CTFS were located in cytoplasm at 24 hours, then mainly appeared in nucleus at 72 hours, and only located in nucleus at 1 week after MCAO (Fig. 7C). The expressions of tight junction proteins (ZO-1 and claudin-5) were decreased significantly after MCAO 24 hours, then gradual recovered 72 hours and 1 week after MCAO ($p < 0.0001$, claudin-5; $p < 0.0001$; ZO-1) (Fig. 8). Furthermore, as shown in Fig. 9, P-YAP expression also significant decreased after MCAO 24 hours ($p < 0.0001$, Fig. 9A-B), accompanied by nucleus YAP expression. Then P-YAP also followed increased after MCAO 72 hours and 1 week, meanwhile, YAP/TAZ expression located in nucleus after MCAO 72 hours and 1 week. In summary, the changes of ZO-1, claudin-5 and P-YAP expressions in brain ECs after MCAO are associated with the dynamic changes of TDP43-CTFS expression.

Effects of TDP43-CTFS overexpression in tight junction protein expression, migration and proliferation of bEnd3 cells.

The associations of tight junction proteins and P-YAP expression changes with the dynamic changes of TDP43-CTFS expression in MCAO imply that TDP43-CTFS may play some roles in regulating brain endothelial cell functions by interfering with tight junction proteins and YAP. To further investigate TDP43-CTFS effects in brain ECs, we successfully constructed the cytomegalovirus immediate-early enhancer and promoter (CMV) plasmid to overexpressing TDP43-CTFS in bEnd3 cells without affecting TDP43 protein expression level ($p < 0.001$) (Fig. 10A, B). We detected there is significant decrease in the expression of tight junction proteins (claudin-5 and ZO-1) after overexpression of TDP43-CTFS in bEnd3 cells ($p < 0.001$, claudin-5; $p < 0.001$, ZO-1) (Fig. 10C, D, E), which are consistent with the same strategy applied in immunofluorescent staining *in vitro* ($p < 0.001$, claudin-5; $p < 0.001$, ZO-1) (Fig. 10F, G, H, I).

Next, cell viability, EDU proliferation and scratch assays were performed to further evaluate the effects of TDP43-CTFS overexpression in bEnd3 cell functions (Fig. 11A-E). As shown in Fig. 11A, B, overexpression of TDP43-CTFS inhibited bEnd3 cells migration ($p < 0.0001$). Notably, overexpression of TDP43-CTFS had no effect on cell viability ($p = 0.2621$) (Fig. 11C), but promoted the bEnd3 cells proliferation as shown by EDU staining assay ($p < 0.001$) (Fig. 11D, E).

Effect of TDP43-CTFS overexpression on Hippo signaling pathway in bEnd3 cells.

To investigate if effects of TDP43-CTFS in ECs is through Hippo signaling pathway, we overexpressed TDP43-CTFS in bEnd3 cells, and detected expression of YAP and P-YAP, and MST1/2 and P-MST1/2. The Fig. 12A shows that TDP43-CTFS overexpression significantly downregulates P-YAP (Ser127) expression in bEnd3 cells ($p < 0.001$), meanwhile, the western blot confirmed that YAP was transferred from cytoplasm to nucleus ($p < 0.001$, cytoplasmic YAP; $p < 0.001$, nucleus YAP) (Fig. 12B). Moreover, we found that the YAP activation, phosphorylation of YAP, was through inactivation of its upstream modulator, PMST1/2 by dephosphorylation. We found that the core effector of hippo signaling pathway, MST1/2, was remarkable deactivated shown as decrease of P-PMST1/2 in TDP43-CTFS overexpression bEnd3 cells ($p < 0.001$) (Fig. 12C).

Discussion

TDP-43 regulates cellular functions through regulation of RNA splicing, stability, transcriptional repression[13, 26]. Recently, TDP43 has been found playing important roles in amyloid deposition, tau hyperphosphorylation, mitochondrial dysfunction, and neuroinflammation[14, 15], which are pathophysiological mechanisms of neurodegenerative diseases [15, 16, 27]. The studies have showed that neurotoxicity of TDP-43 is associated with aberrant phosphorylation, ubiquitination, cleavage and/or nucleus depletion, and cytoplasmic aggregation of TDP43 in neurons of ALS, AD, FTLD, HD[15, 16]. Dysfunction of TDP43 in neurons leads to neurodegeneration and death, which indicates the significance of TDP43 in pathophysiology of neurodegenerative diseases. TDP43 normally expressed in the nucleus[28]. The N-terminal domain of TDP43 has two highly conserved RNA recognition motifs (RRM1 and RRM2) [29], which regulate RNA transcripts and processing pathways, and also control its own messenger RNA (mRNA) transcription through the binding to the 3' untranslated region (UTR)[25, 30–32]. The striking aspect of TDP43 is the discovery of the loss nucleus function of neurons to induce neurotoxicity in ALS, FTLD, and other neurodegenerative diseases[33]. To date, extensive efforts have been made to study the potential functions of TDP43 in brain diseases on neurotoxicity in neurons. There is no study on the role of TDP43 in regulation of brain ECs and the BBB functions. As an important component of neurovascular unit, BBB plays important roles in maintaining CNS homeostasis and regulating neurological functions. The BBB is comprised by ECs with the TJ proteins through interactions with pericytes, astrocytes, neurons and microglia in the neurovascular unit[34, 35], which as a shield to protect brain away from toxic damages[36]. TJ proteins of brain ECs, including claudins, occludin, and zonula occludens (ZO)s, are the principal structural to regulate the BBB integrity and permeability[37, 38]. Meanwhile, vascular ECs undertake angiogenesis via migration and proliferation

during growth and remodeling or reparative process in vascular networks. In the present study, we performed a series of experiments to study the roles of TDP43 and its cytoplasmic CTFS in regulation of brain ECs functions and elucidate the underlying mechanisms, and investigate the impacts of ischemia, OGD and LPS-induced inflammation on TDP43 and TDP43- CTFS in brain ECs. Based on our knowledge, the present study is the first report on the roles of TDP43 and TDP43- CTFS in brain ECs, and ischemic impact on TDP43 and its CTFS, and elucidate the underlying mechanisms.

Our results show that mouse brain-derived vascular ECs, bEnd3 cells, highly express TDP43 as similar scale as tubulin (Fig. 4A). It implies that TDP43 may be an essential gene contributes to regulate brain endothelial cell normal function. Notably, silencing full length TDP43 expression in bend3 cells down-regulates TJs and P-YAP protein expressions, and inhibits migration of bEnd3 cells (Fig. 4). Furthermore, we detected the dynamic changes of TDP43 expression in mice brain 24 hours (acute phase), 72 hours (subacute phase), 1 week (stable phase) after tMCAO (Fig. 7). As shown in Fig. 7B, TDP43 remarkable upregulated and located in brain vascular ECs in peri-infarct region of each phase, but the similar phenomenon did not be detected in contralateral region of each phase. However, we did not find either OGD or LPS condition induced TDP43 protein upregulation in bEnd3 (Fig. 5A, B, C). Notably, the immunofluorescence results show that TDP43 was detected in cytoplasm of bEnd3 cells after LPS (0.1, 0.5 ug/ml) stimulation (Fig. 5D). The findings inspired us a prevalent view that cytoplasmic aggregation of TDP43 play a key adverse effect in in brain ECs. It has been found that there is loss of TDP43 nucleus function in ALS because of TDP43 aggregations in cytoplasm[39–41]. The major striking aggregation of TDP43 is C-terminal fragments (CTFS) 35, which is particularly prone to sequester TDP-43 from nucleus localization into cytoplasmic inclusions[25, 42]. Then, we rechecked the expression of TDP43-CTFS35 in bEnd3 cells with same approach. Notably, we found that LPS increased TDP43-CTFS35 expression in cytoplasm of the bEnd3 cells in a dose-dependent pattern (Fig. 6A, C, D, F). Furthermore, we confirmed that TDP43-CTFS35 main located in cytoplasm co-staining with vascular ECs marker CD31 at acute phase (24H) in MCAO brain slices, and then TDP43-CTFS35 gradual reduced in cytoplasm, but increased nucleus in brain vascular ECs at sub-acute (72H) and stable (1W) phases (Fig. 7C). Thus, taken together, the TDP43-CTFS35 is increased in mice brain ECs after MCAO as well as in LPS-induced inflammation. The dynamic change of TDP43-CTFS35 expression in brain ECs is associated with the changes in P-YAP expression in brain ECs after MCAO. This association in the changes of TDP43-CTFS35 and P-YAP expressions indicates the potential interaction between them in brain ECs after MCAO. The trigger of TDP43-CTFS35 aggregation in cytoplasm is likely post-ischemic inflammation, although OGD did not have an effect on TDP43-CTFS35 aggregation in cytoplasm (Fig. 6A). Inflammation or cytokines induced by OGD is different from post-ischemic inflammation *in vivo*, in which reactive microglia and astrocyte are involved[43]. This may explain the inconsistency of TDP43-CTFS35 aggregation in OGD and MCAO. In order to investigate the role of TDP43-CTFS35 in brain vascular ECs, we designed the TDP43-CTFS35 (90–414) overexpression plasmid and transfected in the bEnd3 cells (Fig. 10A). We found that TJs protein and P-YAP (Ser127) expressions significantly downregulated in the bEnd3 cells after TDP43-CTFS35 overexpression (Fig. 10B). In addition, CTFS35 overexpression delayed migration of bend3 cells (Fig. 11A). The inhibitory effect of TDP43-CTFS35 on P-YAP is through suppression of MST1/2

phosphorylation, the upstream of the core regulatory protein in the Hippo signaling pathway (Fig. 12C), which regulates YAP phosphorylation. The overexpression of TDP43-CTFS35 leads to reduction of YAP phosphorylation in cytoplasm (Fig. 12A), which impairs cell migration [12]. The increase of cell proliferation by TDP43-CTFS35 over-expression can be explained that de-phosphorylated YAP enters nucleus, then induces transcriptional programs of proliferation and growth[44]. This phenomenon may compensate the injury of the brain ECs by promoting their proliferation.

In conclusion, the present study uncovered that TDP43 full-length and TDP43-CTFS35 regulated brain vascular ECs functions by mediating Hippo-YAP signaling pathway. The mechanism of TDP43-CTFS35 regulation is that full length TDP43 important for maintain normal TJ protein level and migration functions, and TDP43-CTFS35 turns off the Hippo signaling pathway leading to de-phosphorylate YAP, activation of YAP, in brain ECs. Activation of YAP promotes ECs proliferation which may compensate the injury. However, the reduction of TJ proteins and P-YAP expression in brain ECs induced by TDP43-CTFS35 may cause the BBB damage and inhibit brain endothelial cell migration impairing repair after MCAO. Our findings demonstrated that ischemia injury induces cytoplasmic TDP43-CTFS35 aggregation in brain vascular ECs, which causes the brain ECs injury and compromised integrity of the BBB by impairing TJs protein expression and YAP phosphorylation[10–12]. The impairment of brain EC migration induced by cytoplasmic TDP43-CTFS35 aggregation will also delay the angiogenesis and repair of the BBB after ischemia, but the injury of brain ECs may be compensated by promoting their proliferation by TDP43-CTFS35. Nevertheless, the exact roles of TDP43 and TDP43-CTFS35 in brain ECs in pathophysiology of cerebral ischemia is still remain to be fully elucidated.

Abbreviations

BBB: blood-brain barrier; ECs: brain endothelial cells; CNS: central nerve system; OGD: Oxygen-glucose deprivation; LPS: lipopolysaccharides, TJ: tight junction; YAP: YES-associated protein; TDP-43: TAR DNA-binding protein 43; MCAO: middle cerebral artery occlusion; CTFS: C-Terminal TDP-43 Fragments; CMV: cytomegalovirus immediate-early enhancer and promoter; ZO: zonula occludens.

Declarations

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Authors' contributions

The experiments were performed by Xiaotian Xu. The manuscript was written through contributions of all authors. All authors have approved the final version of the manuscript. The authors declare no financial conflict of interest.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committees at University of Houston and they complied with pertinent NIH guidelines for care and use of animals.

Consent for publication

All authors of the manuscript have agreed to publish this article.

Competing interests

The authors have declared that no competing interests exist.

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Figures

Figure 1

The density of primary brain ECs impacted on YAP/TAZ expression. (A) Representative images of primary brain ECs stained with anti-CD31 (red) and anti-YAP antibody (green), (immunofluorescence, $\times 40$, scale bar = 50 μm). (B) Western blot analysis of P-YAP, P-TAZ, YAP, TAZ proteins expression. (C) Quantitative analysis of P-YAP, P-TAZ, YAP, TAZ proteins expression. * $p < 0.05$ versus control of each protein. The data are expressed as means $\pm$ SD from three independent experiments.

Figure 2

Loss function of YAP/TAZ inhibited primary brain ECs migration, proliferation. (A) Ki67 assay proliferation imaging assay of YAP/TAZ siRNA transfection in primary brain ECs. (B) MTT assay after YAP/TAZ siRNA transfection in primary brain ECs. (C) Quantitative analysis of Ki67 assay proliferation. (D) The representative images of wound healing assay. * $p < 0.05$ versus control of each protein. The data are expressed as means $\pm$ SD from three independent experiments.

Figure 3

Constitutively nucleus YAP expression plasmid (YAPS112A) upregulated brain primary endothelial cell proliferation. (A) Ki67 assay proliferation imaging assay of YAPS112A transfection in brain primary endothelial cell. (B) Quantitative analysis of Ki67 assay proliferation. * $p < 0.05$ versus control of each protein. The data are expressed as means $\pm$ SD from three independent experiments.

Figure 4

Loss function of TDP43 decreased the expressions of tight junction proteins and P-YAP of bEnd3 cells line. (A) Western blot analysis of TDP43, ZO-1, claudin-5, P-YAP, YAP/TAZ proteins expression. (B) Quantitative analysis of TDP43 protein expression. (C) Quantitative analysis of claudin-5 protein expression. (D) Quantitative analysis of P-YAP protein expression. (E) Quantitative analysis of ZO-1 protein expression. (F) After transfection with TDP43 siRNA for 24 h, bEnd3 cells were subjected to

wound healing assays and images were taken at 0 and 24 h (×4, scale bar = 50 μm). (G) Quantification analysis of the invaded area 24 hours after the scratch demonstrated that knocking down of TDP43 in bEnd3. *p < 0.05 versus control of each protein. The data are expressed as means ± SD from three independent experiments.

Figure 5

The bEnd3 cells were incubated with OGD or LPS (0.1, 0.5 ug/ml) for 24h to examine TDP43 protein expression. (A) Western blot analysis of TDP43 protein expression. (B) Quantitative analysis of TDP43 protein expression after LPS treatment. (C) Quantitative analysis of TDP43 protein expression after OGD treatment. (D) the bEnd3 cells were subjected to immunofluorescent staining by anti-CD31 (green), anti-TDP43(red), and DAPI (blue) (immunofluorescence, $\times 40$, scale bar = 50 μm). Yellow triangle noted that the TDP43 expression detected in cytoplasm of bEnd3 cells. (E) The mean intensity of TDP43 after OGD treatment. (F) The mean intensity of TDP43 after LPS (0.1, 0.5 ug/ml) treatment. The data are expressed as means $\pm$ SD from three independent experiments. * $p < 0.05$ versus control.

Figure 6

The bEnd3 cells were incubated with OGD or LPS (0.1, 0.5 ug/ml) for 24h to examine TDP43-CTFS protein expression. (A) Western blot analysis of TDP43-CTFS protein expression. (B) Quantitative analysis of TDP43-CTFS protein expression after OGD treatment. (C) Quantitative analysis of TDP43-CTFS protein expression after LPS treatment. (D) bEnd3 cells were subjected to immunofluorescent staining by anti-CD31 (green), anti- TDP43-CTFS (red), and DAPI (blue) (immunofluorescence, $\times 40$, scale

bar = 50 μ m). Yellow triangle noted that the TDP43-CTFS expression detected in cytoplasm of bEnd3 cells. (E) The mean intensity of TDP43-CTFS after OGD treatment. (F) The mean intensity of TDP43-CTFS after LPS (0.1, 0.5 ug/ml) treatment. The data are expressed as means $\pm$ SD from three independent experiments. *p < 0.05 versus control.

Figure 7

TDP43 expression in endothelial cell of MCAO mouse model. The mice subjected to MCAO 60 mins and collected the brain samples in 24 hours, 72 hours, and 1week separately. (A) Brain samples collected and prepared schema. (B) Representative images of MCAO brain slices stained with anti-CD31 (green) and anti-TDP43 antibody (red), contralateral region as negative control (immunofluorescence, $\times 40$, scale bar = 50 μm). Yellow triangle noted that the TDP43 expression detected in the nucleus of endothelial cell. (C) Representative images of MCAO brain slices stained with anti-CD31 (green) and anti-TDP43 antibody (red), contralateral region as negative control (immunofluorescence, $\times 40$, scale bar = 50 μm). Yellow triangle noted that the TDP43-CTFS expression detected in the nucleus of endothelial cell. White arrow noted that the TDP43-CTFS expression detected in the cytoplasm of endothelial cell.

Figure 8

Tight junction proteins (ZO-1 and claudin-5) levels were measured in endothelial cell of MCAO mouse model (24 hours, 72 hours, and 1week). (A) Representative images of MCAO brain slices stained with anti-CD31 (green) and anti-claudin-5 antibody (red). (B) The mean intensity of claudin-5 in peri-infarct region of MCAO mouse model (24 hours, 72 hours, and 1week). (C) Representative images of MCAO brain slices stained with anti-CD31 (green) and anti-ZO-1 antibody (red). (D) The mean intensity of ZO-1 in peri-

infract region of MCAO mouse model (24 hours, 72 hours, and 1 week). contralateral region as negative control (immunofluorescence, $\times 20$, scale bar = 50 μm).

Figure 9

Hippo signaling pathway changes in MCAO brain slices (24h, 72h, 1w). (A) Representative images of MCAO brain slices stained with anti-CD31 (green) and anti-P-YAP antibody (red). (B) The mean intensity of P-YAP in peri-infract region of MCAO mouse model (24 hours, 72 hours, and 1 week). (C) Representative

images of MCAO brain slices stained with anti-CD31 (green) and anti-YAP/TAZ antibody (red). The data are expressed as means $\pm$ SD from three independent *p < 0.05 versus control of each protein. contralateral region as negative control (immunofluorescence, $\times 20$, scale bar = 50 μ m).

Figure 10

TDP43-CTFS overexpression plasmid transfected in bEnd3, then TDP43-CTFS and tight junction proteins (ZO-1 and claudin-5) protein levels were measured. (A) Western blot analysis of TDP43-CTFS protein

expression. (B) Quantitative analysis of TDP43-CTFS protein expression. (C) Western blot analysis of ZO-1 and claudin-5 protein expressions. (D) Quantitative analysis of claudin-5 protein expression. (E) Quantitative analysis of ZO-1 protein expression. (F) bEnd3 cells were subjected to immunofluorescent staining by anti-CD31 (green), anti-claudin-5 (red), and DAPI (blue). (G) bEnd3 cells were subjected to immunofluorescent staining by anti-CD31 (green), anti-ZO-1 (red), and DAPI (blue). (H) The mean intensity of claudin-5 after TDP43-CTFS overexpression in bEnd3. (I) The mean intensity of ZO-1 after TDP43-CTFS overexpression in bEnd3. (immunofluorescence, $\times 20$, scale bar = 50 μm). The data are expressed as means $\pm$ SD from three independent $*p < 0.05$ versus control of each protein.

Figure 11

After transfection with TDP43-CTFS O.E plasmid for 24 h, cells were subjected to wound healing assays and images were taken at 0 and 24 h ($\times 4$, scale bar = 50 μm). (A) The Representative images of wound healing assay. (B) Quantification analysis of the invaded area 24 hours after the scratch demonstrated that overexpression of TDP43-CTFS in bEnd3. (C) Cell viability of TDP43-CTFS O.E plasmid transfected in bEnd3. (D) Edu proliferation imaging assay of TDP43-CTFS O.E plasmid transfected in bEnd3. (E)

Quantitative analysis of EDU positive cells after TDP43-CTFS O.E plasmid transfected in bEnd3. *p < 0.05 versus control of each protein. The data are expressed as means $\pm$ SD from three independent experiments.

Figure 12

Hippo signaling pathway changes after transfection with TDP43-CTFS O.E plasmid in bEnd3. (A) Western blot analysis of P-YAP, YAP/TAZ protein expressions. (B) Western blot analysis of cytoplasmic YAP,

nucleus YAP protein expressions. (C) Western blot analysis of P-MST1/2, MST1 protein expressions. (D) Quantitative analysis of P-YAP protein expression. (E) Quantitative analysis of cytoplasmic YAP protein expression. (F) Quantitative analysis of nucleus YAP protein expression. (G) Quantitative analysis of P-MST1/2 protein expression. The data are expressed as means $\pm$ SD from three independent *p < 0.05 versus control of each protein.

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