

Comprehensive analysis of mRNAs, lncRNAs and circRNAs in the early phase of microglial activation

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Abstract. Sepsis-associated encephalopathy (SAE) is a common complication of sepsis that may seriously affect the prognosis and quality of life of patients with sepsis. Microglial activation is vital to the neuroinflammation and the pathology of SAE. In the present study, *in vitro* cultured BV-2 microglial cells stimulated with lipopolysaccharide (LPS) were employed as a model of microglia activation. The altered profiles of long noncoding (lnc)RNAs, circular (circ)RNAs and mRNAs in BV-2 cells after 4 h of LPS exposure were arrayed by using the Agilent competing endogenous (ce)RNA Microarray Chip. Using fold change >2 and P<0.05 as the cutoff criteria, 1,135 mRNAs and 2,488 lncRNAs were determined to be upregulated and 630 mRNAs and 744 lncRNAs to be down-regulated. The number of differentially expressed circRNAs was lower, with 140 upregulated and 123 downregulated. Gene

Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of DE mRNAs suggested that inflammatory responses, as well as lipid metabolism, were involved in microglial activation. Furthermore, analyses of ceRNA networks of the lncRNA-miRNA-mRNA or circRNA-miRNA-mRNA interrelations were performed. The present study revealed a multitude of novel candidate mRNAs, lncRNAs and circRNAs involved in microglial activation, which may improve the current knowledge on neuroinflammation and provide potential therapeutic targets for SAE.

Introduction

Sepsis-associated encephalopathy (SAE) is a common complication of sepsis that is prevalent in hospitalized patients (sepsis happens in ~2% of all hospitalized patients) (1). Furthermore, it has been estimated that SAE affects 9-71% of those patients with severe sepsis (2). The major manifestations of SAE include emotional abnormalities, impaired memory and cognitive impairment. Even after the acute phase of sepsis, these mental manifestations persist, severely affecting the prognosis and quality of life of patients (3). To date, numerous biological processes have been reported to be involved in the pathogenesis of SAE, including inflammatory response, collapse of the blood-brain barrier (BBB), cerebral ischemia and alterations in neurotransmitters. Although the specific mechanism has remained to be established, uncontrolled neuroinflammation is probably the leading cause of SAE (4).

During the early phase of sepsis, brain microglial cells that function as peripheral macrophages are activated and transformed to the M1 cell type (5,6). Thereafter, A great number of inflammatory cytokines, including interleukin-1 (IL-1), IL-6 and tumor necrosis factor- α (TNF- α), are secreted into the brain and lead to uncontrolled neuroinflammation (7). In addition, the activated astrocytes are also capable of releasing multiple types of inflammatory mediators, further facilitating the development of neuroinflammation (8). Overactivation of microglia and other immune cells may cause damage to neuron and endothelial cells, and furthermore deteriorate the BBB and enhance the release of reactive oxygen species, which

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Abbreviations: Agrn, agrin; AMPK, 5'-monophosphate-activated protein kinase; ceRNA, competing endogenous RNA; DE, differentially expressed; DMEM, Dulbecco's modified Eagle's medium; GO, Gene Ontology; FBS, fetal bovine serum; Fpr2, formyl peptide receptor 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; Laccl, laccase domain containing 1; LPS, lipopolysaccharide; Mafk, MAF bZIP transcription factor B; Rapgef2, Rap guanine nucleotide exchange factor 2; Pou2f2, POU class 2 homeobox 2; SAE, sepsis-associated encephalopathy; Socsl, suppressor of cytokine signaling 1; Tarf1, tumor necrosis factor receptor-associated factor 1

Key words: sepsis-associated encephalopathy, long non-coding RNA, circular RNA, microglia activation, neuroinflammation

seriously disturb brain function (9). Recent studies have verified that the suppression of microglial activation and central inflammatory responses may partially reverse the cognitive impairment caused by SAE (7,10). However, there is still a lack of effective treatment for SAE in the clinic, probably due to the detailed pathology of SAE having remained to be fully elucidated.

Non-coding(nc)RNAs, including microRNA(miRNA/miR), long ncRNA (lncRNA) and circular RNA (circRNA), have pivotal roles in the regulation of cell proliferation (11), autophagy (12) and apoptosis (13), as well as inflammatory responses (14), ischemia-reperfusion injury (15) and tumorigenesis (16). Recent studies have indicated that lncRNAs and circRNAs participate in the process of microglial activation (17-19). However, the orchestra of lncRNAs and circRNAs in microglial activation has remained to be fully determined, particularly during the early phase of SAE. In the present study, high-throughput microarray techniques were used to profile the expression of mRNAs, lncRNAs and circRNAs in BV-2 microglial cells after a short stimulation with lipopolysaccharide (LPS). Through the analysis of differentially expressed (DE) RNAs, promising novel signals involving non-coding RNAs were provided in the present study to provide clues for future investigation of microglial activation.

Materials and methods

Cell lines. Murine BV-2 microglial cells were obtained from the National Infrastructure of Cell Line Resource. The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Inc.), 1 mM pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO₂ at 37°C.

Cell treatments. BV-2 microglial cells were seeded in a 96-well plate at a density of 50,000 cells or in a 6-well plate at a density of 500,000 cells in each well 24 h prior to the experiment. During the experiment, the culture medium was changed to high-glucose DMEM supplemented with 0.5% FBS, and then LPS was added into each well (final concentration of 1.00 µg/ml) for incubation for 2, 4, 8, 16 or 24 h. In the control wells, the same volume of PBS was added and the cells were cultured for 24 h. A Cell Counting Kit-8 (CCK-8; BestBio) was used to detect the cell viability. CCK-8 stain (10 µl) was added into the 100 µl culture medium and the cells were further cultured for 4 h in the 37°C incubator prior to the detection of the optical density at the wavelength of 450 nm on a SpectraMax multimode microplate (SpectraMax iD3; Molecular Devices).

The morphology of BV-2 cells with the different incubation times (2, 4, 8, 16 or 24 h) of LPS or incubated with PBS for 24 h in the 6-well plate was examined under an inverted microscope (DMI8; Leica Microsystems) in an independent experiment and the same cells in the 6-well plate were finally collected for RNA extraction and the detection of the expression levels of TNF-α, IL-6 and IL-1β. Furthermore, cells seeded in the 6-well plate were treated with different concentrations of LPS (0, 0.01, 0.10 or 1.00 µg/ml) for 4 h. After washing with PBS, the total RNA in each well was extracted

by RNAiso Plus (Takara Bio, Inc.) for detecting the mRNA levels of TNF-α, IL-6 and IL-1β.

For the microarray analysis, cells were seeded in a 35-mm dish at a density of 500,000 cells 24 h prior to the experiment. BV-2 cells were stimulated with 1.00 µg/ml LPS or PBS for 4 h. Finally, the cells were washed with PBS and collected for the subsequent treatment.

RNA extraction and microarray assay. RNAiso Plus (Takara Bio, Inc.) was used to extract total RNA following the manufacturer's protocol. Total RNA was quantified by a NanoDrop® 2000 (Thermo Fisher Scientific, Inc.) and the RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). The sample labeling, microarray hybridization, washing and scanning were performed based on the manufacturer's standard protocols (Agilent Technologies, Inc.) by OE Biotech. In brief, total RNA was reversely transcribed to double-stranded cDNA (The first strand of cDNA complementary to the RNA was synthesized first and the second strand of cDNA complementary to the first strand was subsequently synthesized). Next, cRNA was synthesized using the double-stranded cDNA as the template and labeled with Cyanine-3-cytidine triphosphate (CTP). The labeled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies, Inc.). Feature Extraction software (version 10.7.1.1; Agilent Technologies, Inc.) was used to analyze array images to obtain raw data. The raw data were submitted to the Gene Expression Omnibus (GEO) database (dataset no. GSE171696).

Bioinformatics analysis. DE genes were then identified by performing Student's t-test to calculate the fold change (FC) and P-value. The threshold set for upregulated and downregulated genes was FC >2.0 and P <0.05.

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis. GO analysis of target genes was performed in the categories of biological process (BP), cellular component (CC) and molecular function (MF) (<http://www.geneontology.org>). KEGG pathway analysis (www.genome.jp/kegg) was also applied to analyze the key regulatory pathways involved in the activation of BV-2 cells. The upregulated or downregulated RNAs were individually analyzed via an online tool (https://cloud.oebio-tech.cn/task/detail/array_enrichment/).

CeRNA network analysis. DE lncRNAs/circRNAs that had a significant positive correlation (correlation coefficient >0.9) with DE mRNAs were selected as the targets for the competing endogenous (ce)RNA analysis. Target prediction of miRNAs to the aforementioned DE lncRNAs, circRNAs and mRNAs was performed by using all known mouse miRNAs in the database Mibase22. The ternary regulatory networks of lncRNA-miRNA-mRNA and circRNA-miRNA-mRNA were established using the predicted miRNAs as a bridge and the top 100 ceRNA network, which contained the top 100 links (ranked by the P-value of each link) of lncRNA-miRNA-mRNA or circRNA-miRNA-mRNA, was drawn by using the R (version 3.5.1) software (20).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from BV-2 cells using RNAiso Plus. For each sample, 1 μ g RNA was reverse-transcribed into cDNA using the Vazyme HiScript III 1st Strand cDNA Synthesis Kit with random hexamer (Vazyme Biotech Co. Ltd.) according to the manufacturer's protocols. For qPCR, 5 μ l reaction mixture composed of 5 μ l Vazyme SYBR qPCR Master Mix (Vazyme Biotech Co. Ltd.), 3 μ l 1:9 diluted cDNA and 2 μ l of 10 nmol/l primers were incubated in a Bio-Rad 96-well PCR plate. The thermocycling conditions in the RT-qPCR System (CFX Connect; Bio-Rad Laboratories, Inc.) was set to 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Data analysis was further performed using Bio-Rad CFX Maestro software (Bio-Rad Laboratories, Inc.). The relative expression levels of RNAs were calculated using the $2^{-\Delta\Delta C_q}$ method with β -actin as a reference (21). The primer sequences of lncRNAs, circRNAs and mRNAs were displayed in Tables SI and SII.

Statistical analysis. Values are expressed as the mean \pm standard error of the mean. An unpaired Student's t-test for parametric data and the Mann-Whitney U-test for nonparametric data were utilized for comparisons between two groups. GraphPad Prism 5 (GraphPad Software, Inc.) was used for all statistical analyses. Pearson correlation analysis was used to confirm the co-expression of lncRNAs/circRNAs and mRNA with an online tool (<https://cloud.oebiotech.cn/task/detail/correxpress/>). $P < 0.05$ was considered to indicate statistical significance.

Results

Differential expression of mRNAs, lncRNAs and circRNAs in LPS-treated BV-2 cells. Treatment with LPS (1 μ g/ml) for 2-24 h had no impact on the viability of BV-2 cells (Fig. S1A and B) and 4 h of treatment with LPS concentration-dependently (0.01, 0.1 and 1 μ g/ml) induced the transcription of TNF- α , IL-6 and IL-1 β (Fig. S1C). The expression levels of TNF- α , IL-6 and IL-1 β mRNAs changed after treatment with LPS for different durations (Fig. S1D). However, the transcription of these inflammatory factor genes was already induced after 4 h of treatment. Therefore, the LPS concentration of 1 μ g/ml and the incubation time of 4 h was used in the subsequent experiments.

The expression profiles of mRNAs, lncRNA and circRNAs in BV-2 cells were analyzed using microarray technology. A total of 1,135 mRNAs were identified to be upregulated and 630 mRNAs were downregulated. There were 2,488 DE lncRNAs, including 1,744 upregulated and 744 downregulated lncRNAs. Furthermore, 140 circRNAs were upregulated and 123 circRNAs were downregulated. The top 30 DE mRNAs ranked by FC, including both upregulated and downregulated, were present as a heatmap (Fig. 1). Volcano plot analysis was used to assess the variations in mRNA (Fig. 2A), lncRNA (Fig. 2B) and circRNA (Fig. 2C) expression profiles between the two groups.

GO and KEGG analysis of the DE mRNAs. The results of the GO functional enrichment analysis indicated that the upregulated DE mRNAs were involved in 585 BP, 54 CC

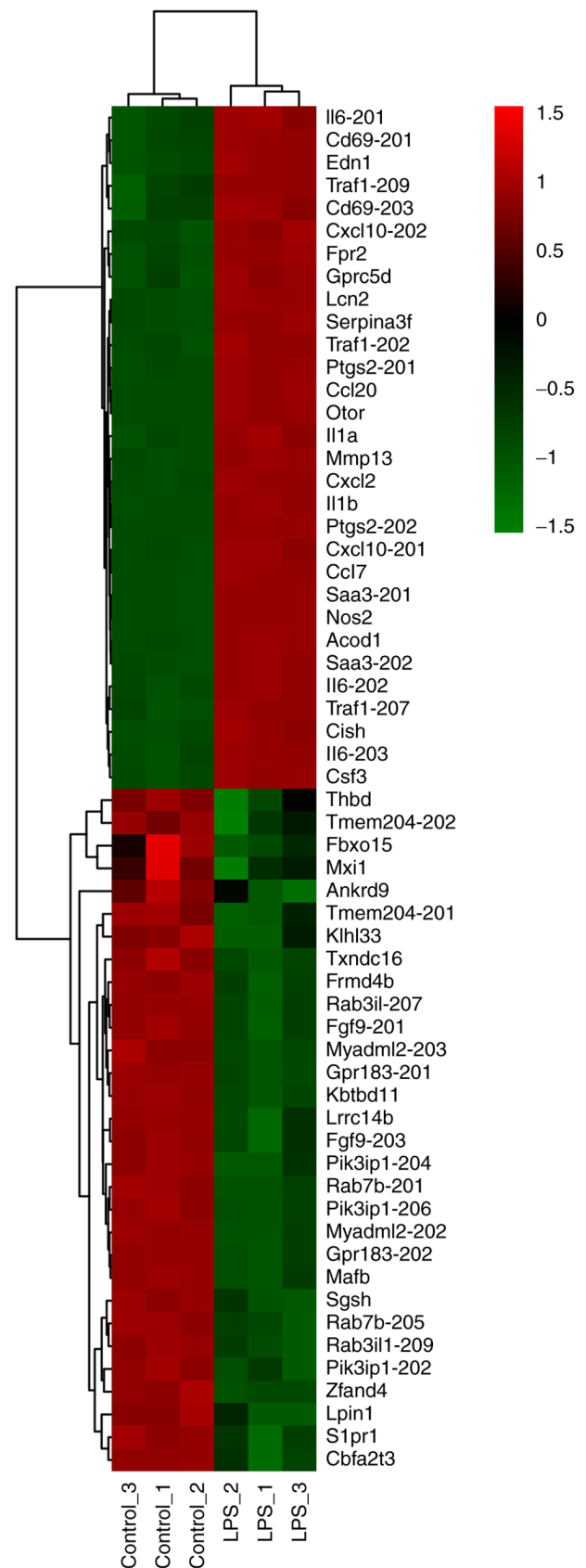


Figure 1. Differentially expressed mRNAs in LPS-stimulated BV-2 cells compared to vehicle. Heatmap with hierarchical clustering of top 30 dysregulated mRNAs (both up- and downregulated) between LPS and vehicle treatment. Green and red columns refer to high and low relative expression, respectively. 'Control_1, Control_2 and Control_3' represent the three repeated samples treated with PBS (vehicle) and 'LPS_1, LPS_2 and LPS_3' represent the three repeated samples treated with 1 μ g/ml LPS. LPS, lipopolysaccharide.

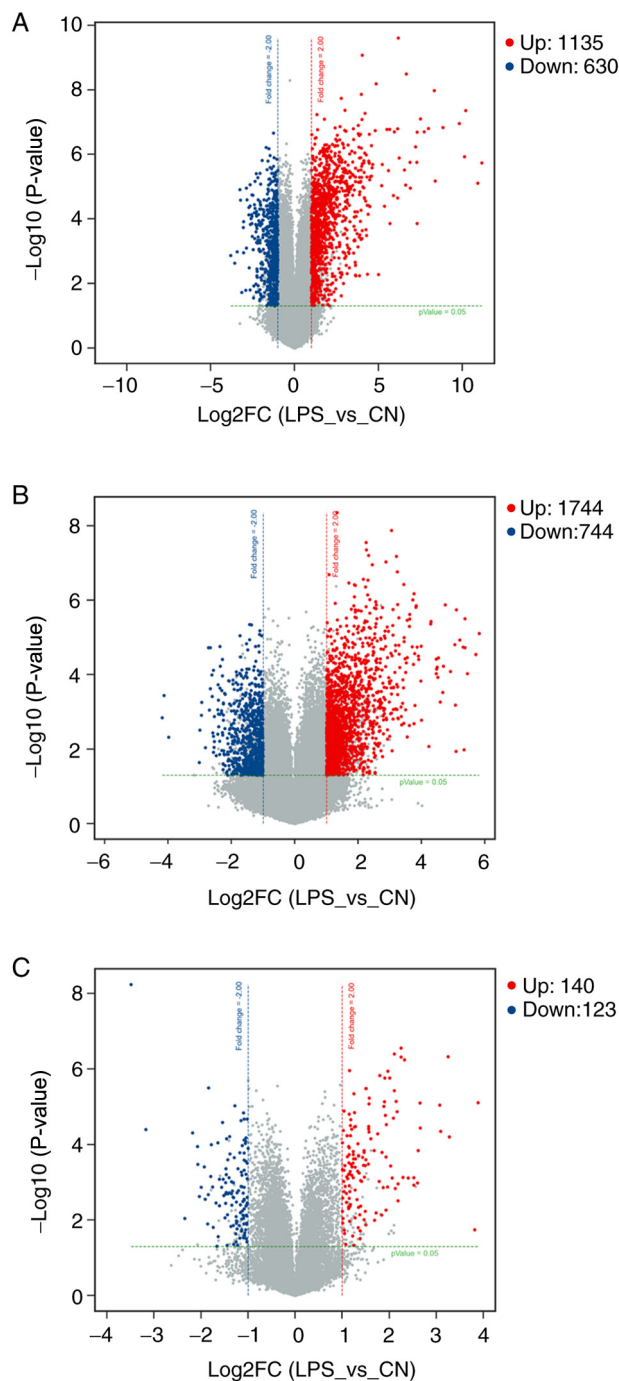


Figure 2. Volcano plots of differentially expressed (A) mRNAs, (B) long noncoding RNAs and (C) circular RNAs between LPS and vehicle treatment. Values plotted on the x- and y-axes represent the averaged normalized signal values of each group (\log_2 -scaled). FC, fold change; LPS, lipopolysaccharide; CN, control.

and 112 MF terms. Furthermore, the downregulated mRNAs were involved in 182 BP, 19 CC and 58 MF terms. The top 10 results with the most significant P-values of the upregulated DE mRNAs in BP and MF were presented in the bar blot (Fig. 3A). GO term analysis of upregulated DE mRNAs revealed that the most enriched GO terms were cytokine activity (MF) and inflammatory response (BP). The upregulated mRNAs in BV-2 cells were most significantly enriched in the TNF signaling pathway, Herpes simplex infection, NOD-like receptor signaling pathway, IL-17 signaling pathway

and cytokine-cytokine receptor interaction (Fig. 3B). For the downregulated mRNAs, the most enriched GO terms were GTPase activator activity (MF) and negative regulation of transcription by RNA polymerase II (BP), which were shown in Fig. 4A. KEGG pathway analysis of DE mRNAs determined 79 pathways for upregulated mRNAs and 33 pathways for downregulated mRNAs. Furthermore, the pathways most significantly enriched by the downregulated mRNAs were ABC transporters, transcriptional misregulation in cancers, endocrine resistance, osteoclast differentiation and T-cell receptor signaling pathway (Fig. 4B). In addition, pathways associated with lipid metabolism, including bile secretion, regulation of lipolysis in adipocyte and insulin resistance, were among the top 30 pathways enriched by downregulated mRNAs (Fig. 4B).

CeRNA network analysis. Co-expressed lncRNAs/circRNAs and mRNAs were selected to predict the association with miRNAs. In total, >300,000 lncRNA-miRNA-mRNA links were predicted. The top 100 links were chosen according to the P-value of their correlation and were present in the top 100 ceRNA network (Fig. 5). Furthermore, 4,053 circRNA-miRNA-mRNA links were predicted, with the top 100 ceRNA network presented in Fig. 6.

Validation of DE mRNAs, lncRNAs and circRNA. DE mRNAs, lncRNAs and circRNAs from the expression profile with a high FC or in the hub of the ceRNA networks were selected for validation (Fig. 7). RT-qPCR validated that seven selected mRNAs, including TNF receptor (TNFR)-associated factor 1 (Traf1), formyl peptide receptor 2 (Fpr2), POU class 2 homeobox 2 (Pou2f2), suppressor of cytokine signaling 1 (Socs1), agrin (Agrn), laccase domain containing 1 (Laccl) and Rap guanine nucleotide exchange factor 2 (Rapgef2), were increased after LPS stimulation, and that the mRNA expression of α/β -hydrolase domain containing protein 15 (Abhd15) was decreased. Furthermore, four lncRNAs (ENSMUST00000155949.1, NONMMUT144861.1, ENSMUST00000181460.2 and NONMMUT141017.1) and three circRNAs (CIRCpedia_38964, CIRCpedia_37520 and CIRCpedia_216004) were demonstrated to be upregulated and one lncRNA (ENSMUST00000203956.1) was proven to be downregulated.

Discussion

Neuroinflammation is proposed to have a central role in the pathogenesis of SAE, which increases the burden on the health system and may cause major damage to patients' quality of life (3,22). Microglial cells, which act as residential macrophages, are activated during the pathological process of neuroinflammation (5,6). The modulation of microglial cell activation has become a promising therapeutic direction in the treatment of SAE, as well as neuropathic pain (10,23). The murine microglial cell line BV-2 is commonly used for study of neuroinflammation and microglial activation (24,25). Henn *et al* (26) indicated that the cell line has a high transcriptome homology with primary microglial cells and a similar response to inflammatory challenges. Therefore, in the present study, BV-2 cells stimulated with

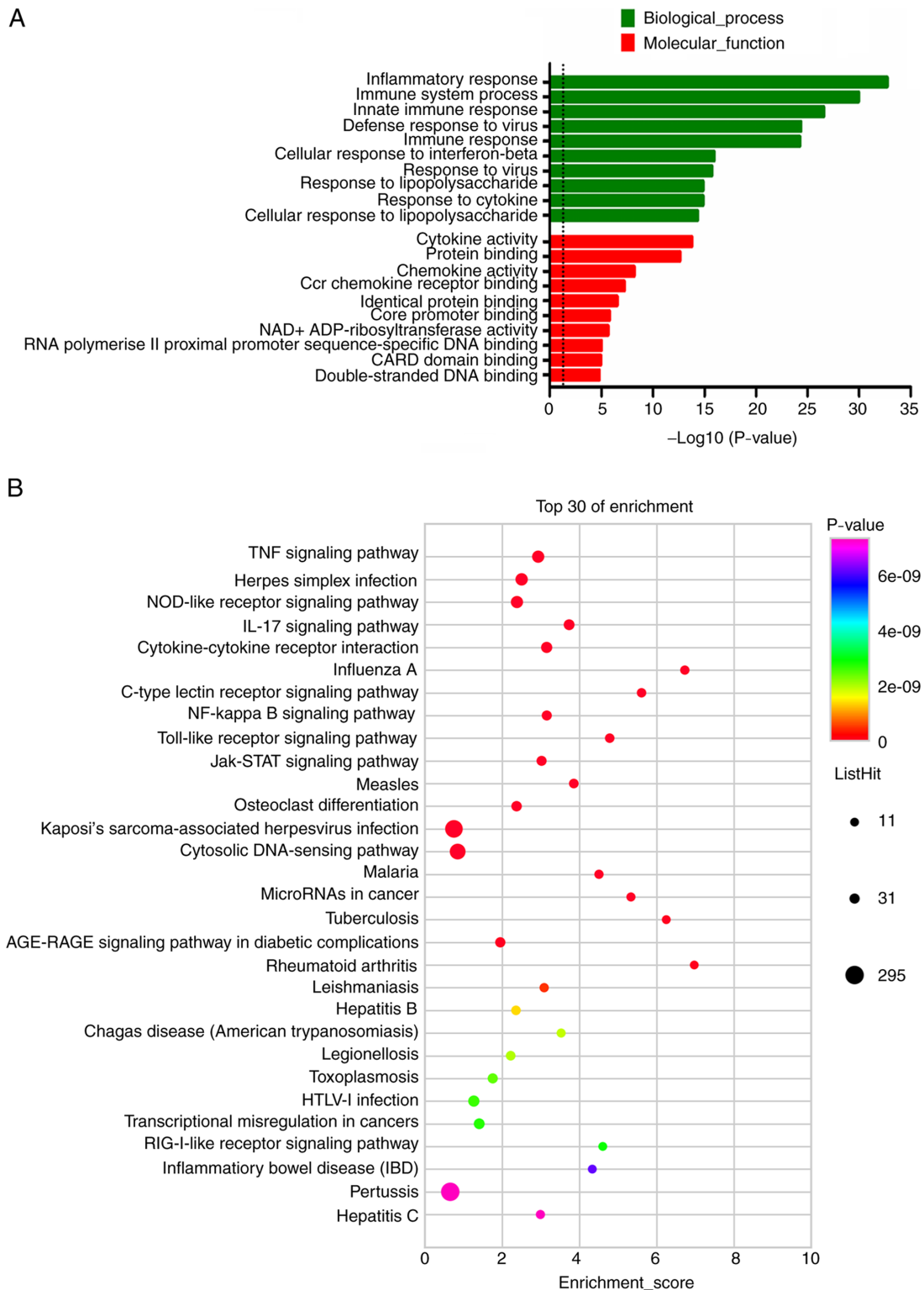


Figure 3. GO and KEGG analysis of upregulated mRNAs in lipopolysaccharide-stimulated BV-2 cells compared to vehicle. (A) GO analysis (top 10 functional terms) of the upregulated mRNAs in the categories of molecular function and biological process. (B) KEGG analysis presenting the top 30 enriched pathways for the upregulated mRNAs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

LPS were employed as the model of microglial activation. Microarrays of mRNAs, lncRNAs and circRNAs of stimulated or vehicle-treated BV-2 cells were performed and the data were analyzed.

The results of the present study indicated that the expression profiles of mRNAs and lncRNAs were acutely altered

after 4 h of LPS exposure. A total of 1,135 mRNAs were determined to be significantly upregulated and 630 mRNAs were downregulated. The top upregulated mRNAs included IL-6, IL-1 β , IL-1 α , neutrophil gelatinase-associated lipocalin (Lcn2), C-X-C motif chemokine 2 (Cxc12), Fpr2, Traf1 and CD69 molecule. Compared with the study from

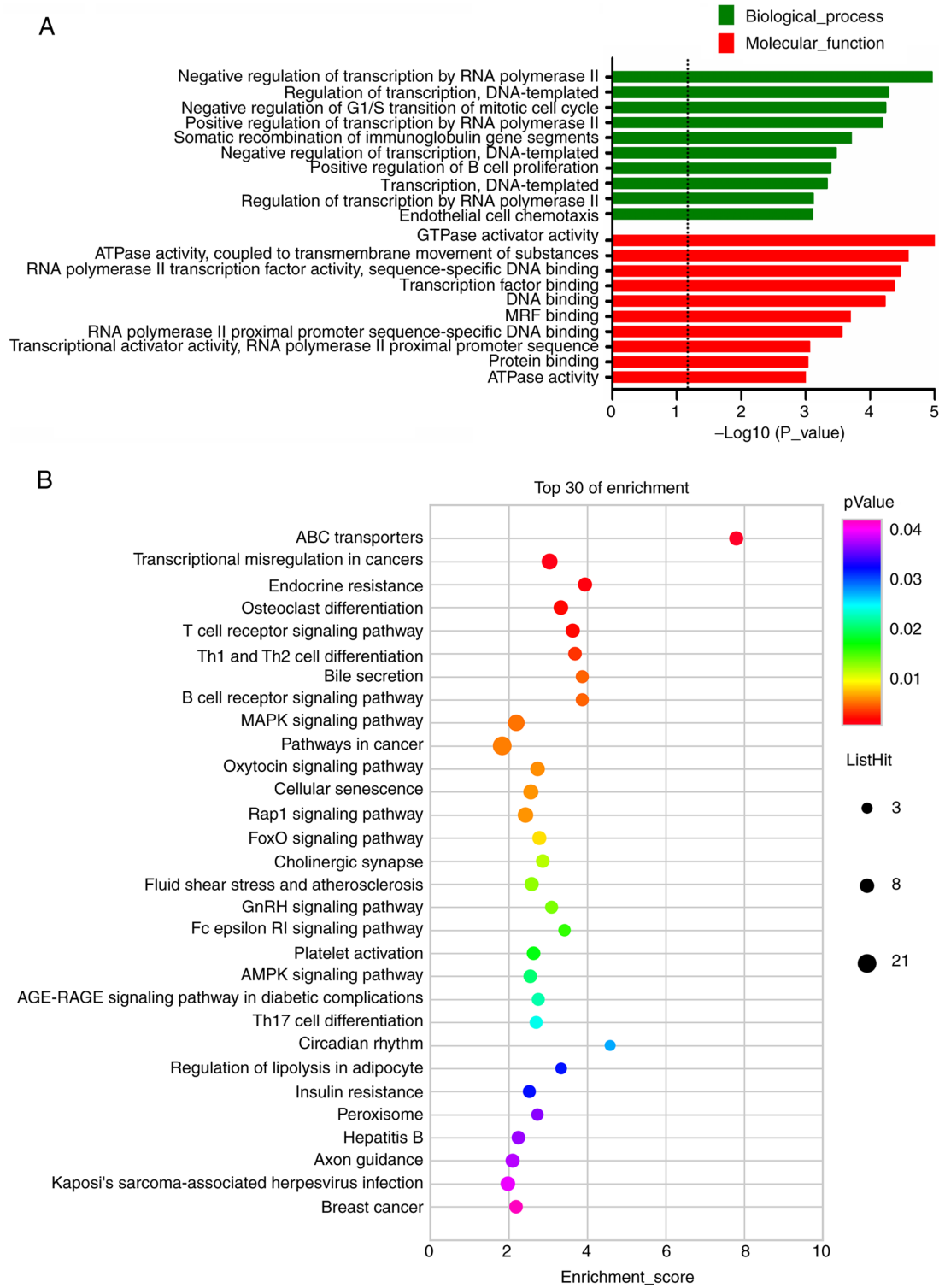


Figure 4. GO and KEGG analysis of downregulated mRNAs in lipopolysaccharide-stimulated BV-2 cells compared to vehicle. (A) GO analysis (top 10 functional terms) of the downregulated mRNAs in the categories of molecular function and biological process. (B) KEGG analysis presenting the top 30 enriched pathways for the downregulated mRNAs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Li *et al* (17), who prolonged the exposure to LPS to 24 h, there were slight variations in the top changed mRNAs. Traf1, Fpr2 and CD69 were among the top 30 upregulated mRNAs in the early phase of LPS exposure, while IL-6, IL-1 β , Lcn2, serum amyloid A-3 protein and Cxcl2 were the top upregulated genes in both the early and late phase. Furthermore, the probes contained in the chip used in the present study

were designed to discern the different transcripts of genes. For instance, two transcripts of the IL-6 gene (202 and 203), recorded in the Ensemble Database, were among the top 30 upregulated mRNAs, while IL6-201 was upregulated to a lesser degree (FC=71.15). The different expression of the alternative transcripts of the same gene requires further exploration to elucidate their potential function.

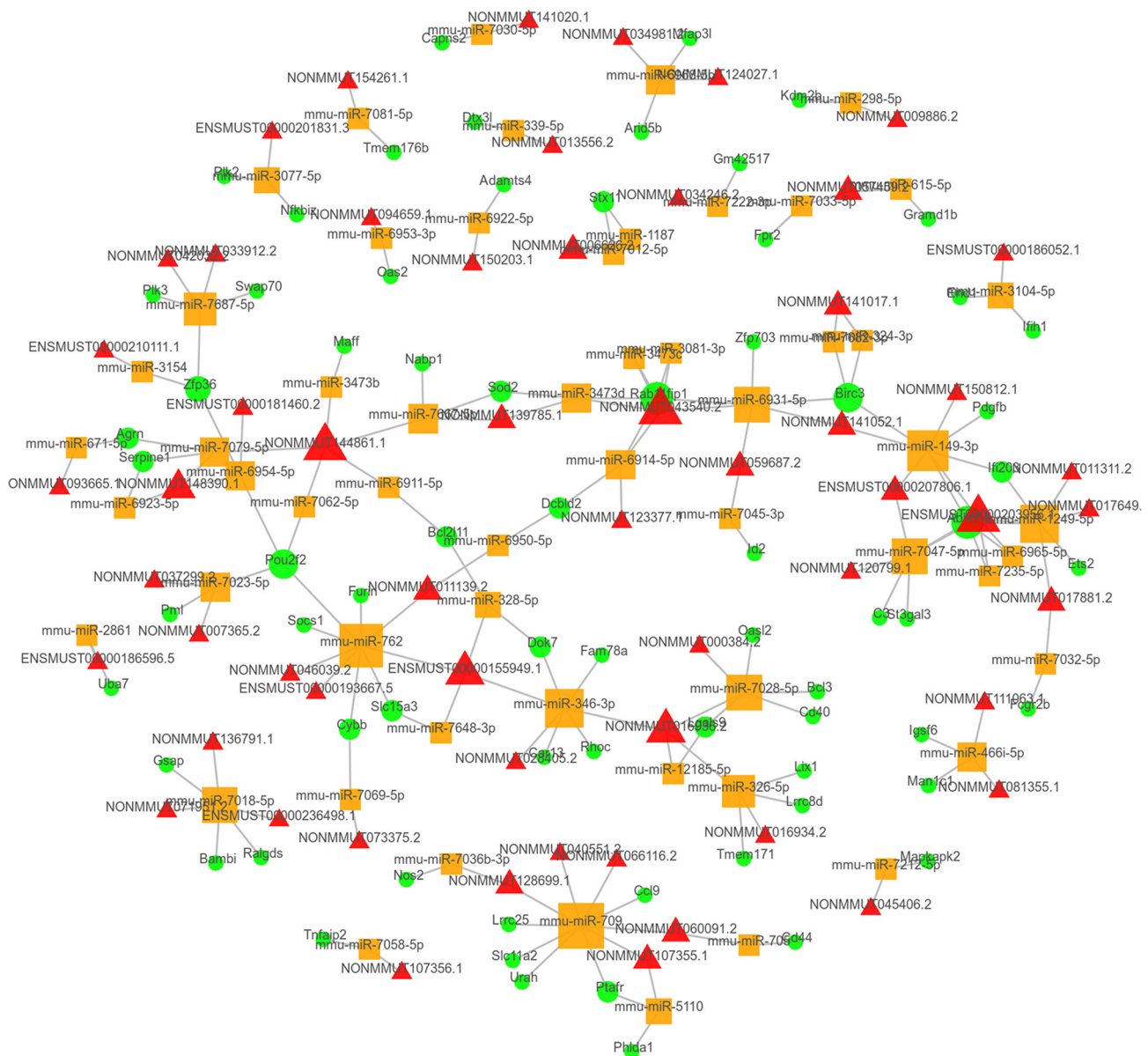


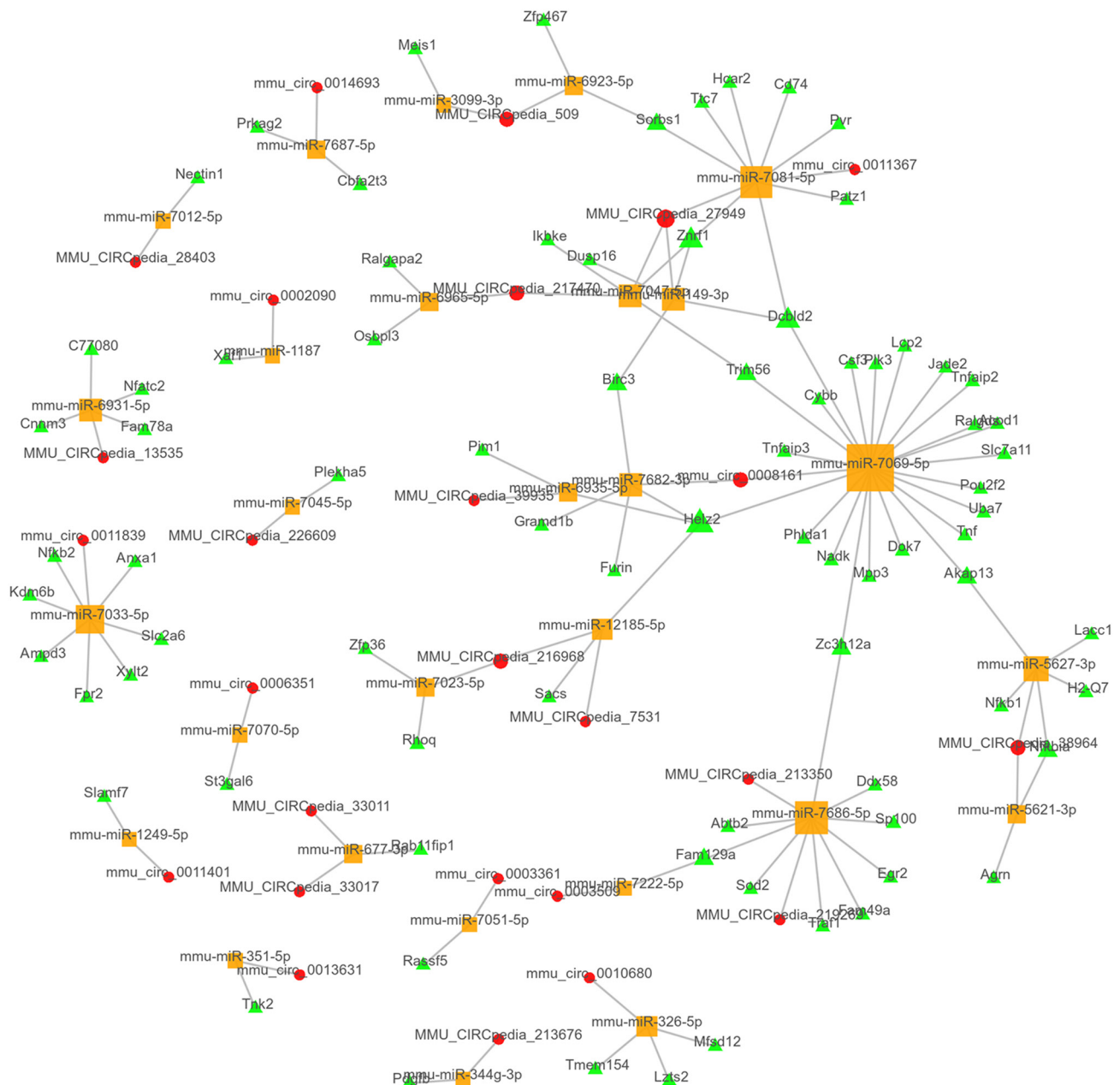
Figure 5. Ternary regulatory network of lncRNA-miRNA-mRNA. Yellow square nodes represent miRNAs; green circle nodes represent mRNAs; red triangle nodes represent lncRNAs. The larger the node in the diagram, the more links this node is connected to. miR, microRNA; lncRNA, long noncoding RNA.

Recently, Sun *et al* (27) also employed transcriptome sequencing approaches to characterize the effects of LPS on lncRNA and mRNA expression patterns in brain tissue isolated from Sprague Dawley rats. In this study, Sprague Dawley rats were exposed to LPS for 6 or 24 h and a large number of lncRNAs and mRNAs were indicated to be changed. However, no behavioral observation of these rats was made, which is required to diagnose SAE. The upregulated gene regulator of G-protein signaling 14 identified by Sun *et al* (27) was also verified in the BV-2 cells of the present study. Furthermore, the difference in the lncRNA expression signature between the present study and the study by Sun *et al* (27) was huge, possibly due to the gap between different species and different cell types.

GO analysis of these early upregulated DE mRNAs in the category BP included inflammatory response, immune system process, response to LPS and response to cytokine, which were all related to inflammation and with a \log_{10} (P-value) >10.

This differed from downregulated DE mRNAs, which mainly involved the regulation of transcription, with \log_{10} (P-value) <5. Among the top downregulated coding RNAs, several had the function of gene transcription or cooperation with transcription factor. For instance, MAF bZIP transcription factor B (Mafk), an important transcription factor gene strongly expressed in macrophages, is a member of the Maf transcription factor family and regulates gene transcription via the binding to the Maf recognition element (28). A study demonstrated that Mafk determined the anti-inflammatory properties of macrophages (29). Consistently, in the present study of BV-2 cells, short-time exposure to LPS markedly decreased the expression of Mafk.

In the KEGG analysis of the downregulated mRNAs, it was noted that pathways associated with lipid metabolism were prevalent. Bile secretion, regulation of lipolysis in adipocyte and insulin resistance were among the top 30



terms enriched by downregulated mRNAs. Accumulating evidence has indicated that different metabolic pathways or energy homeostasis are required for programming M1 and M2 macrophages (30). Furthermore, it has been indicated that LPS treatment may induce lipid droplet accumulation and increased cellular triglyceride content in microglia. Lipid metabolism was linked with inflammatory signaling and microglial activation (31). Wang *et al* (32) indicated that exogenous recombinant human fibroblast growth factor 21, which may suppress lipid accumulation through the activation of adenosine 5'-monophosphate-activated protein kinase (AMPK) (33), ameliorated LPS-induced depressive-like behavior by inhibiting microglial expression of proinflammatory cytokines. In addition, it has been indicated that the activation of AMPK by ENERGI-F704 (a chemical

Although the validated expression levels of Fpr2 and TNF receptor-associated factor 1 (Tar1) after LPS exposure were

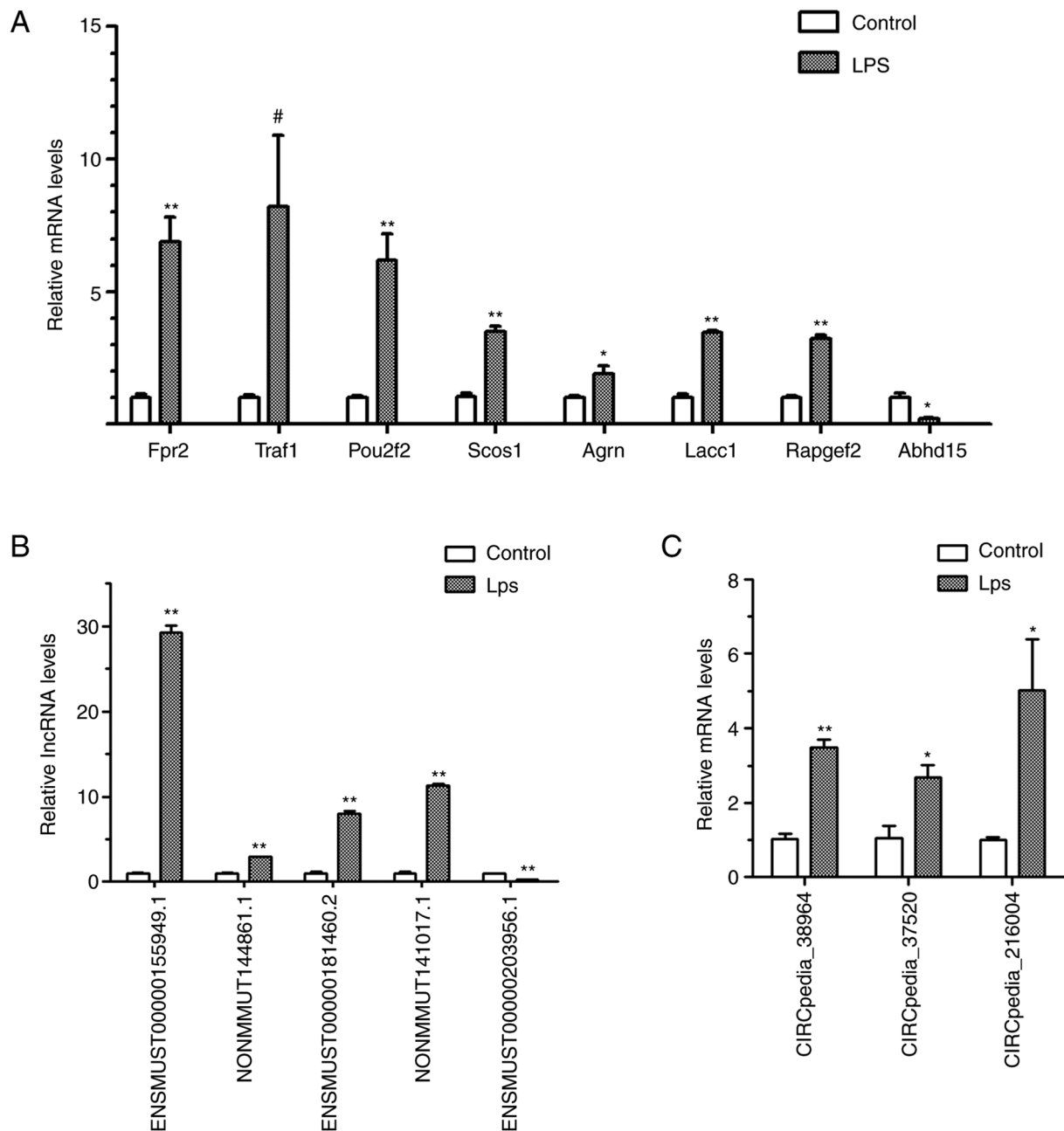


Figure 7. Reverse-transcription-quantitative PCR of (A) mRNAs, (B) lncRNAs and (C) circRNAs in BV2 microglial cells exposed to LPS. Values are expressed as the mean \pm standard error of the mean (n=3). *P<0.05, **P<0.01, compared with the control group (two-tailed Student's t-test); #P<0.05, compared with the control group (one-tailed Student's t-test). lncRNA, long noncoding RNA; circRNA, circular RNA; LPS, lipopolysaccharide.

not as high as those detected by microarray in the present study, their functions in inflammation signaling were striking (35). Fpr2, a G-protein-coupled receptor, has an important role in host defense and inflammation. Fpr2 inhibition is able to markedly reduce the activation of transforming growth factor beta-activated kinase 1 induced by LPS and decrease inflammation and oxidative stress in LPS-induced lung injury (36). Mounting evidence has indicated that Fpr2 participates in the pathogenesis of sepsis (37,38). Traf1 is a signaling adaptor first identified as part of the TNFR2 signaling complex (39). Traf1 has a key role in pro-survival signaling downstream of TNFR superfamily members, such as TNFR2 and CD40. Moreover, it has been demonstrated that Traf1 negatively regulates Toll-like receptor and Nod-like receptor signaling, through

sequestering the linear ubiquitin assembly complex (40). In a study of acute lung injury, the interferon of Traf1 markedly attenuated LPS-induced mortality, alleviated lung injury and markedly reduced inflammation (41).

In 2011, Salmena *et al* (42) proposed the ceRNA hypothesis, which aimed to explain how RNAs 'talk' to each other to establish interactions to modify functional genetic information, and function in pathological conditions. Through miRNA response elements, miRNAs recognize and bind to regions with partial complementary sequences on target RNA transcripts, resulting in the repression of the target gene. Thereby, miRNAs mediate regulatory crosstalk between various components of the transcriptome. On the contrary, lncRNAs or circRNAs competitively bind and absorb miRNAs via specific nucleotide

elements, acting like a ‘sponge’, and release the coding mRNAs from repression. Based on the expression profiles and the ceRNA analysis, several lncRNAs, circRNAs and mRNAs were selected for validation in the present study. For mRNAs, *Traf1*, *Fpr2*, *Pou2f2*, *Socs1*, *Agrn*, *Laccl*, *Rapgef* and *Birc3* were demonstrated to be upregulated and *Abhd15* was downregulated. lncRNA ENSMUST00000155949.1, also named 6530402F18Rik, was demonstrated to have high transcript levels. The gene of 6530402F18Rik is located on chromosome 2 and does not encode any protein. In the ceRNA network analysis, it was predicted that 6530402F18Rik is able to combine with miR-762 and miR-7648-3p, with the predicted scores of 750 and 735, respectively. Both miR-762 and miR-7648-3p were predicted to target *Pou2f2*, with a predicted score >600. *Socs1* was also predicted to be associated with 6530402F18Rik through miR-762, as well as miR-7648-3p, with high scores. Unlike 6530402F18Rik, ENSMUST00000203956.1 (2010008C14Rik) was demonstrated to be reduced in expression. In the prediction, 2010008C14Rik was able to ‘sponge’ miR-149-3p. Thus, it is probable that the downregulated 2010008C14Rik released more free miR-149-3p that had a predicted domain for *Abhd15*, leading to the decreased expression of *Abhd15*. However, functional research on 6530402F18Rik and 2010008C14Rik is scarce. lncRNAs functioning in the activation of BV-2 cells and modulating BV-2 cell activation via ‘sponging’ are worth further investigation.

The present study detected thousands of DE lncRNAs and mRNAs, while the number of significant DE circRNAs was lower, with only 140 circRNAs determined to be upregulated and 123 circRNAs were downregulated. The circRNAs with a high FC were not all included in the circRNA-miRNA-mRNA network. For instance, CIRCpedia_216004, the top increased circRNA, was determined to be highly expressed, but was not predicted in the ceRNA network. For CIRCpedia_37520, there was only one predicted association with *Rapgef2*, which was also validated in the present study, likely via miR-5620-3p. Furthermore, it was predicted that CIRCpedia_38964 was able to sequester miRNA and lead to the increased translation of *Agrn* and *Laccl* that were indicated to moderately increase. It remains elusive whether these circRNAs are only biomarkers of inflammation or have other potential functions.

Despite the results obtained, the present study has certain limitations. First, the pathogenesis of SAE involves not only microglial cells, but also neurons, endothelial cells and other cell types. Furthermore, although LPS is the primary inflammatory substance in sepsis, other components of microorganisms and the substances of damaged host cells are capable of inducing inflammatory responses. The *in vitro* microglial cells stimulated by LPS cannot completely imitate the pathogenesis of SAE. Furthermore, the genes and links validated in the present study require further validation in human microglial cells and *in vivo* SAE models and their functions in inflammation should also be explored in the future.

In conclusion, the present study revealed the ceRNA networks involved the interrelation of lncRNA-miRNA-mRNA or circRNA-miRNA-mRNA in the early phase of the microglial activation. Furthermore, most of the DE genes were mainly involved in inflammatory responses, immune responses and lipid metabolism. Although more *in vivo* and *in vitro* functional studies

are required, the present study may improve the present knowledge on neuroinflammation and provide potential therapeutic targets for SAE.

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Availability of data and materials

The datasets of the microarray used in the present study are available from the GEO database under accession no. GSE171696 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171696>). Other datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

JW and QX conceived and designed the experiments. YL and SC contributed to the cell culture and treatments. JW, JG, ZZ and BH analyzed the data and drafted the manuscript. JG and QX contributed to manuscript editing. JW, JG and QX confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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