Construction of Chronic glomerulonephritis-related lncRNA-mRNA regulatory network and lncRNA-miRNA-mRNA ceRNA network by bioinformatics analysis

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Research Article

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Construction of Chronic glomerulonephritis-related lncRNA-mRNA regulatory network and lncRNA-miRNA-mRNA ceRNA network by bioinformatics analysis

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Abstract

Background Long non-coding RNAs (lncRNAs), generally known as transcripts longer than 200nt, are important genetic regulators. Lately, researches on lncRNA-mRNA regulatory network and lncRNA-miRNA-mRNA competitive endogenous RNA (ceRNA) network has been the hotspots in the field of non-coding RNAs. The present study explored the functional roles and regulatory mechanisms of lncRNAs in chronic glomerulonephritis (CGN).

Methods In the current study, the RNA-seq was performed to study the differences in the expression profiles of lncRNAs in lipopolysaccharide (LPS) -induced glomerular mesangial cells (GMCs). LncRNA-mRNA regulatory network and lncRNA-miRNA-mRNA competitive endogenous RNA (ceRNA) network were constructed to examine the role and mechanism of proliferation-related lncRNAs. To reveal the biological functions of lncRNAs, GO biological process and KEGG pathway analysis were we performed on all mRNAs involed in lncRNA-mRNA regulatory network and ceRNA network.

Results 1532 differentially expressed lncRNAs, including 594 upregulated lncRNAs and 938 downregulated lncRNAs were screened out by RNA-seq in LPS-induced GMCs. Furthermore, the lncRNAs-mRNAs regulatory network, including 236 lncRNAs and 556 mRNAs and lncRNA-miRNA-mRNA ceRNA network, including 6 lncRNAs, 18 miRNAs, and 419 mRNAs were successfully constructed. The GO biological process and KEGG pathway analysis demonstrated that lncRNAs were mainly related to inflammatory response and substance metabolism.

Conclusions This study first identified key proliferation-related lncRNAs in LPS-induced GMCs, and further revealed a global view of lncRNAs-mRNAs regulatory network and lncRNA-miRNA-mRNA ceRNA network involved in CGN. Our findings offered novel insights into the roles of lncRNAs in the pathogenesis of CGN and provided promising diagnostic biomarkers.

Keywords LncRNAs; lncRNA-mRNA regulatory network; ceRNA network; Chronic glomerulonephritis; RNA-seq

Introduction

Chronic glomerulonephritis (CGN) is an autoimmune glomerulopathy, characterized by the proliferation of mesangial cells, accumulation of extracellular matrix and infiltration of circulating inflammatory cells [1]. Glomerular mesangial cells (GMCs), distributing in the mesangial matrix of the glomerulus, constitute the mesangial region of the glomerulus together with the mesangial matrix. GMCs have multiple physiological functions, including stabilizing the structure of glomerular capillaries, maintaining mesangial matrix homeostasis, regulating filtration surface area, phagocytosis of apoptotic cells and immune complexes, etc. Under the influence of some pathological factors such as hyperglycemia and inflammation, GMCs will proliferate abnormally, increase intracellular protein synthesis, and increase secretion of the extracellular matrix [2,3]. Therefore GMCs' proliferation is an important pathological feature of many human kidney diseases, including chronic glomerulonephritis and diabetic nephropathies. Knowledge of the GMCs' responses to pathological stimuli is crucial to the understanding pathogenesis of chronic glomerulonephritis. Thus a deeper understanding of the GMCs' proliferation is required to devise more effective prevention and therapies for CGN.

Competing endogenous RNAs (ceRNA) theory, first proposed by professor Salmena in 2011, provides a new perspective for studying the role of RNA biological behavior in the occurrence and development of disease [4]. According to the theory of ceRNA, RNA transcripts, including non-coding

RNAs, circular RNAs, and pseudogene transcripts, cloud function as miRNA sponges and subsequently regulate miRNA expression. Studies have indicated that lncRNAs could affect mRNA stability though ceRNA theory. For example, research shows that long non-coding RNA linc00673, acting as a ceRNA by sponging miR-150-5p and regulated ZEB1 expression, might play an essential role in regulating non-small cell lung cancer proliferation, migration, invasion [5]. Lately, researches on ceRNA theory between lncRNAs and miRNAs have been the hotspots in the field of lncRNAs [6]. However, the construction of lncRNA-mRNA regulatory network and lncRNA-associated ceRNA network in CGN have not yet been fully perceived.

In the present study, we set up the lncRNA-mRNA regulatory network and lncRNA-miRNA-mRNA ceRNA network based on proliferation-related lncRNAs, which were identified by lncRNA expression sequencing and identification. Moreover, the connection between CGN and lncRNA-associated network was assessed to provide a bioinformatics basis for discovering possible molecular pathways.

Materials and methods

Glomerular mesangial cell lines and culture conditions

Mouse glomerular mesangial cells (GMCs) SV40-MES-13 were obtained from BNCC Biological Technology (Beijing, China) and cultured in DMEM medium (Solarbio) supplemented with 10% fetal bovine serum (FBS; BI) and penicillin/streptomycin at 37℃ in 5% CO_2-humidified atmosphere.

Cell Proliferation Assay

Cell proliferation rate was measured with Cell Counting Kit-8 (CCK-8) (BestBio, CHN). At the indicated times, GMCs with blank wells (medium only) were treated with CCK8 (10 μL/well, BestBio) for an additional 1.0 h. The OD value (absorbance at 450 nm) was detected using an enzyme-linked immunosorbent assay (ELISA) (Rayto, CHN).

LncRNA sequence analysis

To ensure the quality of paired-end sequencing reads, FastQC software v0.10.1 was used to evaluate the quality of the original sequencing data. The input reads were considered good data quality, when the Q20 base percentage in Reads ≥ 90% and the Q30 base percentage in Reads ≥ 80%.

High-throughput RNA-seq was performed by Genesky Biotechnologies Inc. (Shanghai, China). RNA fragmentation was performed using Bioruptor Pico (Diagenode, Belgium) with sonication RNase-free water. RNA integrity was detected by denaturing gel electrophoresis and quantified by

NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The purified RNA fragments were then used to construct the TruSeq RNA Sample Prep Kit (Illumina, USA). Libraries underwent quality control and were quantified using an Agilent 2100 bioanalyzer system (Agilent Technologies, Inc., USA). Sequencing was carried out on Illumina HiSeq 2500 (Illumina, USA) with pair-end 150-bp read length.

Isolation of RNA and RT-qPCR validation of RNA sequencing data

According to the manufacturer's instructions, total RNA was extracted from the cells using TRIzol Reagent (Life Technologies). Primers were designed based on cDNA sequence using Primer Premier 5 software (Premier Biosoft, Palo Alto, CA, USA), and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The concentration and purity of the isolated RNA was determined using OD1000+ Ultra Micro Spectrophotometer (WuYi technogies, Nanjing, China) and reversing transcription was performed according to the PrimeScript™RT reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd, Beijing, China). Quantitative real-time PCR was performed using an StepOne Plus fluorescence quantitative PCR instrument (ABI, Massachusetts, USA) with the SYBR Green qPCR Master Mix (Takara, Japan). For quantitative results, the expression of lncRNA was expressed as fold change using the $2^{-\Delta\Delta CT}$ method and processed by SPSS22.0 with a one-way analysis of variance.

Construction of the lncRNA-mRNA regulatory network

To identify interactions among LncRNAs and mRNAs, we used LncTar (http://www.cuilab.cn/lnctar) to predict lncRNA-mRNA interactions utilizing free energy minimization [7,8]. LncTar utilized a variation on the standard "sliding" algorithm approach to calculate the normalized binding free energy (ndG $\leq$ 0.1) and found the minimum free energy joint structure. The ndG was regarded as a cutoff that determined the paired RNAs as either interacting or not. Subsequently, the *Cytoscape 3.8.1* was used to visualize the lncRNA-mRNA network after screening the mRNAs for differentially expressed LncRNAs.

Construction of the lncRNA-associated ceRNA network

The interactions between miRNAs and LncRNAs were predicted with miRNA target prediction software miRanda (http://www.miranda.org/), and the putative mRNA targets of the miRNAs mentioned above were predicted by TargetScan (https://www.targetscan.org/) [9]. Then *Cytoscape 3.8.1* was used to delineate the LncRNA–miRNA–mRNA ceRNA network.

GO ＆ KEGG analysis

To uncover the role of differentially expressed lncRNAs in LPS-induced GMCs, we analyzed the Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, respectively [10]. GO terms with $P<0.05$ were considered statistically significant. KEGG pathway enrichment analysis was used to identify significantly enriched signal transduction pathways or metabolic pathways ($P<0.05$). GO and KEGG enrichment analysis was performed using OmicShare tools (https://www.omicshare.com/tools).

Results

Concentration and intervention time screening of LPS inducing cell proliferation

The proliferation ability of GMCs induced by different concentrations (0.5 μg/mL, 1.0 μg/mL, 3.0 μg/mL, 5.0 μg/mL and 10.0 μg/mL) of LPS were detected with CCK-8 assay at 24h and 48h. The relationship between cell proliferation, the concentration of LPS, and intervention time was presented in Figure 1. The results of the CCK-8 proliferation assay demonstrated that LPS could induce cell proliferation on GMCs, the maximum absorbance of OD 450nm was observed when the concentration of LPS was 3.0 μg/mL (Supplementary file 1), which means that 3.0 μg/mL was the optimal concentration of LPS to induce cell proliferation. Meanwhile, there was no significant difference between intervention time of 24h and 48h ($p=0.62$), when the concentration of LPS was 3.0 μg/mL. Therefore, 3.0 μg/mL LPS and intervention 24h were chosen for follow-up studies.

Characteristics of differentially expressed lncRNAs

In this study, six groups of cells were involved in subsequent experiments, including three groups of LPS-induced GMCs as the model group (LPS1-3) and three groups of normal GMCs as the control group (CON1-3).

After deduplication, quality trimming, and quality filtering, the sequencing datas at both ends of R1 and R2 were shown good quality (Q20 base and Q30 base were both greater than 95%). The clean reads of the six samples were all greater than 95%, which met the quality requirements of sequencing. The quality control result of sequencing data was presented in Tab. 1 (Supplementary file 2).

Violin plot showing the relative abundance of lncRNAs in each sample was presented in Fig. 2A. In the violin plot, the white dot represents the median; the violin shape represents the kernel density curves, and the black line represents the 95% confidence interval. Violin plot demonstrating that there were significant differences in lncRNAs' expression between LPS induced GMCs as compared with

control GMCs. The threshold for differentially expressed lncRNAs was set at absolute Fold Change (FC)≥1.5 and $P<0.05$ [11,12], 1532 differentially expressed lncRNAs, including 594 upregulated lncRNAs and 938 downregulated lncRNAs, were selected from 46879 lncRNAs (Fig. 2B; Supplementary file 3) eventually. The heat map of the top 50 differentially expressed lncRNAs was shown in Fig. 2C. The top 10 upregulated and downregulated expressed lncRNAs were presented in Tab. 2, and the radar map (Fig.2D) demonstrated the top 10 upregulated and downregulated expressed lncRNAs in LPS induced GMCs as compared with control GMCs.

Construction of the lncRNA-mRNA regulatory network

To explore the functions of differentially expressed lncRNAs, 556 target mRNAs of 236 lncRNAs out of 1532 differentially expressed lncRNAs were screened out from LncTar after deleting the duplicate data (Supplementary file 4). The highly coordinated expression between lncRNAs and target mRNAs may be due to complementary base pairing between lncRNA and mRNA [13]. In this lncRNA-mRNA regulatory network, we can see the interaction relationship between lncRNA and mRNA. For example, lncRNA NONMMUG029023.2 can regulate mRNA Stoml2 expression. Ptdss2 could be affected by lncRNA NONMMUG039651.2 and lncRNA NONMMUG095401.1 at the same time. The lncRNAs-mRNAs regulatory network was presented in Fig. 3.

To reveal the biological functions of differentially expressed lncRNAs in LPS-induced GMCs, we further performed GO biological process and KEGG pathway analysis on all mRNAs in the lncRNA–mRNA regulatory network. The results of GO and KEGG analysis were presented in Fig. 4.

Within the biological processes (BP) category of GO classification, cellular macromolecule metabolic process, negative regulation of biological process, and nitrogen compound metabolic process were the top 3 over-represented terms (Fig. 4A). Within the cellular components (CC) category of GO classification, intracellular, intracellular part, and membrane-bounded organelle were the top 3 over-represented terms (Fig. 4B). Within the molecular function (MF) category of GO classification, galactoside 2-alpha-L-fucosyltransferase activity, alpha-(1,2)-fucosyltransferase activity, and chromatin insulator sequence binding were the top 3 over-represented terms (Fig. 4C). The KEGG metabolic pathway analysis demonstrated that GnRH secretion, Melanoma, Ras signaling pathway, Glycosphingolipid biosynthesis-globo and isoglobo series, and beta-Alanine metabolism were the top 5 most significant enriched KEGG pathways (Fig. 4D).

RT-qPCR validation of 6 selected lncRNAs

To verify the accuracy of the sequencing results, we used RT-qPCR to detect the expression of 6 selected lncRNAs which are highly conservative with homo sapiens (Tab. 3) [14]. All primers used for PCR amplification were presented in supplementary file 5. RT-qPCR was done as triplicates with the standard deviation for each PCR shown in Fig. 5. The results of RT-qPCR showed the same expression trend as the RNA-Seq results, and 6 selected lncRNAs were all upregulated in LPS-induced GMCs.

Construction of the lncRNA-associated ceRNA network

Except for lncRNA-mRNA regulatory network, lncRNAs can also participate in multiple gene networks that regulate diverse biological processes, like the ceRNA network [15].

To construct a lncRNA-associated ceRNA network, We utilize 6 selected lncRNAs above that are conservative with homo sapiens and have been verified to build ceRNA networks. The ceRNA network consisted of the top 3 miRNAs combined with screened lncRNAs and mRNAs with high confidence (cumulative weighted context score cutoff level less than -0.8) bound to the miRNAs, including 6 lncRNAs, 18 miRNAs, and 419 mRNAs (Fig. 6; Supplementary file 6). It can be clearly seen in Fig. 6 that lncRNAs can be competing targets of shared miRNAs with other mRNAs and form a complex regulatory ceRNA network. For example, the lncRNA NONMMUG089165.1 could act as a sponge for mmu-miR-3960 to affect EVX1 expression. PARD3B could be affected by lncRNA NONMMUG039651.2/mmu-miR-7081-5P and lncRNA NONMMUG028702.2/mmu-miR-7044-5P axis at the same time.

To reveal the biological functions of lncRNAs in ceRNA network, we performed GO biological process and KEGG pathway analysis on all mRNAs involed in ceRNA network. The results of GO and KEGG analysis were presented in Fig. 7.

Within the biological processes (BP) category of GO classification, system development, animal organ development, and multicellular organism development were the top 3 over-represented terms (Fig. 7A). Within the cellular components (CC) category of GO classification, neuronal cell body membrane, cell body membrane, and alpha-beta T cell receptor complex were the top 3 over-represented terms (Fig. 7B). Within the molecular function (MF) category of GO classification, DNA-binding transcription activator activity, RNA polymerase II-specific, alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase activity, and DNA-binding transcription factor activity were the top 3 over-represented terms (Fig. 7C). The KEGG metabolic

pathway analysis demonstrated that Collecting duct acid secretion, Circadian rhythm, Proteoglycans in cancer, Pathways in cancer, and Oxytocin signaling pathway were the top 5 most significant enriched KEGG pathways (Fig. 7D).

Discussion

Chronic inflammation is an essential factor in the occurrence and development of CGN, which may activate cell proliferation and induce deregulation of cells [17,18]. Excessive proliferation of GMCs, commonly observed in glomerulonephritis, is an important pathological basis of kidney disease [19,20]. LPS is a major constituent of the outer membrane of Gram-negative bacteria, which can stimulate the activation of inflammatory factors in cells, leading to systemic inflammatory response and immune activation. LPS has been often used as an inducer of cell proliferation according to many literature reports. For example, LPS can induce THP-1 cell [21] and naive B cell [22] proliferation. Therefore, in this study RNA-seq was used to study proliferation-related lncRNAs in LPS-induced GMCs, the lncRNA–mRNA regulatory network and lncRNA-miRNA-mRNA ceRNA network were constructed to explore the possible molecular mechanism of CGN.

Long non-coding RNAs (lncRNAs), generally known as transcripts longer than 200nt, are important genetic regulators. LncRNAs have been proven to be critical determinants of epigenetic regulation, regulation of chromatin structure, scaffolding or decoy function of mRNAs, and post-transcriptional regulation of mRNA [23]. The regulatory role of lncRNA can be summarized as cis action on neighboring genes, or trans action through affecting mRNA stability, mRNA translation, or the regulation of microRNA-mRNA interactions and RNA binding proteins [24-26]. Over the years, ceRNA appeared to be a vital mechanism for lncRNA and miRNA regulatory network. As an important member of the ceRNA network, lncRNAs play regulatory roles in various diseases and are the most frequently reported mechanism of lncRNAs. In this study, the RNA-seq was performed to study the differences in the expression profiles of lncRNAs in LPS-induced GMCs to examine the role and mechanism of lncRNAs in the occurrence and development of CGN.

Analysis of the GO and KEGG pathways of lncRNA targeted genes could give a peripheral description of the lncRNA function we focused on. In this study, the GO biological process, and KEGG pathway analysis were performed on all mRNAs involed in lncRNA–mRNA regulatory network and lncRNA-miRNA-mRNA ceRNA network. The KEGG pathway analysis revealed that some classical inflammatory signaling pathways such as MAPK, Rap1, Ras, and mTOR were significantly enriched.

When the renal function gets impaired, the pro-inflammatory cytokines and various small and medium-sized molecular toxins cannot be cleared in time, which increases inflammatory cytokines. Meanwhile, the accumulation of small and medium molecular toxins in the body can promote more inflammatory cytokines [27]. This explains why so many inflammatory signaling pathways were enriched. Furthermore, the substances metabolism appeared to be affected, since pathways such as N-Glycan biosynthesis, Glycosphingolipid biosynthesis, Galactose metabolism, Glycosaminoglycan degradation, beta-Alanine metabolism, beta-Alanine metabolism, Riboflavin metabolism and Histidine metabolism were significantly enriched. Normally, kidneys are important organs for removing metabolic waste products from the blood and maintaining homeostasis levels of electrolytes and metabolites while eliminating toxic waste from the body. When the loss of kidney function occurred, the metabolic balance of certain substances in the body will be disrupted. For example, previous studies indicated that there were significant differences in the plasma amino acid profile of patients with chronic kidney disease compared with healthy people, usually manifested as changes in the levels of endogenous amino acids and essential amino acids [28,29]. The results corroborate the findings of a great deal of the previous work in the CGN study.

In this study, we screened out 1532 differentially expressed lncRNAs, including 594 upregulated lncRNAs and 938 downregulated lncRNAs in LPS-induced GMCs, then constructed lncRNA–mRNA regulatory network and lncRNA-miRNA-mRNA ceRNA network. We must admit there are certain limitations in our study should be acknowledged. First, the number of samples tested in RNA-seq should be increased to reduce possible bias in sequencing results. Secondly, both lncRNA-miRNA and miRNA-mRNA regulatory relationships were predicted by bioinformatics analysis software, all hypotheses and relevant mechanisms need to be verified by further experimental molecular studies.

Conclusions

In conclusion, 1532 differentially expressed lncRNAs, including 594 upregulated lncRNAs and 938 downregulated lncRNAs were screened out by RNA-seq in LPS-induced GMCs. Furthermore, the lncRNAs-mRNAs regulatory network including 236 lncRNAs and 556 mRNAs and lncRNA-miRNA-mRNA ceRNA network including 6 lncRNAs, 18 miRNAs, and 419 mRNAs were successfully constructed. The KEGG pathway analysis revealed that some classical inflammatory signaling pathways and substances metabolism were significantly enriched. Our study revealed a global

view of lncRNA-associated network, and offered novel insights into the roles of lncRNAs in the pathogenesis of CGN and provided promising diagnostic biomarkers.

Declarations

Competing interests

All authors declare that they have no conflict of interest.

Ethics approval and consent to participate

As this article is about cell experiments and bioinformatic analysis, ethical approval was unnecessary.

Authors' contributions

The manuscript was written with contributions of all authors. Jiarong Gao undertook the conception and designed the experiment. Xingxing Zhuang and Weifeng Zhang implemented the cell experiment, acquisition of data, and drafted this manuscript. Tao Liu interpreted and analyzed the sequencing data. Liangbing Wei and Yanyan Guo revised this manuscript critically for important intellectual content. All authors read and finally approved the manuscript to be submitted and declared that all datas were generated in-house and that no paper mill was used.

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Figures

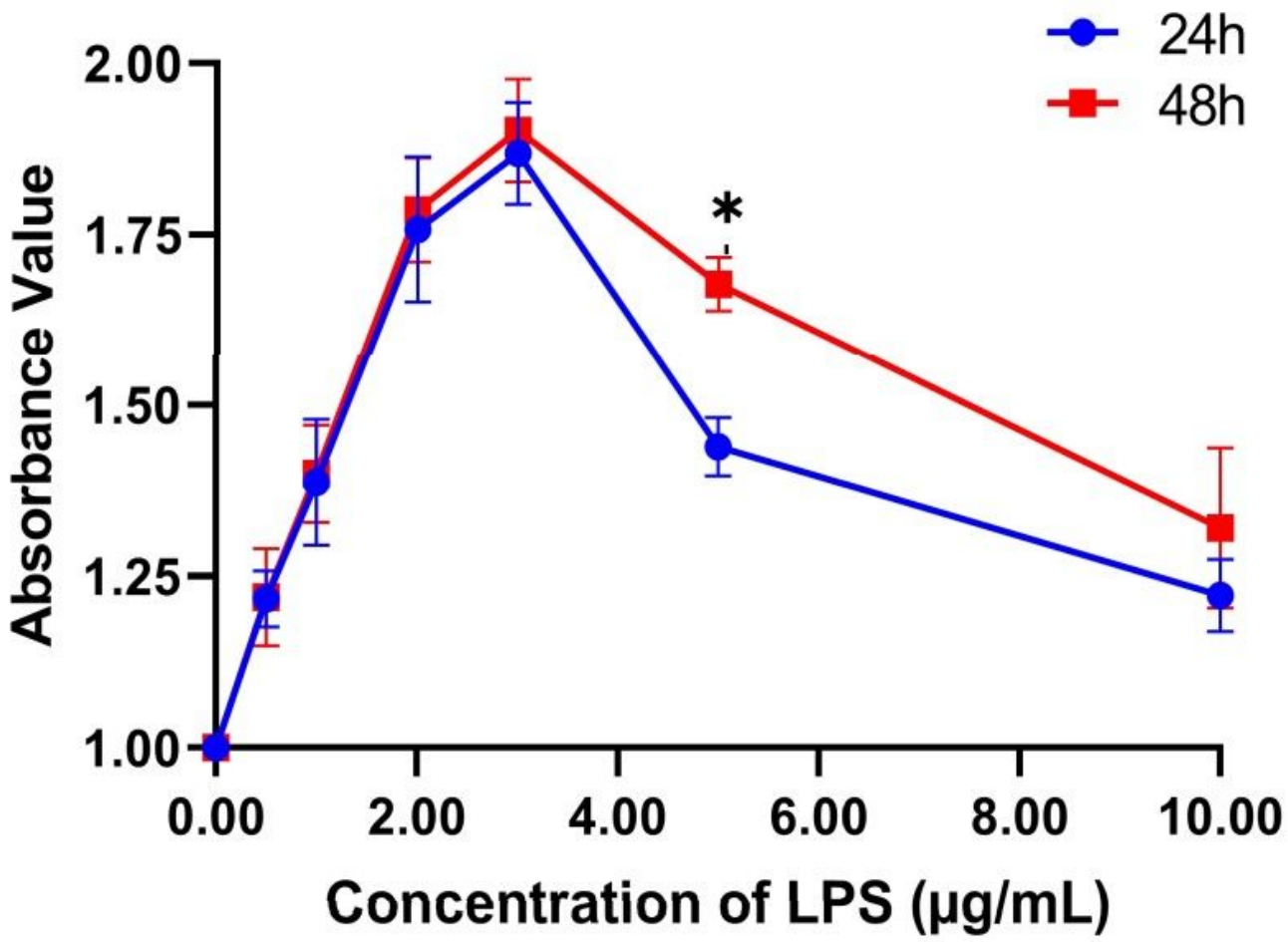

Figure 1

The result of CCK-8 proliferation assay

Figure 2

The features of differentially expressed lncRNAs. A Violin plot of relative abundance of lncRNAs in each samples. B General numbers of differentially expressed lncRNAs. C Heat map of the top 50 differentially expressed lncRNAs. D Radar map of top 10 upregulated and downregulatedexpressed lncRNAs.

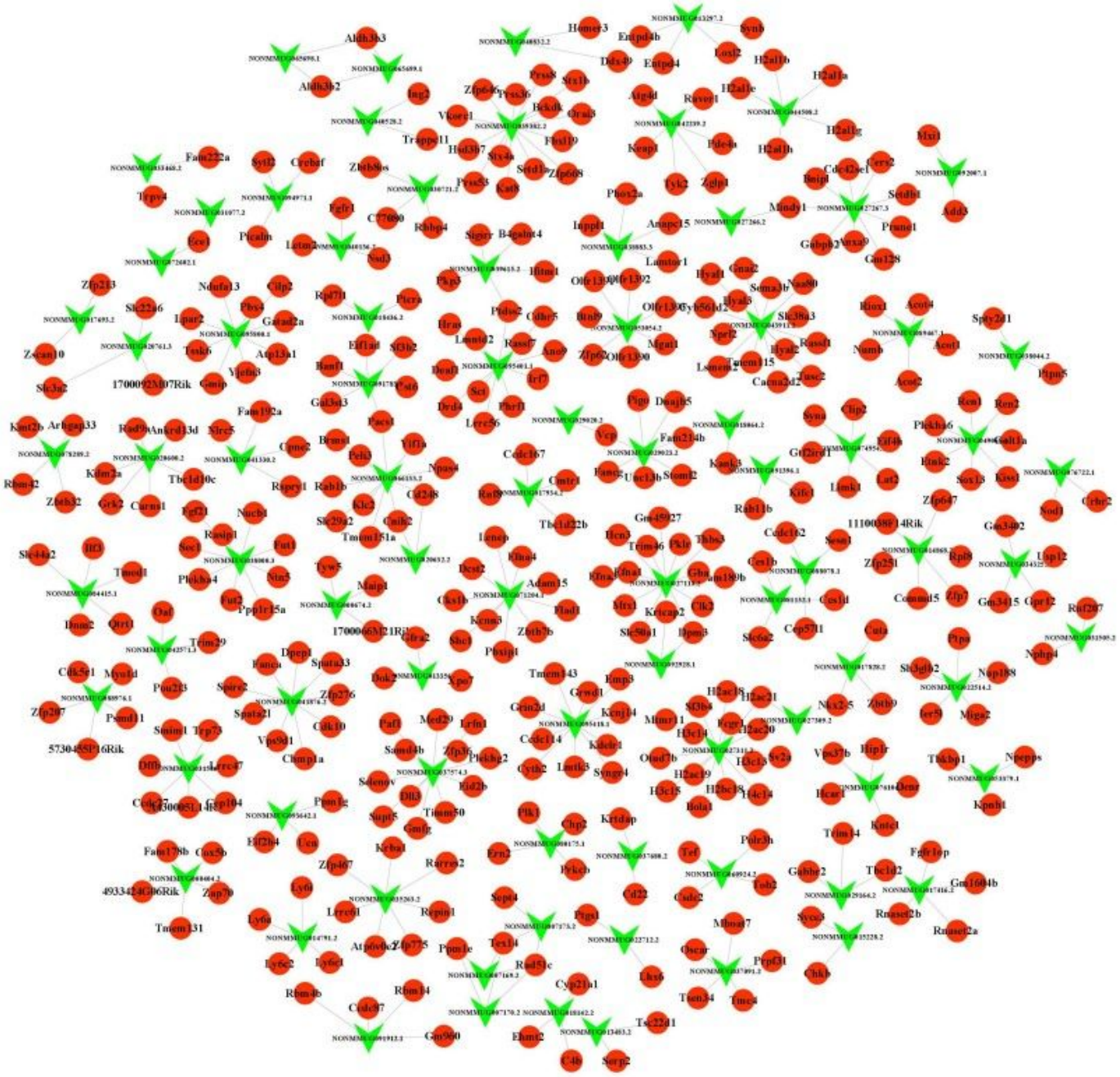

Figure 3

The lncRNA–mRNA regulatory network. The circles in red represent mRNAs, the arrows in green represent lncRNAs.

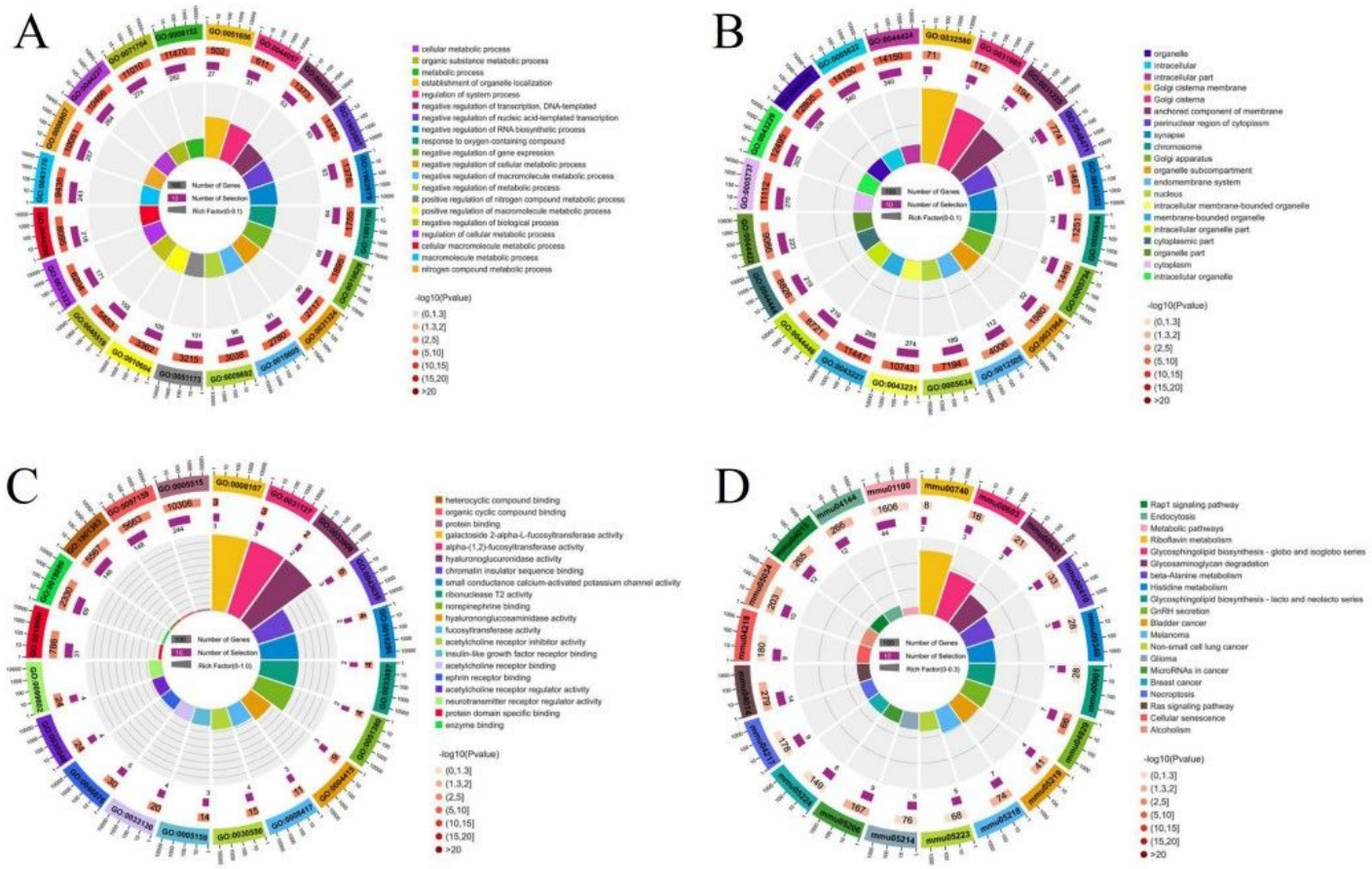

Figure 4

GO and KEGG pathway and enrichment analysis of mRNA in lncRNA-mRNAregulatorynetwork. A GO terms for biological process (BP). B GO terms for cellular component (CC). CGOterms for molecular function (MF). D KEGG pathway and enrichment analysis.

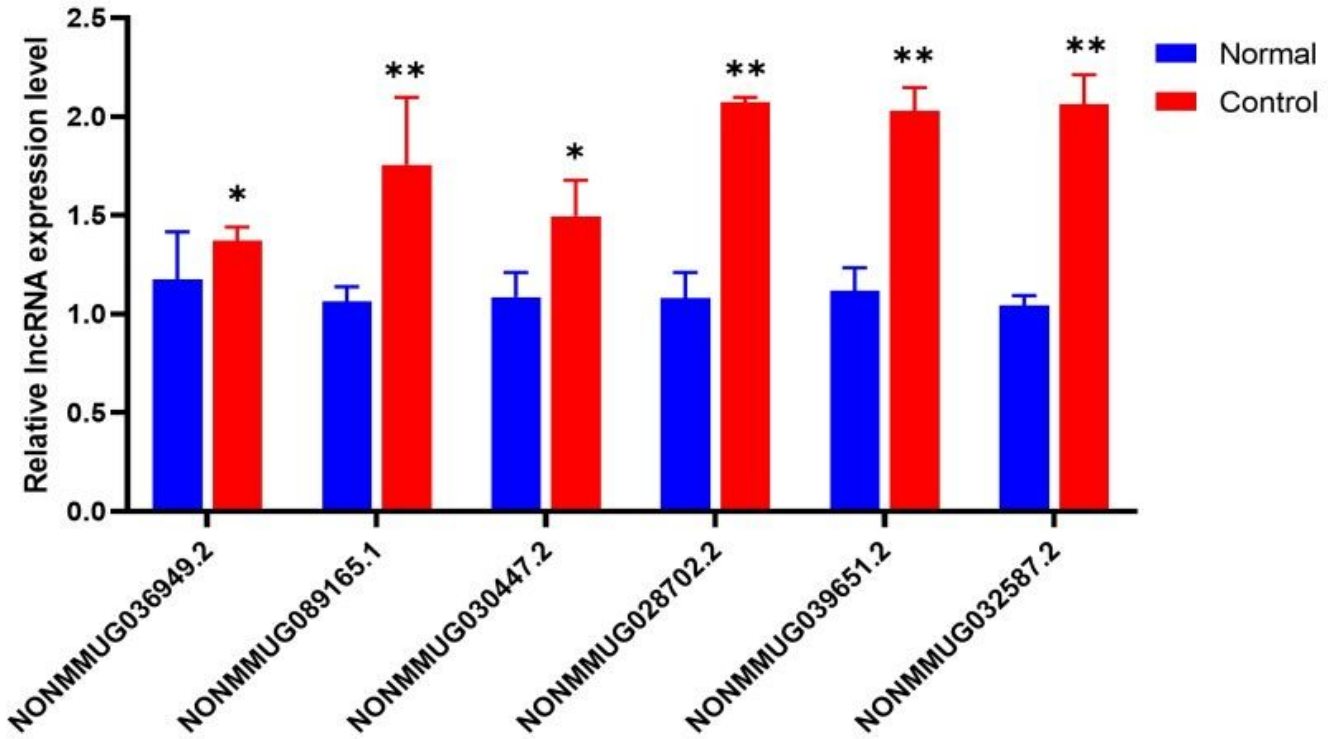

Figure 5

The result of RT-qPCR validation experiments

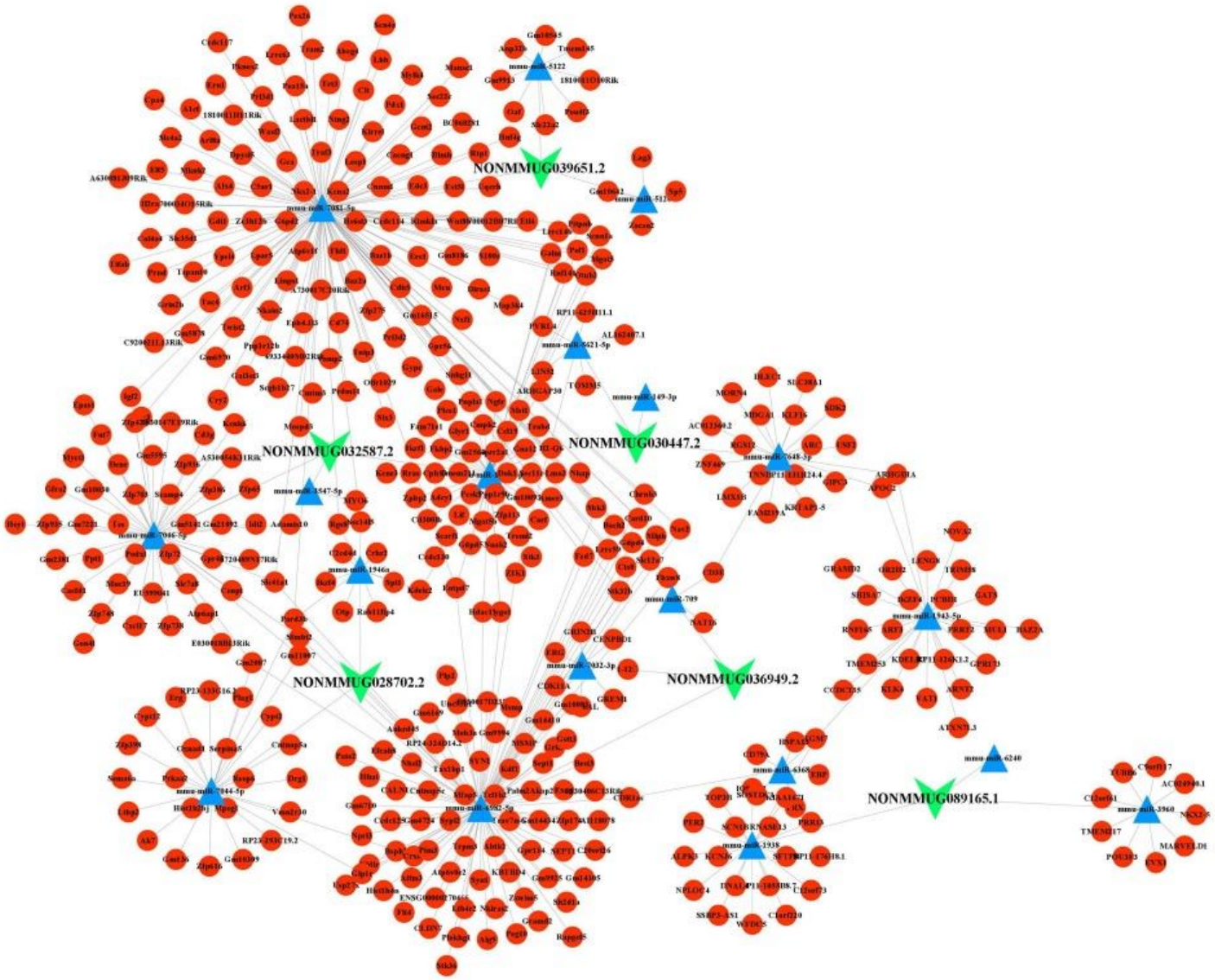

Figure 6

The lncRNA-miRNA-mRNA ceRNA network. The circles in red represent mRNAs, the triangles in blue represent miRNAs, the arrows in green represent lncRNAs.

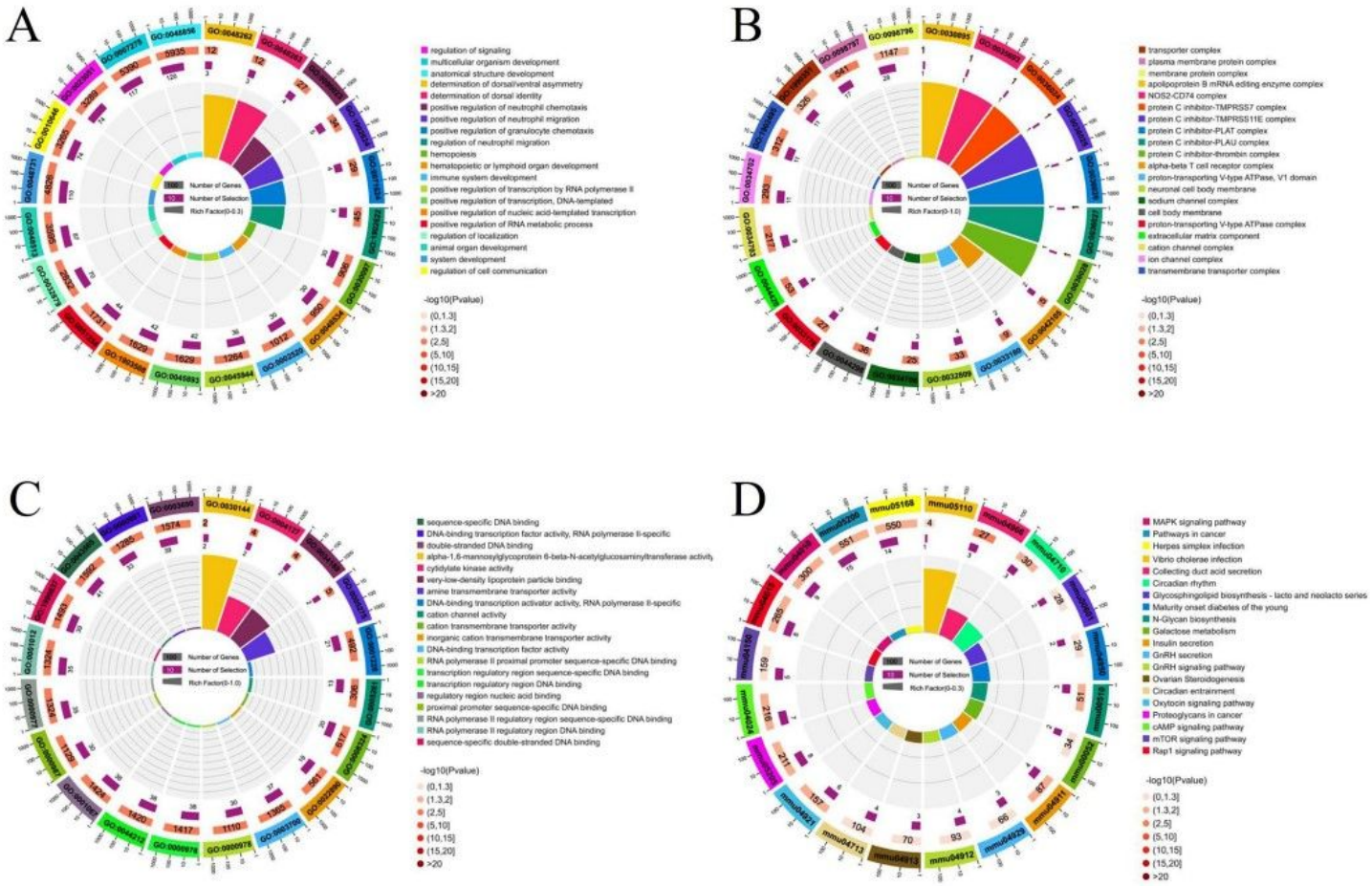

Figure 7

GO and KEGG pathway and enrichment analysis of mRNA in ceRNA network. AGOterms for biological process (BP). B GO terms for cellular component (CC). C GO terms for molecular function (MF). D KEGG pathway and enrichment analysis.

Supplementary Files

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