Original Article

TAK-242 protects against oxygen-glucose deprivation and reoxygenation-induced injury in brain microvascular endothelial cells and alters the expression pattern of lncRNAs

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Background: Deep hypothermic circulatory arrest (DHCA) is a technique used during the surgical treatment of aneurysms of the thoracic aorta in adult patients, and complex congenital heart disease in neonates. And brain microvascular endothelial cells (BMECs) are essential components of the cerebrovascular network and participate in maintaining the blood-brain barrier (BBB) and brain function. In our previous study, we found that oxygen-glucose deprivation and reoxygenation (OGD/R) activated Toll-like receptor 4 (TLR4) signaling in BMECs, and induced pyroptosis and inflammation. In this study, we further investigated the potential mechanism of ethyl(6R)-6-[N-(2-Chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242) on BMECs under OGD/R, as in patients with sepsis, the TAK-242 was tested in clinical trials.

Methods: To confirm the function of TAK-242 on BMECs under OGD/R, cell viability, inflammatory factors, inflammation-associated pyroptosis, and nuclear factor-κB (NF-κB) signaling were determined using Cell Counting Kit-8 (CCK-8) assay, enzyme-linked immunosorbent assay (ELISA), and western blotting, respectively. To investigate the lncRNAs associated with TLR4 during OGD/R, long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) expression patterns were profiled with RNA deep sequencing. Moreover, to confirm whether lncRNA-encoded short peptides, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used.

Results: Relative control group, OGD/R inhibited the cell viability, increased the section of inflammatory factors secretion, including IL-1β, IL-6, and TNF-α, and promoted the pathways of TLR4/NLRP3/Caspase-1 and TLR4/NF-κB. However, TAK-242 + OGD/R group promoted OGD/R cell viability, decreased OGD/R-induced inflammatory factors secretion, and inhibited the pathways of TLR4/NLRP3/Caspase-1 and TLR4/NF-κB. In addition, AABR07000411.1, AABR070006957.1, and AABR070008256.1 were decreased in OGD/R cells compared with controls, but TAK-242 restored their expression under OGD/R condition. AABR07000473.1, AC130862.4, and LOC10254972.6 were induced by OGD/R, but were suppressed in TAK-242 + OGD/R cells compared with OGD/R. Moreover, AABR07049961.1, AC127076.2, AABR07066020.1, and AABR07025303.1-encoded short peptides were dysregulated in OGD/R cells, and TAK-242 attenuated the dysregulation of AABR07049961.1, AC127076.2, and AABR07066020.1-encoded short peptides.

Conclusions: TAK-242 alters the expression pattern of lncRNAs in OGD/R cells, and differently expressed lncRNAs may exert a protective effect against OGD/R injury through a mechanism of competing endogenous RNA (ceRNA) and encoding short peptides. These findings maybe provide a new theory basis for the treatment of DHCA.

Keywords: Oxygen-glucose deprivation and reoxygenation (OGD/R); TAK-242; TLR4/NLRP3/Caspase-1 axis; TLR4/NF-κB axis; long non-coding RNAs (lncRNAs)

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Introduction

Deep hypothermic circulatory arrest (DHCA) is a technique used during the surgical treatment of aneurysms of the thoracic aorta in adult patients, and complex congenital heart disease in neonates (1,2). It involves cooling the patient's core body temperature and stopping blood circulation (3). The cessation of bold flow provides an optimal visual field during surgery, and deep hypothermia protects cerebral and other vital organs from ischemia (3). Although the hypothermic state limits cerebral damage by lowering the global cerebral metabolism and oxygen consumption, DHCA-induced hypoxic-ischemic insult is still the leading cause of neuronal injury after surgery involving DHCA (3). Ischemia and hypoxia results in the excessive and hyperactivity of neuronal cells, which increase the expression of genes that promote oxidation and the caspase family genes to induce the apoptosis, and the adenosine triphosphate deletion leads neuronal cells necrosis (4). Therefore, efforts to develop effective adjunctive neuroprotective therapies are necessary.

Brain microvascular endothelial cells (BMECs) are essential components of the cerebrovascular network and participate in maintaining the blood-brain barrier (BBB) and brain function (5). They are highly responsive and sensitive to oxygen deprivation (6). Cerebral ischemia causes BMECs degeneration and dysfunction, resulting in degradation of endothelial tight junction and increased BBB permeability and the injury of neuronal cells (7,8). Moreover, BMECs have been shown to release various vasoactive and proinflammatory/inflammatory factors in response to ischemia. These factors induce platelet and neutrophil activation and adhesion, and enhance leukocyte adhesion and transmigration into the brain, resulting in the exacerbation of ischemic damage (9). Additionally, protection the BMECs contributed to reduced neuronal apoptosis (10,11). The important roles of BMECs in brain ischemic injury highlight the potential of BMECs-targeted therapy as an adjunctive neuroprotective therapy during DHCA.

In our previous studies, oxygen-glucose deprivation and reoxygenation (OGD/R)-treated rat primary BMECs were used to mimic the BMECs exhibiting from hypoxia-ischemia after DHCA, and we found that OGD/R treatment stimulated Toll-like receptor 4 (TLR4) protein expression, caspase-1-mediated pyroptosis, and the release of pro-inflammatory factors (12,13). TLR4, belonging to the family of pattern recognition receptors, plays an important role in innate and inflammatory responses (14). It can recognize pathogen-associated molecular patterns and damage-associated molecular patterns, and activate the production of pro-inflammatory nuclear factor κB (NF-κB) and subsequent inflammatory cytokines (15). A previous study found that TLR4/NF-κB inflammatory pathway maybe the signal pathway for treating epilepsy (16). Accumulating studies suggest that TLR4 plays a key role in

Highlight box

Key findings
- TAK-242 alleviated OGD/R-induced injury in BMECs by altering expression of lncRNAs.

What is known and what is new?
- Previous study showed that OGD/R activated TLR4 signaling and induced pyroptosis and inflammation in BMECs.
- TAK-242 changed the expression pattern of lncRNAs in OGD/R cells and suppressed OGD-R-mediated NF-κB and NLRP3/Caspase-1 pathway activation in BMECs.

What is the implication, and what should change now?
- TAK-242 regulated AABR07049961.1, AC127076.2, AABR07066020.1, and AABR07025303.1-encoded short peptides during OGD/R.

cell death and inflammation injury in the brain following ischemia-reperfusion, and suppression of TLR4 could remarkably improve cerebral ischemia injury in animal models (17,18). Additionally, TLR4 affected many other pathways, such as MyD88/mTOR pathway, TRIF/TBK1/IRF3 pathway, and MAPK pathway etc. Therefore, TLR4 play an important role in different diseases (19-21).

Long non-coding RNAs (lncRNAs) are defined as RNA transcripts >200 nt in length that are not translated into functional proteins (22). They participate in multiple biological processes and exhibit various regulatory functions through epigenetic modification, transcriptional, chromatin remodeling, and mRNA integrity, and post-translational modification to regulate the mRNA expression or protein expression, which expression changes affected the downstream signaling networks (22). LncRNAs play dual role in cancer, for example, lncRNA-PART1 inhibits the progression of glioma, but promotes the lung cancer development (23). Recently, TLR4 has been shown to regulate inflammation via lncRNAs (24). LincRNA-EPS has emerged as a transcriptional brake to restrain inflammation (25). Research has shown that TLR4 suppresses lincRNA-EPS expression, which increases the chromatin accessibility at the promoters of immune response genes (IRGs), enhances IRGs expression, and promotes inflammation (25). Another study showed that TLR4 regulates lncRNA-F630028O10Rik in the secondary phase of spinal cord injury (SCI) mice (24). The study showed that lncRNA-F630028O10Rik was induced via the TLR4/MyD88/STAT1 signal complex, and that lncRNA functioned as a competitive endogenous RNA (ceRNA) for the miR-1231-5p/Col1a1 axis and enhanced microglial pyroptosis after SCI by activating the PI3K/AKT pathway (24). Therefore, we hypothesized that some lncRNAs were associated with pyroptosis and inflammation in OGD/R BMECs, and that TLR4 participated in these lncRNAs expression.

MicroRNAs (miRNAs) are small non-coding RNAs of around 18–24 nt in length. They regulate gene expression post-transcriptionally by binding to complementary sequences in the target messenger RNAs (mRNAs) (26). For example, the miR-200a-3p expression was promoted in oxygen-glucose deprivation-induced apoptosis of SHSY5Y, and inhibited the expression of miR-200a-3p promoted its' target gene corin expression to promote the cell proliferation in oxygen-glucose deprivation treated SHSY5Y (27). Several lncRNAs have been found to harbor miRNAs binding sites; these lncRNAs can function as ceRNA to sponge miRNAs and regulate gene expression by sequestering miRNA from their targets (26,28). Moreover, several lncRNAs have been confirmed to have small open reading frames <300 nt in length, and could code for short peptides with biological functions (29,30). Emerging evidence indicates that some lncRNAs may have dual functions, RNA, and peptides (29,31). Thus, we speculated that several lncRNAs involved in OGD/R injury may function as ceRNAs or encode peptides.

Ethyl(6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242) is a small-molecule inhibitor of TLR4 which selectively binds to TLR4 and interferes with interactions between TLR4 and its adaptor molecules, blocking the signaling transduction and downstream signals activation (32). In the preclinical animal models, TAK-242 play an effective drug for the ischaemia-reperfusion injury in myocardial and liver (33,34). And a previous study showed in patients with sepsis, the TAK-242 was tested in clinical trials; however, due to the cost-effectiveness, its development has been terminated (35). Another study indicated that TAK-242 maybe a novel drug for the treatment of IR when combined with a nanotechnology-based drug delivery system (33).

In this study, we evaluated the role of TLR4 signaling in OGD/R-induced injury in BMECs by shutting off the signaling with TAK-242, and profiled the expression patterns of lncRNAs and mRNAs in BMECs (control group), OGD/R BMECs (OGD/R group), and TAK-242-treated OGD/R BMECs (TAK-242+OGD/R group) by next-generation sequencing. Further, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to detect and characterize the lncRNA-encoded peptides. These results maybe provide a new theory basis for the treatment of DHCA. We present this article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-360/rc).

Methods

Preparation and cultivation of primary rat BMECs

Rat primary BMECs were isolated and cultured as previously described (12,13). Briefly, BMECs were isolated from 6-week-old rats with a Percoll (Pharmacia, Uppsala, Sweden) gradient, and cultured in collagen type IV/fibronectin-coated 35-mm plastic dishes with Dulbecco's modified Eagle's medium (DMEM) low-glucose (Gibco, Grand Island, NY, USA) at 37 ℃ and 5% CO_2.

The rats were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All animal experimental procedures complied with local and international guidelines for the care and use of animals and were approved by the Animal Ethics Welfare Committee of First Affiliated Hospital of Gannan Medical University (approval No. GFAC-AEWC-121).

Cell treatment

BMECs were divided into 3 groups: the control, OGD/R BMECs, and TAK-242+OGD/R groups. In the control group, cells were cultured in a growth culture medium under normoxic conditions. In the OGD/R group, the cells were rinsed twice with phosphate-buffered saline (PBS), and then cultured with glucose-free DMEM in a hypoxia chamber for 4 hours. Thereafter, an equal volume of DMEM high-glucose (Gibco) containing 10% fetal bovine serum (FBS; Gibco) was added to the cells, and culturing continued for 12 hours under normoxic conditions. In the TAK-242 + OGD/R groups, the cells were exposed to glucose-free DMEM and hypoxia condition for 4 hours. Subsequently, an equal volume of DMEM high-glucose medium containing 10% FBS and TAK-242 was added to the wells, and the cell was further cultured for 12 hours under normoxic conditions. The TAK-242 working concentration was 30 μM, same as previous study (36).

Cell viability assay

The cells were seeded in triplicate, allowed to adhere overnight, and then subjected to OGD/R or TAK-242 + OGD/R treatment. To detect cell viability, Cell Counting Kit-8 (CCK-8) assays were performed using a CCK-8 Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay

To detect the inflammatory factors secreted by BMECs, the cell supernatants of each group were collected after OGD/R treatment. The concentrations of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) were measured using the enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, Wuhan, China) according to the manufacturer's instructions. The assays were performed in triplicate.

Western blot assay

The proteins were extracted from the cells with radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology), and the concentration was measured using a bicinchoninic acid (BCA) kit (Beyotime Biotechnology). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins and the cells were then transferred to the polyvinylidene fluoride membranes (PVDF; Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour and incubated with TLR-4 (1:500, Beyotime Biotechnology), NLRP3 (1:500, Beyotime Biotechnology), p-p65 (1:500, Beyotime Biotechnology), Caspase-1 (1:500, Beyotime Biotechnology), or GAPDH (1:500, Beyotime Biotechnology) primary antibodies at 4 ℃ overnight. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:500, Beyotime Biotechnology) at room temperature for 1 hour, and then the bounded secondary antibody was visualized using chemiluminescence (Beyotime Biotechnology).

High-throughput sequencing and bioinformatics analysis

Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The RNA integrity was evaluated using denaturing agarose gel electrophoresis. Subsequently, the RNA samples were sent to Novogen company (Beijing, China) for library construction, and then subjected to RNA deep sequencing on HiSeq3000 (Illumina, San Diego, CA, USA). Fold change >2 and adjusted P value <0.05 were used as thresholds to select the differentially expressed genes. The ceRNA networks were plotted using Cytoscape software V.3.8.2 (https://cytoscape.org/). To explore the associated biological pathways of the differentially expressed genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted using the online platform of Database for Annotation, Visualization and Integrated Discovery (DAVID).

Quantitative reverse transcription polymerase chain reaction

The lncRNAs expression in the cells was validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The total RNA was extracted from cells

Table 1 Primer sequences in this study

Primer name	Sequence (5'-3')
AABR07000411.1-F	ACTGGGCACAGATGAGAACG
AABR07000411.1-R	CTGGTTGCTGACAAAGCACC
AABR07006957.1-F	AAACCACACCGAGGCAAAGT
AABR07006957.1-R	GGGCTCAACTCTGAGCACTG
AABR07008256.1-F	ATAGAACAGTGGCCTGGTGC
AABR07008256.1-R	GTCTGTTAGGTAGCGGAGCC
AABR07069473.1-F	CGCCCAGTAGCCATAGAGTG
AABR07069473.1-R	TCCAGCCTAAATCCAAGCCG
AC130862.4-F	CGGAGTCCAGAGAATTGGGG
AC130862.4-R	CCTTCCCTGCTTCTCCGTTT
LOC102549726-F	ATGGACTCTGGGGGACAAGA
LOC102549726-R	CTTCCGGAGTCCACAGACG
GAPDH-F	GAGTCAACGGATTTGGTCGT
GAPDH-R	GACAAGCTTCCCGTTCTCAG

and reverse-transcribed into cDNA with PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan). The qRT-PCR was performed using ChamQTM SYBR Color qPCR Master Mix (Vazyme, Jiangsu, China) following the manufacturer's protocols. GAPDH was used as an internal reference and relative expression levels of the lncRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in *Table 1*.

LC-MS/MS

The total proteins from control, OGD/R, and TAK-242+OGD/R BMECs were subjected to trypsin digestion, and Fitgene Biotech Co., Ltd. (Guangzhou, China) for LC-MC/MS analysis. Briefly, the proteins were separated by SDS-PAGE. The target bands were harvested and subjected to in-gel trypsin digestion and the digested peptides were extracted using C18 Zip Tip (Millipore, Burlington, MA, USA) and dissolved in 0.1% formic acid and 2% acetonitrile. The resulting peptides were loaded on an Acclaim PepMap RSLC C18 column (300 μm × 5 mm, 5 μm, 100 Å, Thermo Fisher, Waltham, MA, USA, 160454) and separated on an Acclaim PepMap C18 column (75 μm × 150 mm, 3 μm, 100 Å, Thermo, 160321) with a linear gradient (5–90% B for 50 min and 90% B for 5 min, A: 0.1% formic acid, B: 0.1% formic acid and 80% acetonitrile) at a flow rate of 300 nL/min. The separated peptides were online detected in QExative mass spectrometer (Thermo). The raw data were converted into MGF files using MM file conversion. Protein identification was performed using ProteinPilot™ 4.5 (AB Sciex, Framingham, MA, USA) and compared against the putative lncRNA-encoded proteins and UniProt protein database (https://www.uniprot.org/).

To quantify protein, parallel reaction monitoring (PRM) analyses were performed using a QExactive mass spectrometer (Thermo). The PRM method employed an orbitrap resolution of 17,500, a target automatic gain control value of 5e4, and maximum fill times of 120 ms. The PRM proteomics data were analyzed with Skyline (MacCoss, Seattle, WA, USA).

Statistical analysis

All experiments were replicated at least 3 times. Data were represented as the mean ± standard deviation of these replicates. All statistical analyses were performed using SPSS version 19.0 (IBM Corp., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to determine the statistically significant differences among multiple groups. Statistical significance was considered when $P<0.05$.

Results

TAK-242 alleviated OGD/R-induced injury in BMECs

Given that our previous study suggested that the TLR4 pathway was activated in OGD/R-impaired BMECs (12), the BMECs were exposed to TAK-242, shutting off the TLR4 signaling, during OGD/R just before reoxygenation. As shown in *Figure 1A*, OGD/R impaired the cell viability of BMECs as expected, and TAK-242 significantly promoted the cell viability of OGD/R cells. Since the TLR4 pathway is well known to regulate the expression of multiple proinflammatory and inflammatory factors, the impact of TAK-242 on OGD/R-induced IL-1β, IL-6, and TNF-α were evaluated. The results of ELISA suggested that TAK-242 suppressed IL-1β, IL-6, and TNF-α inductions in OGD/R cells (*Figure 1B-1D*).

In our previous study (12), OGD/R was demonstrated to stimulate TLR4/NLRP3/Caspase-1 axis and induce pyroptosis in BMECs. To explore the effect of TAK-242 on Caspase-1-dependent pyroptosis, western blot was performed. As shown in *Figure 1E*, NLRP3 protein and Caspase-1 cleavage in OGD/R cells were remarkably

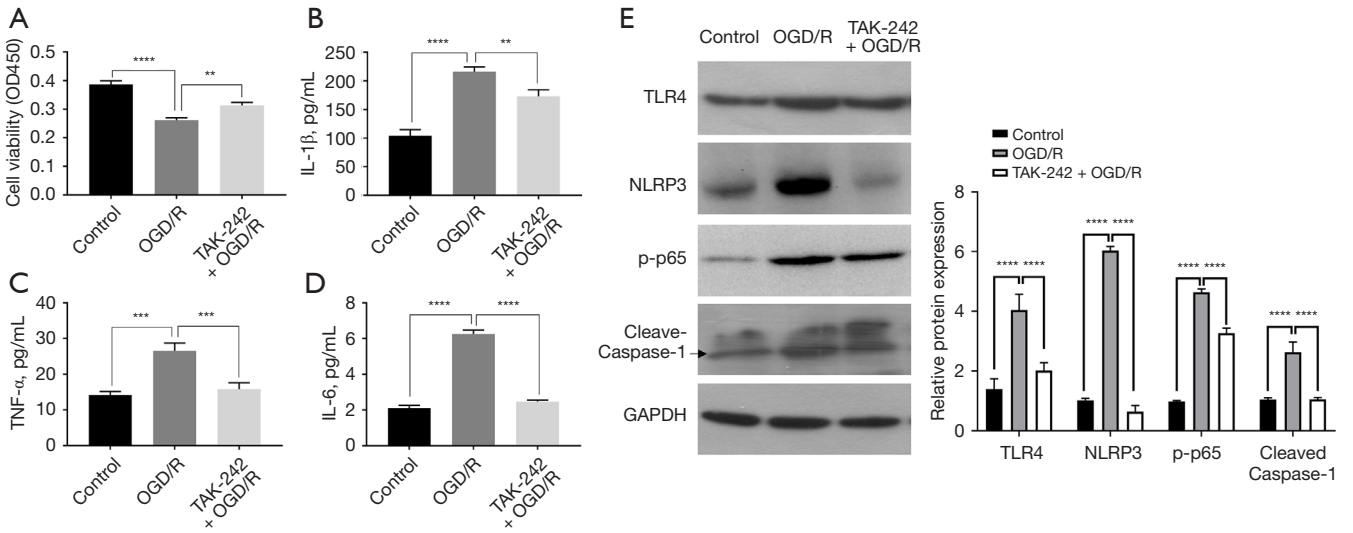

Figure 1 TAK-242 exerted protective effect against OGD/R in BMECs. To mimic ischemic insults during DHCA, BMECs were exposed to OGD/R, the cells cultured at normoxic conditions were employed as controls. TAK-242 was used to block TLR4 signaling, after oxygen-glucose deprivation, BMECs were treated with TAK-242 prior to reoxygenation. Subsequently, cell viability was determined using a CCK-8 assay (A), the IL-1β, TNF-α, and IL-6 in the supernatant were evaluated with an ELISA kit (B-D), and the TLR4, NLRP3, p-p65, and Caspase-1 protein levels were determined with western blot (E). Black arrow indicated the band of cleave Caspase-1. **, P value less than 0.01; ***, P value less than 0.001; ****, P value less than 0.0001. OD, absorbance; OGD/R, oxygen-glucose deprivation and reoxygenation; DHCA, deep hypothermic circulatory arrest; BMECs, brain microvascular endothelial cells; TLR4, Toll-like receptor 4; CCK-8, Cell Counting Kit-8; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; ELISA, enzyme-linked immunosorbent assay; NLRP3, NLR family pyrin domain containing 3.

decreased by TAK-242, implying that TAK-242 could alleviate OGD/R-induced Caspase-1-dependent pyroptosis. NF-κB is an important downstream gene of TLR4 signaling and a vital transcription factor that controls the transcription of a variety of pro-inflammatory genes, including IL-1β, IL-6, and TNF-α (37). Our results showed that the phosphorylation of p65 (p-p65) in BMECs was increased after OGD/R treatment, whereas p-p65 in TAK-242+OGD/R was lower than OGD/R cells, indicating that TAK-242 suppressed OGD-R-mediated NF-κB activation (*Figure 1E*).

TAK-242 altered the lncRNAs expression pattern in OGD/R BMECs

Besides controlling the protein-mediated signaling transduction, TLR4 also regulates lncRNAs expression, contributing to the immune and inflammation responses (24). To investigate the lncRNAs associated with TLR4 during OGD/R, the RNA from the cells was subjected to RNA deep sequencing. The lncRNAs 612, 507, and 628 were respectively identified in control, OGD/R, and TAK-242+OGD/R BMECs, and 413 lncRNAs were commonly expressed in these 3 groups cells (*Figure 2A*). These commonly expressed lncRNAs were distributed on all of the chromosomes, with chromosome 1 having the most expressed lncRNAs and chromosome 11 having the least expressed lncRNAs (*Figure 2B*). The length of the lncRNAs was mainly ranged from 200 to 1000 bp (*Figure 2C*). The differential expression analysis suggested 18 lncRNAs were increased in OGD/R cells (*vs.* control cells) but decreased in TAK-242+OGD/R cells (*vs.* OGD/R), and 20 lncRNAs were suppressed in OGD/R cells (*vs.* control cells) but elevated in TAK-242+OGD/R cells (*vs.* OGD/R) (*Figure 2D*). These 38 differently expressed lncRNAs are listed in Table S1. The top 3 increased OGD/R-impaired lncRNAs (AABR07000411.1, AABR070006957.1 and AABR070008256.1) and top 3 decreased OGD/R-induced lncRNAs (AABR07000473.1, AC130862.4 and LOC10254972.6) in TAK-242+OGD/R BMECs were selected and validated using qRT-PCR. Our results confirmed that these 6 lncRNAs were significantly differentially expressed among the groups, and the

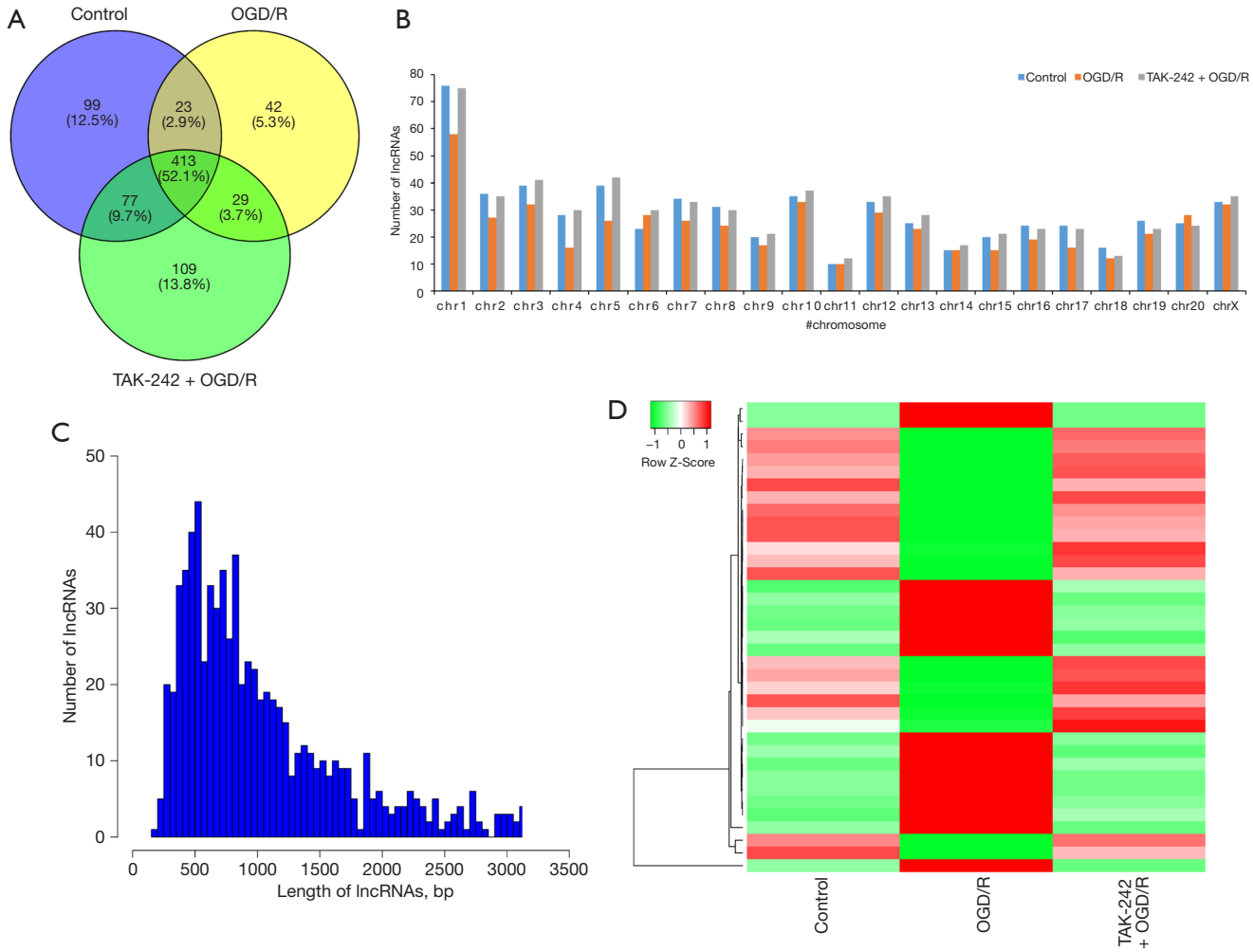

Figure 2 The lncRNAs expression patterns in control, OGD/R, and TAK-242+OGD/R cells. (A) Venn diagram representing the identified lncRNAs in control, OGD/R, and TAK-242+OGD/R cells. (B) Chromosomal distribution of the commonly expressed lncRNAs. (C) Length distribution of the commonly expressed lncRNAs. (D) Heat map showing the differently expressed lncRNAs. The color bar depicts the color contrast level of the heat map. Red and green indicate high and low expression levels, respectively. lncRNAs, long non-coding RNAs; OGD/R, oxygen-glucose deprivation and reoxygenation.

expression changes were consistent with RNA sequencing data (*Figure 3*). These results indicated that TAK-242 could restore some OGD/R-impaired lncRNAs and inhibit some OGD/R-induced lncRNAs, implying that TLR4 may regulate the expression of these lncRNAs and that these lncRNAs may be involved in OGD/R injury.

TAK-242 altered the mRNAs expression pattern in OGD/R BMECs

Besides lncRNAs, the mRNAs expression pattern of each group was profiled. Our results suggested that 1,336 genes had opposite expression trends in OGD/R (*vs.* control) and TAK-242+OGD/R (*vs.* OGD/R). A total of 632 genes were increased in the OGD/R group versus control group but decreased in the TAK-242+OGD/R group versus OGD/R group; 704 genes were decreased in the OGD/R group versus control group but increased in the TAK-242+OGD/R group versus OGD/R group (*Figure 4A* and available online: https://cdn.amegroups.cn/static/public/10.21037jtd-23-360-1.pdf). The KEGG pathway analysis indicated that these differently expressed genes were significantly enriched in the pathways linked to inflammatory response, cell fate, and cell proliferation,

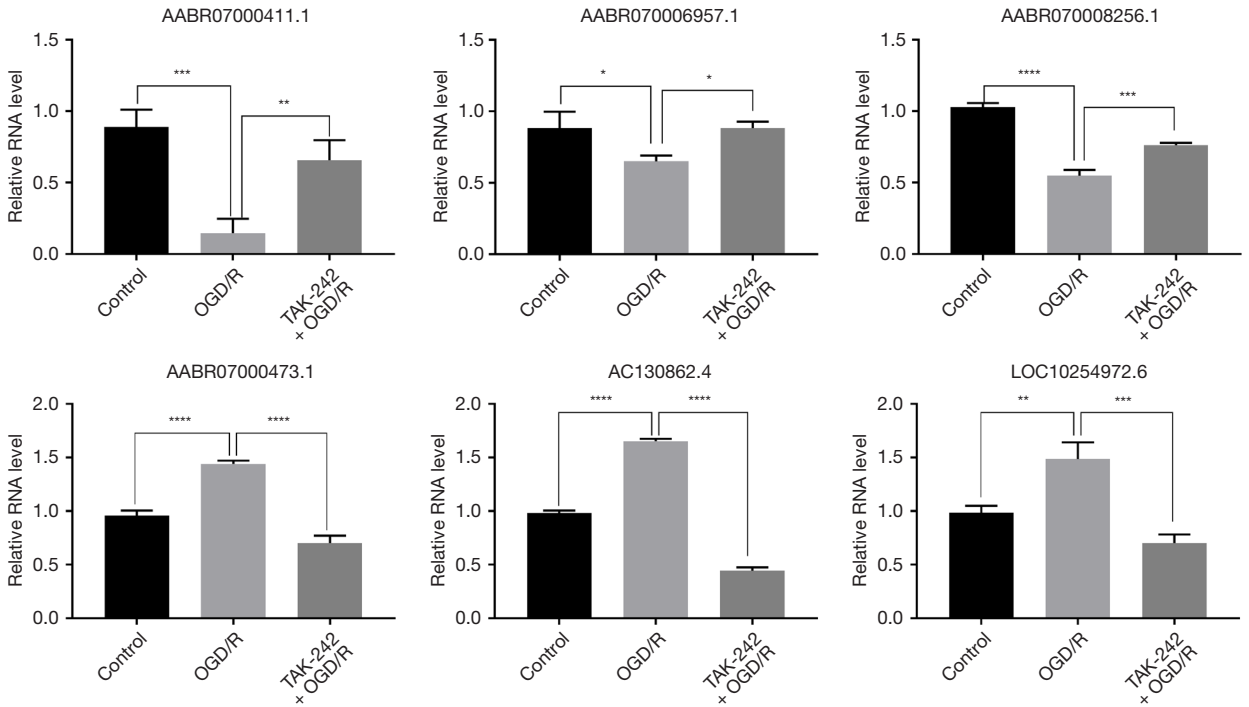

Figure 3 qRT-PCR validation of differently expressed lncRNAs. The expressions of AABR07000411.1, AABR070006957.1, AABR070008256.1, AABR07000473.1, AC130862.4, and LOC10254972.6 in control, OGD/R, and TAK-242+OGD/R cells were detected by qRT-PCR. *, P value less than 0.05; **, P value less than 0.01; ***, P value less than 0.001; ****, P value less than 0.0001. qRT-PCR, quantitative reverse transcription polymerase chain reaction; OGD/R, oxygen-glucose deprivation and reoxygenation.

including NOD-like receptor, Wnt, p53, TNF, and the MAPK signaling pathway (*Figure 4B*).

ceRNAs regulatory network

Some lncRNAs have miRNAs binding sites and function as ceRNAs to regulate gene expression. Thus, we analyzed the correlation between the differentially expressed mRNAs and lncRNAs and predicted miRNAs target sites in mRNAs and lncRNAs, in differentially expressed mRNAs. Only those that were involved in inflammatory response, cell fate, and cell proliferation, including NOD-like receptor, Wnt, p65, TNF, and MAPK signaling pathway, were included. Next, a lncRNAs-miRNAs-mRNAs interaction network was constructed (*Figure 5*). This ceRNA network was composed of 15 mRNAs nodes, 107 miRNAs nodes, and 28 lncRNAs nodes. Notably, AABR07000411.1 interacted with 29 miRNAs and may regulate the expression of 11 genes by ceRNAs mechanism, implying the great role of this lncRNA in OGD/R injury.

Identification of lncRNA-encoded small peptides

As some functional lncRNA-encoded small peptides have been found by several studies (29,30), the protein-coding potential of differently expressed lncRNAs was assessed. The results of LM-MS identified the 133 peptide fragments derived from 59 putative lncRNA-encoded proteins (available online: https://cdn.amegroups.cn/static/public/10.21037jtd-23-360-2.xlsx). And after analysis by Skyline software, 71 peptide fragments derived from 53 putative lncRNA-encoded proteins were further subjected to RPM assay to evaluate proteins expression levels in control, OGD/R, and TAK-242+OGD/R cells (available online: https://cdn.amegroups.cn/static/public/10.21037jtd-23-360-3.xls). The results showed that 12 lncRNA-encoded proteins were confirmed, and 4 of them (AABR07049961.1, AABR07025303.1, AC127076.2, AABR07066020.1) were commonly expressed in control, OGD/R, and TAK-242+OGD/R cells (*Figure 6* and Table S2). AABR07049961.1-encoded

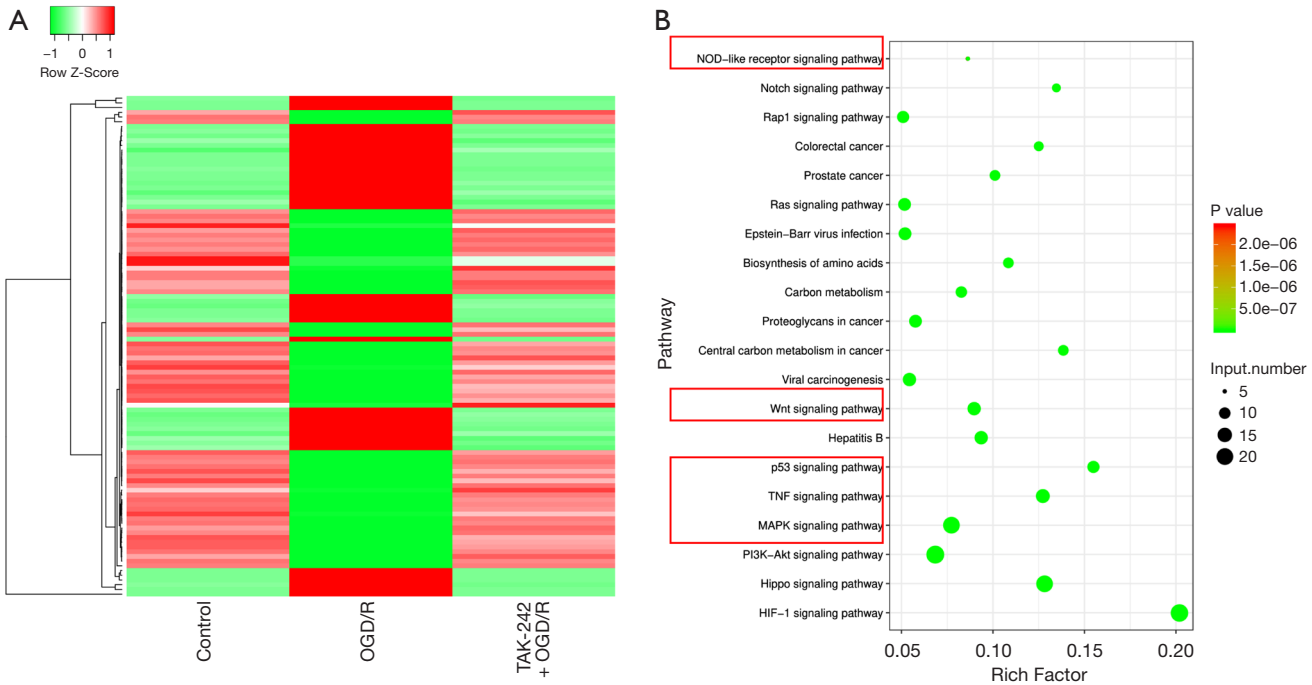

Figure 4 TAK-242 altered mRNAs expression pattern in OGD/R cells. (A) Heat map showing the differently expressed genes. The color bar depicts the color contrast level of the heat map. Red and green indicate high and low expression levels, respectively. (B) KEGG pathway enrichment results of the differently expressed genes. Red Box indicated the pathways that differently expressed genes enriched in the inflammatory response, cell fate, and cell proliferation, including NOD-like receptor, Wnt, p53, TNF, and the MAPK signaling pathway. OGD/R, oxygen-glucose deprivation and reoxygenation; KEGG, Kyoto Encyclopedia of Genes and Genomes.

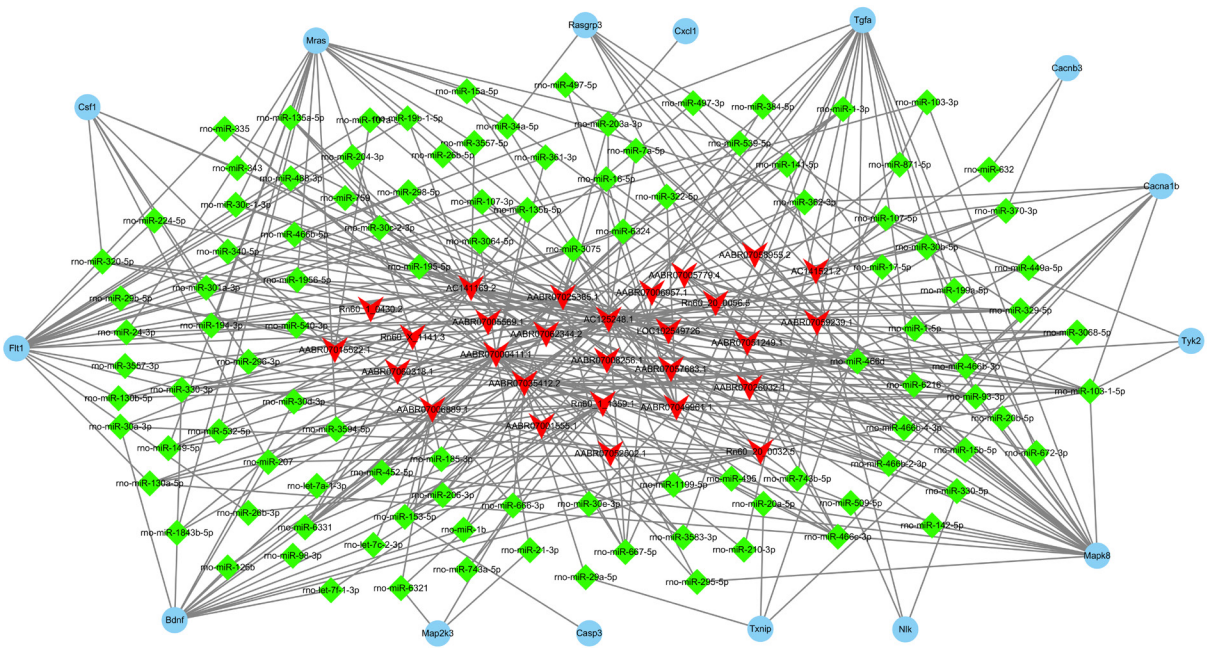

Figure 5 lncRNAs-miRNAs-mRNAs interaction network. Red concave quadrilateral, green rhombus, and blue circles represent lncRNAs, miRNAs, and mRNAs, respectively. lncRNAs, long non-coding RNAs; miRNAs, microRNAs; mRNAs, messenger RNAs.

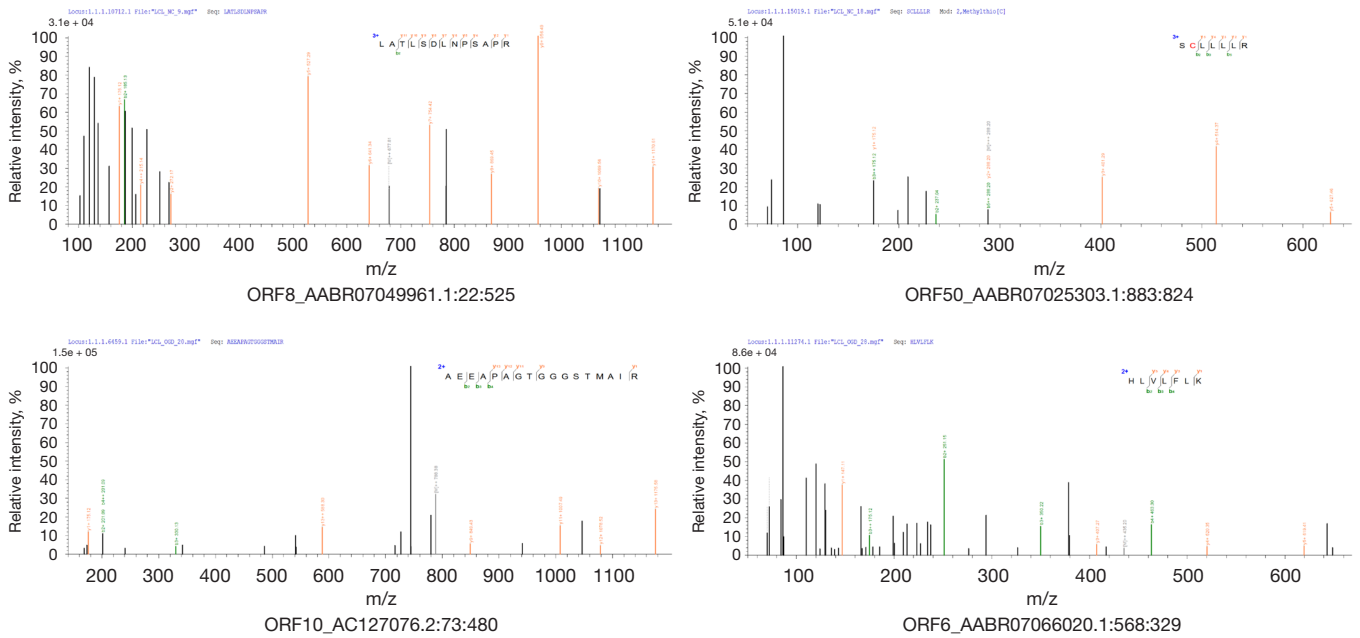

Figure 6 LC-MS/MS identified lncRNAs-encoded short peptides. The amino acid sequences of the four lncRNAs-encoded peptides identified by LC-MS/MS. LC-MS/MS, liquid chromatography-tandem mass spectrometry; lncRNAs, long non-coding RNAs.

protein was increased in OGD/R cells versus control but decreased in TAK-242+OGD/R cells versus OGD/R cells (Table S3). AC127076.2 and AABR07066020.1-encoded proteins were decreased in OGD/R cells versus control but were somewhat restored by TAK-242 in OGD/R cells (available online: https://cdn.amegroups.cn/static/public/10.21037jtd-23-360-2.xlsx). AABR07025303.1-encoded protein was decreased in OGD/R cells, and TAK-242 did not affect the expression levels of this protein (Table S3). These results suggested that TLR4 regulated lncRNA-encoded proteins expression during OGD/R, and these small peptides may participate in OGD/R injury.

Discussion

TLR4 is activated by endogenous proteins released from ischemia-damaged tissues, including heat-shock proteins, high mobility group box 1, hyaluronic acid, and fibronectin, among other, that trigger NF-κB activation, leading to the inflammatory response and exacerbation of ischemia injury (18,38-40). Suppression of TLR4 with TAK-242 has been documented to improve ischemic injury in the brain, liver, heart, kidney, and epilepsy (16,33,41-43). In this study, we reported that TAK-242 enhanced cell viability, suppressed pyroptosis, and decreased inflammatory factors release in OGD/R BMECs. The protective effect of TAK-242 against OGD/R-induced injury implied the possibility of adjunctive neuroprotective therapies during DHCA.

RNA deep sequencing was performed to explore the mechanism underlying the TAK-242 protective effect. Our results suggested that OGD/R altered some lncRNAs expression via TLR4 signaling, and TAK-242 could alleviate OGD/R-induced changes in lncRNAs expression pattern. AABR07000411.1 showed the greatest decrease in OGD/R cells in comparison to the control (*Figure 3*). Meanwhile, TAK-242 almost restored AABR07000411.1 to normal values (*Figure 3*), hinting that this lncRNA was closely regulated by TLR4 signaling.

We further comprehensively analyzed differently expressed lncRNAs and genes, and plotted lncRNAs-miRNAs-mRNAs networks. In the networks, we identified 15 co-expressed protein-coding genes with lncRNAs via miRNAs (*Figure 5*), including brain-derived neurotrophic factor (BDNF), Caspase-3 (Casp3), C-X-C motif chemokine ligand 1 (CXCL1), fms-related receptor tyrosine kinase 1 (FLT1), mitogen-activated protein kinase kinase 3 (MAP2K3), mitogen-activated protein kinase 8 (MAPK8), muscle RAS oncogene homolog (MRAS), and transforming growth factor alpha (TGF-α). All of BDNF, MAP2K3, MAPK8, MRAS, and TGF-α are closely connected to cell proliferation,

survival, and differentiation (44-47): Casp3 mediates cell apoptosis (48), CXCL1 functions as a chemoattractant for neutrophils and is associated with inflammation (49), and FLT1 plays an important role in angiogenesis and vasculogenesis (50). These results indicated that some TLR4-related lncRNAs may function as ceRNAs to regulate gene expression, and participate in cell survival, inflammation, and angiogenesis during ischemic injury.

In lncRNAs-miRNAs-mRNAs networks, AABR07000411.1 was predicted to regulate 11 protein-coding genes via interacting with 29 miRNAs. Notably, this lncRNA contained 3 miR-130a-5p, 2 miR-20a, and 4 miR-466-3p family binding sites. MiR-130a-5p and miR-20a have been reported to be increased during cerebral ischemia (51,52). Ischemia-induced miR-130a is predominantly from BMECs (51). Suppressing miR-130a could attenuate brain edema, lower BBB permeability, reduce infarct volume, and improve neurologic function in cerebral ischemia/reperfusion model rats (51). Inhibiting miR-20a promoted MAT2B and Bcl-2 expression, while inhibited Bax expression in hypoxic-ischemic brain damage animal model (52). Although research on the role of miR-466-3p in ischemic injury is limited, this miRNA is predicted to have more than 300 putative target genes, and these target genes are involved in many biological processes, indicating the multiple biological functions of miR-466-3p (53). Given that TAK-242 restored the decreased AABR07000411.1 in OGD/R cells, this lncRNA may play a crucial role in the protective effect of TAK-242.

Considering the emerging evidence on lncRNAs-encoded short peptides with biological functions, we explored the protein-coding potential of the differently expressed lncRNAs with LS-MS and assessed the lncRNAs-encoded short peptides expression levels among the control, OGD/R, and TAK-242+OGD/R cells with RPM assay. Our results suggested that AABR07049961.1, AC127076.2, AABR07025303.1, and AABR07066020.1 could encode short peptides, and these short peptides were associated with OGD/R and/or TLR4 signaling. However, these findings come from the rat-origin BMECs, cannot reflect the situation of human. In our future work, we will further confirm these short peptides expression with western blot assay, explore the biological function of these lncRNAs and related key miRNAs in human BMECs.

Conclusions

Taken together, our results suggest that TAK-242 attenuates OGD/R injury in BMECs. TAK-242 inhibits the TLR4/NLRP3/Caspase-1 and TLR4/ NF-κB pathways to decrease OGD/R-induced pyroptosis and inflammation. Moreover, TAK-242 alters lncRNAs expression pattern in OGD/R cells, and the differently expressed lncRNAs may exert a protective effect against OGD/R injury by ceRNA mechanism and encoding short peptides.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-360/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-360/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-360/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experimental procedures complied with local and

international guidelines for the care and use of animals and were approved by the Animal Ethics Welfare Committee of First Affiliated Hospital of Gannan Medical University (approval No. GFAC-AEWC-121).

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Table S1 The 38 differently expressed lncRNAs in OGD/R cells (*vs.* control cells) and in TAK-242+OGD/R cells (*vs.* OGD/R)

geneID	RBMEC_OGD-RPKM	RBMEC_NC-RPKM	log2 Ratio (RBMEC_NC/RBMEC_OGD-R)	Up-Down-Regulation(RBMEC_NC/RBMEC_OGD)	P-value	FDR	RBMEC_OGD-RPKM	RBMEC_OGD_inTLR4-RPKM	log2 Ratio (RBMEC_OGD_inTLR4/RBMEC_OGD)	Up-Down-Regulation (RBMEC_OGD_inTLR4/RBMEC_OGD-R)	P-value	FDR
AABR07006957.1	0.001	459.8774408	18.8108899	Up	1.69E-08	2.89E-07	0.001	615.2208002	19.23074476	Up	2.62E-11	5.50E-10
AABR07008256.1	0.001	367.5983121	18.48777062	Up	3.77E-05	0.000378314	0.001	575.0838634	19.13341283	Up	8.37E-08	1.14E-06
AABR07000411.1	0.001	117.1615011	16.83813906	Up	1.58E-06	1.99E-05	0.001	90.07921307	16.4589066	Up	3.20E-05	0.000284373
AABR07068285.1	208.652833	2303.675949	3.464761377	Up	2.16E-21	1.34E-19	208.652833	2981.091598	3.836664291	Up	5.97E-30	5.17E-28
AABR07062344.2	137.8422071	1091.007342	2.984571199	Up	4.17E-09	7.70E-08	137.8422071	904.4637679	2.714045004	Up	2.89E-07	3.40E-06
AABR07025385.1	217.4753085	951.8986821	2.129956413	Up	8.26E-13	2.01E-11	217.4753085	1114.452207	2.357411231	Up	8.61E-17	2.98E-15
Rn60_1_0430.2	404.8714128	1509.373909	1.898414555	Up	1.30E-06	1.67E-05	404.8714128	1838.909526	2.183314815	Up	3.37E-09	5.56E-08
Rn60_1_1359.1	339.2832302	810.8070592	1.256868526	Up	4.21E-05	0.000416838	339.2832302	1353.994805	1.996660175	Up	8.44E-14	2.34E-12
Rn60_20_0032.5	99.51823224	456.6242885	2.197974837	Up	1.68E-06	2.09E-05	99.51823224	388.3756099	1.964419838	Up	3.42E-05	0.000299687
Rn60_X_1141.3	289.6148181	880.3655038	1.603967196	Up	4.33E-07	6.17E-06	289.6148181	1103.419223	1.929773695	Up	8.09E-11	1.56E-09
AABR07035412.2	332.7975293	776.5095771	1.222358995	Up	6.77E-08	1.08E-06	332.7975293	1004.588978	1.593888724	Up	3.65E-14	1.05E-12
AABR07005569.1	642.3620236	1691.39452	1.396754701	Up	8.23E-06	8.92E-05	642.3620236	1884.969286	1.553082508	Up	2.61E-07	3.12E-06
AABR07049961.1	716.940132	2358.560245	1.717981896	Up	1.39E-19	6.76E-18	716.940132	1961.303735	1.451888417	Up	1.33E-13	3.17E-12
AABR07052502.1	4921.40516	15971.43789	1.698352005	Up	2.79E-41	2.72E-39	4921.40516	13172.97404	1.420438899	Up	1.47E-27	9.26E-26
AABR07015522.1	9422.424737	23333.89523	1.308256892	Up	4.62E-94	1.05E-91	9422.424737	24193.61994	1.360456375	Up	1.16E-105	2.68E-103
AABR07006889.1	508.058485	1049.017125	1.045971743	Up	5.78E-06	6.36E-05	508.058485	1278.504436	1.331390679	Up	9.04E-10	1.53E-08
AC125248.1	209.0756827	575.5872027	1.461009243	Up	1.58E-10	3.38E-09	209.0756827	492.3237707	1.235582125	Up	1.37E-07	1.72E-06
AC141169.2	188.0410882	464.3530707	1.304174241	Up	1.35E-07	2.05E-06	188.0410882	439.475481	1.224734743	Up	7.77E-07	8.83E-06
AABR07001555.1	3288.455587	7322.398551	1.154906115	Up	5.50E-08	9.16E-07	3288.455587	7301.680888	1.150818433	Up	4.37E-08	6.18E-07
Rn60_20_0056.5	2637.798599	5471.627017	1.052635474	Up	1.24E-07	1.93E-06	2637.798599	5722.855241	1.117400698	Up	9.40E-09	1.45E-07
AC130862.4	700.7585712	100.4788824	-2.802025163	Down	7.87E-05	0.000726505	700.7585712	46.35170375	-3.918223211	Down	2.27E-06	2.50E-05
LOC102549726	3477.195238	862.4098404	-2.011478529	Down	1.46E-17	5.53E-16	3477.195238	430.9895261	-3.012199359	Down	7.64E-29	5.88E-27
AABR07025303.1	219.7508349	46.43456073	-2.242597755	Down	0.00015605	0.001332279	219.7508349	36.7210807	-2.581188224	Down	2.56E-05	0.000233182
AABR07069473.1	532.2640739	67.83924541	-2.971950165	Down	1.53E-05	0.000157996	532.2640739	93.88434263	-2.503185712	Down	5.73E-05	0.000484375
AABR07051249.1	363.6442977	127.4568938	-1.512518546	Down	7.47E-05	0.00069878	363.6442977	69.48721472	-2.387708495	Down	3.06E-08	4.51E-07
AABR07002969.1	3680.050728	676.4965402	-2.443571192	Down	5.10E-16	1.51E-14	3680.050728	773.9415575	-2.24942912	Down	4.05E-15	1.28E-13
AABR07027407.1	23106.87538	6520.255492	-1.825321783	Down	2.26E-56	3.85E-54	23106.87538	5036.392412	-2.197859584	Down	6.46E-75	1.12E-72
AABR07021465.2	197307.3352	65019.18376	-1.601507242	Down	0	0	197307.3352	47101.47549	-2.066600432	Down	0	0
AABR07060318.1	654.4913817	58.39233295	-3.486523338	Down	9.70E-14	2.55E-12	654.4913817	177.0135554	-1.886514352	Down	1.16E-07	1.52E-06
AABR07057683.1	7080.1043	2175.400639	-1.70248949	Down	1.64E-17	5.91E-16	7080.1043	1953.537492	-1.857681669	Down	2.49E-20	1.08E-18
AABR07026032.1	2178.802597	312.4095214	-2.802025163	Down	1.59E-35	1.36E-33	2178.802597	650.3740863	-1.744193811	Down	1.16E-20	5.73E-19
AABR07059239.1	2073.360918	672.1354363	-1.625147401	Down	2.35E-06	2.76E-05	2073.360918	739.3771773	-1.487588857	Down	6.68E-06	6.71E-05
AABR07021998.1	10720.88371	4474.417859	-1.260651931	Down	1.18E-17	4.73E-16	10720.88371	4254.799693	-1.333260711	Down	8.82E-20	3.60E-18
AC141521.2	2961.708691	1347.033534	-1.13664398	Down	1.25E-20	7.13E-19	2961.708691	1262.759032	-1.229850385	Down	5.48E-24	2.92E-22
AABR07024833.1	3845.604983	1753.867917	-1.132670475	Down	1.73E-06	2.11E-05	3845.604983	1642.663673	-1.227173452	Down	2.05E-07	2.54E-06
AC127076.2	2802.120479	992.0605001	-1.498018976	Down	8.30E-17	2.84E-15	2802.120479	1217.336661	-1.202790779	Down	9.99E-13	2.23E-11
AABR07058955.2	1168.635738	459.0845487	-1.347993532	Down	2.52E-07	3.74E-06	1168.635738	525.2127535	-1.15385146	Down	3.55E-06	3.72E-05
AABR07066020.1	643.263633	252.9872097	-1.34634568	Down	4.54E-07	6.33E-06	643.263633	296.6252142	-1.116768891	Down	9.82E-06	9.45E-05

RBMEC: Rat brain microvascular endothelial cells; RBMEC_NC indicates control group; RBMEC_OGD-R indicates OGD/R group; RBMEC_OGD_inTLR4 indicates TAK-242+OGD/R group. FDR, false discovery rate.

Table S2 Information of twelve lncRNA-encoded proteins

Protein	Protein Sequence
ORF10_Rn60_13_0829.4:50:394	MMACKHCSDIFSKEGAGKPVAISREDQGLEADDEKDSLKKQLREMELELAQTKLQLVEAKCKIQELEHQRGALMNEIQAAKNSWFSKTLNSIKTATGTQPLQPPQAPQPPKEST
ORF148_AABR07006894.1:5153:4980	MFLLAICFLRGHLTSSWFLYSCLGVLGELVRVPGQVNRDRIPVQLLKLGEGRGASPV
ORF45_AABR07019085.1:65:3250	MLRKILSEGVDHCMVKLDNDPQGYLSQPLSLLESVLQECHNTFTACFHSFYPTPALQWACLCDLLNCLDQDIQEANFKTSSSRLLAAVMSALCHTSVKLTSLFPIAYDGEVLLRSIVKQVSTENESTLVHRFPLLVGHMEKLSQSEENIS GMTSFREVLEKMLVIVVLPVRNSLRRESELFSSHLVSNTCGLLASIVSELTASALGSEVDGLNSLHSVKASANRFTKTSQGRSWNTGNGSPDAICFAVDKPGIVVVGFSVYGGGGIHEYELEVLVDDSEHSGDSTHSHRWTSLELVKGTY TTDDSPSDIAEIRLDKVVPLKENVKYAVRLRNYGSRTANGDGGMTTVQCPDGVTFTFSTCSLSSNGTNQTRGQIPQILYYRSEFDGDLQSQLVSKANEEDKNCSRALSVVSTVVRAAKDLLHRALAVDADDIPELLSSSSLFSMLLPLII AYIGPVAAAIPKVAVEVFGLVQQLLPSVAVLNQKYAPPAFNPNQSTDSTTGNQPEQGLSACTTSNHYAVIESEHPYKPACVMHYKVTFPECVRWMTIEFDPQCGTAQSEDVLRLLTPVRTIQNAGYGAKVTSAHENLNSWIELKKYSGSS GWPTMVLVLPGNEALFSLETASDYVKDDKASFYGFKCFAIGYEFSPGPDEGVIQLEKELANLGGVCAAALMKKDLALPIGNELEEDLEILEEAALQVCKTHSGILGKGLALSHSPTILEALEGNLPLQIQSNEQSFLDDFIACVPGSSGG RLARWLQPDSYADPQKTSLILNKDDIRCGWPTTITVQTKDQYGDVVHVPNMKVEVKAVPVSQKKTSLQQEQVKKSQRVPGSPAAAAAASCTTDMTFGGLASPKLDVSYEPMIVKEARYIAITMMKVYENYSFEELRFASPTPKRPSENML IRVNNDGTYCANWTPGAIGLYTVHVTIDGIEIDAGLEVKVKDPPKGMIPPGTQLVKPKADPQPNKIRKFVAKDSAGLRIRSHPSLQSEQIGIVKVNGTITFIDEIHNDDGVWLRLNEETIRKYVPNMNGYTEAWCLSFNQHLGKSLLVPVDVSERWILKAS
ORF50_AABR07025303.1:883:824	MFPRRSCLLLLRLFNFMVR
ORF8_AABR07049961.1:22:525	MYHHSWLTLYDLHVQAQQGKIAAEEEDVGEDSQFMMLAKKVTAKALQKNASRAVAVQESKSVLSNPFETIRAGGAHQLKTGSLLNQPKAVLQKLATLSDLNPSAPRNSRNFVFHTLSPTKAETAKSSSKPQVRRRGPSSMMSPSPKRLKT NDSSPGPKRSIFRYLES
ORF20_AABR07037943.1:929:1615	MFAGEEASESLMNLSPFDMKSLLHHILSGKEFGVERSVRPIHSSMSSPAISIHEVGHTGVTKTERSGISRLRSEMKQRSSTPSSPTGTSSTDSGGHHLGWGEQQGQWLRRRRLDGAINRVPVGFYQRVWKILQKCHGLSIDGYVLPSSTT QEMTPCEIKFAVHVESVLNRVSQPEYRQLLVEAIMVLTLLSDTEMDSIGGIIHVDQIVQLANQLFLQDQVGVTGPMAGX
ORF6_AABR07066020.1:568:329	MVSDAQVRGPQRKVAHLRVVHTQGLDFVKWKQDADEKHLVLFLKGQGKAIDDAAEDLQELSNAIVMLSLINEPKGRRPS
ORF18_AABR07015040.1:701:486	MSSRQLFNTRPVLNSGGCTSRCCSLGGALSQPAGYCDCVFSAILGSAACRRIPFQRDGPSGLRSDLQKRRA
ORF10_AC127076.2:73:480	MAFFFFFFFFPGRPGGCVLAVGWEVKPQRAAVVGLCGAASGRRRRQRGGEQPRARSRAEEAPAGTGGGSTMAIRKKSNKNPPVLSHEFVLQNHADIVSCLAMLFLLGLMFEITAKGAIIFVALQYNVTRPATGEY
ORF10_AABR07027870.2:106:402	MENLDEMDNFLNRYQVPKLNQDQINHLNSSITPKEIEAVIRSLPTKKSPGSDGFSAEFYQTFKEEIVTILLKLLYKIETEGKLPNYFYEATVTLISNP
ORF63_AABR07033564.1:1925:2047	MLSLLFLSLVSTLSRLLETFCSLTFLVPWRKKLGFRLKQP
ORF95_Rn60_10_0635.1:1957:1892	MSHLSKGLLFLSRIFDSELKS

Table S3 TLR4 regulated lncRNA-encoded proteins expression in control, OGD/R, and TAK-242+OGD/R group

geneID	RBMEC_OGD-R RPKM	RBMEC_NC-RPKM	RBMEC_OGD_inTLR4-RPKM	Protein Sequence
AABR07049961.1	716.940132	2358.560245	1961.303735	MYHHSWLTLYDLHVQAQQGKIAAEEEDVGEDSQFMMLAKKVTAKALQKNASRAVAVQESKS VLSNPFETIRAGGAHQLKTGSLLNQPKAVLQKLATLSDLNPSAPRNSRNFVFHTLSPTKAETA KSSSKPQVRRRGPSSMMSPSPKRLKTNDSSPGPKRSIFRYLES
AABR07025303.1	219.7508349	46.43456073	36.7210807	MFPRRSCLLLLRLFNFMVR
AC127076.2	2802.120479	992.0605001	1217.336661	MAFFFFFFFFPGRPGGCVLAVGWEVKPQRAAVVGLCGAASGRRRRQRGGEQPRARSRAEE APAGTGGGSTMAIRKKSNKNPPVLSHEFVLQNHADIVSCLAMLFLLGLMFEITAKGAIIFVALQ YNVTRPATGEY
AABR07066020.1	643.263633	252.9872097	296.6252142	MVSDAQVRGPQRKVAHLRVVHTQGLDFVKWKQDADEKHLVLFLKGQGKAIDDAAEDLQELS NAIVMLSLINEPKGRRPS

RBMEC: Rat brain microvascular endothelial cells; RBMEC_NC indicates control group; RBMEC_OGD-R indicates OGD/R group; RBMEC_OGD_inTLR4 indicates TAK-242+OGD/R group.